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Screening of green AgNPs against the larvicidal activity of *Anopheles stephensi*

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ABSTRACT

Over the last decades, climate change, population growth, deforestation, habitat invasion and insecticide resistance have contributed to the emergence, reemergence and dispersion of various vector-borne diseases including malaria, filariasis, chikunguniya and dengue. The larvicidal activity of aqueous extracts of neem and green-synthesized silver nanoparticles (AgNPs) was served against third instar larvae of *Anopheles stephensi*. Synthesized AgNPs were characterized UV-Visible spectrum analysis and high resolution TEM. AgNPs (150µl, 200µl and 250µl) and plant extracts (600µl, 800µl and 1000µl) were tested against twenty third larval instar of *A. stephensi* at different concentrations. All the treated and control samples were analyzed after 24 hrs. The data were analyzed by using One-Way ANOVA test and the results were found to be significant.

Figure : 01 References : 19 Tables : 03
KEY WORDS : AgNPs, ANOVA, *A. stephensi*, Malaria, Mosquito, TEM.

Introduction

Mosquito are the vectors of many diseases, including malaria, filariasis, dengue and Japanese encephalitis. Among these kinds of malaria, spread by the bite of female causes these vectors borne diseases of the tropical region and are considered major public health concerns. According to WHO[19], two fifth of world population is under risk of dengue infection and in year 2010, 28,292 cases of infection and 108 deaths were reported to be caused by dengue in India[19]. Figure of malaria is much higher than dengue affecting 225 million and 7,81,000 deaths worldwide in 2009: whereas, 1.49 million infection and 767 deaths were reported in India in 2010[15].

In recent years, repeated use of synthetic insecticides for mosquito control have disrupted natural biological control systems and led to resurgences in mosquito populations leading to major outbreaks of mosquito borne diseases. Moreover, development of resistance and undesirable effects at various trophic levels in food chains have fostered environmental and human health concerns which initiated a need to search for alternative control measures. Nanoparticles have attracted considerable attention, owing to their various applications particularly silver nanoparticles, which are reported to possess antibacterial, antifungal, anti-inflammatory, anti-cancerous and anti-viral activity. Nanotechnology is mainly concerned with synthesis of nanoparticles, nanocomposites of variable size, shapes and most predominantly studied nanoparticles today are those made from noble metals, *viz.* Ag, Pt, Au and Pd. Among these, silver nanoparticles (AgNPs) play a significant role in the field of biology and medicine. It is well known that due to a similar size to cellular proteins and components of nanoparticles, nanoparticles are able to cross some of the barriers of biological systems. Nanoparticles can enter cells by diffusing through cell membranes, endocytosis and pinocytosis. Most internalization of nanoparticles will probably occur *via* endocytosis (particles up to 100 nm)[14].

Synthesis of silver nanoparticles has been reported by a number of methods involving chemical, physical and biological synthesis. Researchers synthesized and characterized silver nanoparticles using transmission electron microscopy (TEM) and X-ray diffractometry[7]. Some created nanometer sized silver particles by inert gas condensation and co-condensation techniques[4]. The silver nanoparticles exhibit antibacterial effects at low concentrations. It was found that smaller particles with a

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TABLE- 1: The larval density of breeding site

Breeding site	LD 1	LD2	LD3	LD4	LD 5	LD6	LD7	LD8	LD9	LD10
LD	8.67	8.67	8	9.33	8.33	11.3	12	12	8	9
pH	6.7	7	6.8	7	7	6.5	6.8	6.6	7	7.1
T	27°C	28°C	27°C	29°C	29°C	27°C	26°C	27°C	25°C	29°C

LD: Larval density, T: Temperature

larger surface to volume ratio provided a more efficient means for antibacterial activity. Chemical methods are usually employed for nanoparticles synthesis at industrial level which involve the use of toxic reducing and capping agents for synthesis thus limiting their use in biomedical applications due to risk of health hazard. Therefore, recently the interest is shifted towards utilizing potential of biological agents (plants, bacteria, fungi *etc.*) for nanoparticles production.

The researchers observed assessments of the antiparasitic activities to determine the efficacies of synthesized silver nanoparticles (AgNPs) using aqueous leaf extract of *Mimosa pudica* against the larvae of malaria vector, *Anopheles*, filariasis vector *Culex quinquefasciatus* and *Rhipicephalusmicroplus*[13]. Larvicidal activity of silver nanoparticles: synthesized using *Plumeria rubra* plant latex against *Aedes aegypti* and *Anopheles stephensi* was also monitored[16].

Toxicity of NPs mechanisms include disruption of membranes, oxidation of proteins, genotoxicity, interruption of energy transduction, formation of reactive oxygen species, and release of toxic constituents. Silver nanoparticles (AgNPs) may cause toxicity *via* multiple mechanisms such as adhering to the surface, altering the membrane properties, so it affects the permeability and the respiration of the cell. The present study is focused upon the screening of the AgNPs against the test insect *A. stephensi* for their larvicidal activity. The conducted experiment was successful in controlling the test insect at their respective doses.

Materials and Methods

Rearing and larval density: For the conduction of various experiments breeding sites were spotted for the collection of mosquito larvae in the University Campus, University of Rajasthan, Jaipur. The larvae were collected with the help of ladle and dropper. At every breeding spot pH and temperature of the water source was taken using thermometer and pH probe. Larval density of *Anopheles stephensi* of different samples were determined using the collecting ladle.

Larval Density (LD) =Number of larvae in each ladle/Number of ladle

The unit of measuring LD is larvae/ml. The collected larvae were placed in clean beaker containing fresh water and reared in the BOD incubator in the laboratory conditions. Their adult and larval stages were identified using key in the laboratory[6,12,17]. The larvae were cultured in the beakers kept in the cooling incubator at the temperature of 27ºC in clean distilled water of pH 6-7. The larvae were fed by dried brewer's yeast[9].The adults were fed by cotton soaked in 10% glucose solution and changed daily.

Preparation of neem nano-formulation

Azadirachta indica leaves were collected from the Department of Zoology, Rajasthan University campus in Jaipur, Rajasthan. The collected leaves were washed with running tap water to remove dust materials and rinsed in large volumes of deionized water and allowed to shade dry at room temperature (RT:27±2°C). Dried leaves were cut in fine pieces[10]. A plant leaf broth was prepared by placing 25g of the leaves (finely cut) in a flask with 100mL of sterilized distilled water. The flask was placed on magnetic stirrer set with temperature 80°C-90°C. The broth was observed till the color changed to light green. The extract was then filtered, decanted through Whatman-1 filter paper, stored at - 4°C, for the use of experiments within a week.

Magnetic stirrer was again set up at 60°C -70°C with a conical flask having silver nitrate solution. Leaf broth solution was poured drop-wise in $AgNO_3$ solution till light yellow color appeared and later to yellowish-brown which indicates silver nanoparticle formation.

Characterization of nanoparticle: UV-Visible spectrum analysis was performed for freshly prepared nanoparticle sample and also for sample kept after 24 hours of incubation and absorption maxima analysis was done.

TABLE- 2: Comparison of leaf extract & Green silver nanoparticles on mosquito larvae (III instar)

S.No.	Sample	N	Number of larvae dead				%	Mean		
			R1	R2	R3	Mean	Mortality	(ö-value)		
Group I	C	20	3	1	0	1.33	6.65	11.90	Treat. Mean	47.83
Group II (i)	AgNP (150µl)	20	8	9	8	8.33	41.65	40.20	G.M.	42.70
Group II (ii)	AgNP (200µl)	20	10	11	9	10	50	45.00	For C v/s Treat	
Group II (iii)	AgNP (250µl)	20	20	20	20	20	100	**90.00**	**S.Em±**	**2.87**
Group III (i)	LE (600µl)	20	3	3	3	3	15	22.79	**C.D.5%**	**8.69**
Group III (ii)	LE (800µl)	20	6	7	6	6.33	31.65	34.23		
Group III (iii)	LE (1000µl)	20	13	13	14	13.33	66.65	54.75	**C.V. %**	**10.77**
	S.Em±	2.65								
	C.D.5%	**8.05**								

C: Control; AgNP: silver nanoparticles of neem; LE: leaf extract; N: total number of larvae taken

Prepared nanoparticles were analyzed at a wavelength spectrum of 350-510 nm at a resolution of 1nm. Analysis was performed at different time intervals.

High resolution transmission electron microscope, The Tecnai G2 20(FEI)S-Twin was used for obtaining micrographs of prepared nanoparticle.

Larval toxicity test[1]: To study the toxicity of prepared nanoparticle and leaf extract in comparison against mosquito larva, standard methods were used. The third instar larvae of *A.stephensi* were taken from the reared generation and tested against different concentrations of silver nanoparticle (150µl, 200µl and 250µl) and leaf extract (600µl, 800µl and 1000µl) for toxicity studies against larvae.

Experimental design and Bioassay: Each experiment was run in a 250ml flask and in triplicates.

Group I Control: 100ml deionized water + 20 larvae.

Group II Nanoparticle test samples:

i. 150µl nanoparticle+ 100ml deionized water+ 20 larvae
ii. 200µl nanoparticle +100ml deionized water + 20 larvae
iii. 250µl nanoparticle+ 100ml deionized water+ 20 larvae

Group III Leaf extract test samples:

i. 600µl leaf extract solution+100ml deionized water+ 20 larvae
ii. 800µl leaf extract solution+100ml deionized water+ 20 larvae
iii. 1000µl leaf extract solution +100ml deionized water+ 20 larvae

All the samples were analyzed after 24hr for mortality studies.

Mortality percentage was calculated using formula:

% mortality = Number of dead larvae/ Number of larvae introduced x100

Result and Discussion

The adult and larvae were reared in the laboratory condition upto 2-3 generations for the screening test of this experiment. The larval densities of different samples in the local area were calculated using the Abott formula[1] (Table 1). Prepared silver nanoparticles using aqueous

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TABLE- 3 : One-way ANOVA Test

ANOVA	(One way-CRD)				
Source	**d f**	**S S**	**M S**	**Fcal**	**p Value**
Sample	6	11431.898	1905.316	90.175	2.24E-10
Treated	5	8113.301	1622.660	76.797	1.13E-09
C v/s Treat	1	3318.598	3318.598	157.063	5.35E-09
Error	14	295.808	21.129		
Total	20	11727.706			

extract of shade dried leaves of *Euphorbia hirta* were evaluated[8]. In the recent past, several plants have been screened successfully for silver nanoparticles synthesis like *Azadirachta indica*[18], *Emblica officinalis*[3], *Aloe vera*[5]. Some researchers highlighted the scope of green chemistry in context to potential health effects of nanoparticles, alongside medical applications of nanoparticles including imaging, drug delivery, disinfection, and tissue repair[2]. Preparation of green silver nanoparticle of *A. indica* was indicated by dark-brown coloration of the solution. In UV-Visible spectrum analysis the maximum absorption of silver nanoparticle was shown between 438-442nm with average maximum absorbance at 440nm while absorption maxima peak steadily increased after 24 hours of incubation of silver nanoparticle and got saturated indicating complete reduction of silver nitrate. The absorption peak varied as a function of reaction time and silver nitrate concentration. Further, TEM micrographs showed that the high density of AgNPs synthesized without aggregation by using *A. indica* aqueous leaf filtrate. The average size of silver nanoparticle ranged between 15-25nm (Figure 1 a,b) and spherical or rounded in shape (Figure 1 c). Analysis by Selected Area Electron Diffraction Pattern revealed that the nanoparticles are polynanocrystalline in nature (Figure d). Prepared silver nanoparticles of different shapes (spheres, rods, and dendrites) by using a pulse nanoelectrochemical technique from an aqueous solution of $AgNO_3$ in the presence of nitrilotriacetate N(CH2COOH)3-NTA are also reported[11]. The silver nanoparticles were characterized by using TEM, X-ray diffraction and absorption spectroscopy. It was found that the concentration of AgNO3 and NTA affects the shape of the nanoparticles.

The leaf broth alone was tested and found that they can achieve 66% mortality with 1000µl leaf indicating the medicinal value of *Azadirachta indica.* Effect of silver nanoparticle on larval density of mosquito larvae suggests that 200µl nanoparticle solution indicating LC_{50} dose while 250µl was LC_{100} conferring 100% mortality rate (Table 2). Thus, confirming the use of silver nanoparticle for larvicidal activity against various pathogenic mosquito vector. One way Analysis of Variance test determined the values S.Em± was to be 2.65 and C.D. at 5% was 8.05.

Conclusion

For many mosquitoes borne diseases, the age and abundance of female adult mosquitoes are key in determining the ability of a mosquito population to vector the disease effectively; malaria is one such disease.The larval density counted from the local breeding sites in University of Rajasthan, Jaipur were found to be higher than the critical larval density. Effect of leaf extract on larval density of mosquito suggests that larvicidal activity is effective alone in controlling the test insect.

But green-synthesized AgNPs using a medicinal plant, *A. indica* aqueous leaf filtrate exhibits a more effective rapid process, quick process for large scale production, cost effective, stable, non-toxic, bio-degradable, recyclable and environmentally acceptable green approach and serves as a strong larvicidal against*A. stephensi.* This novel approach would be a boon for the event of other biopesticide by utilizing natural resources

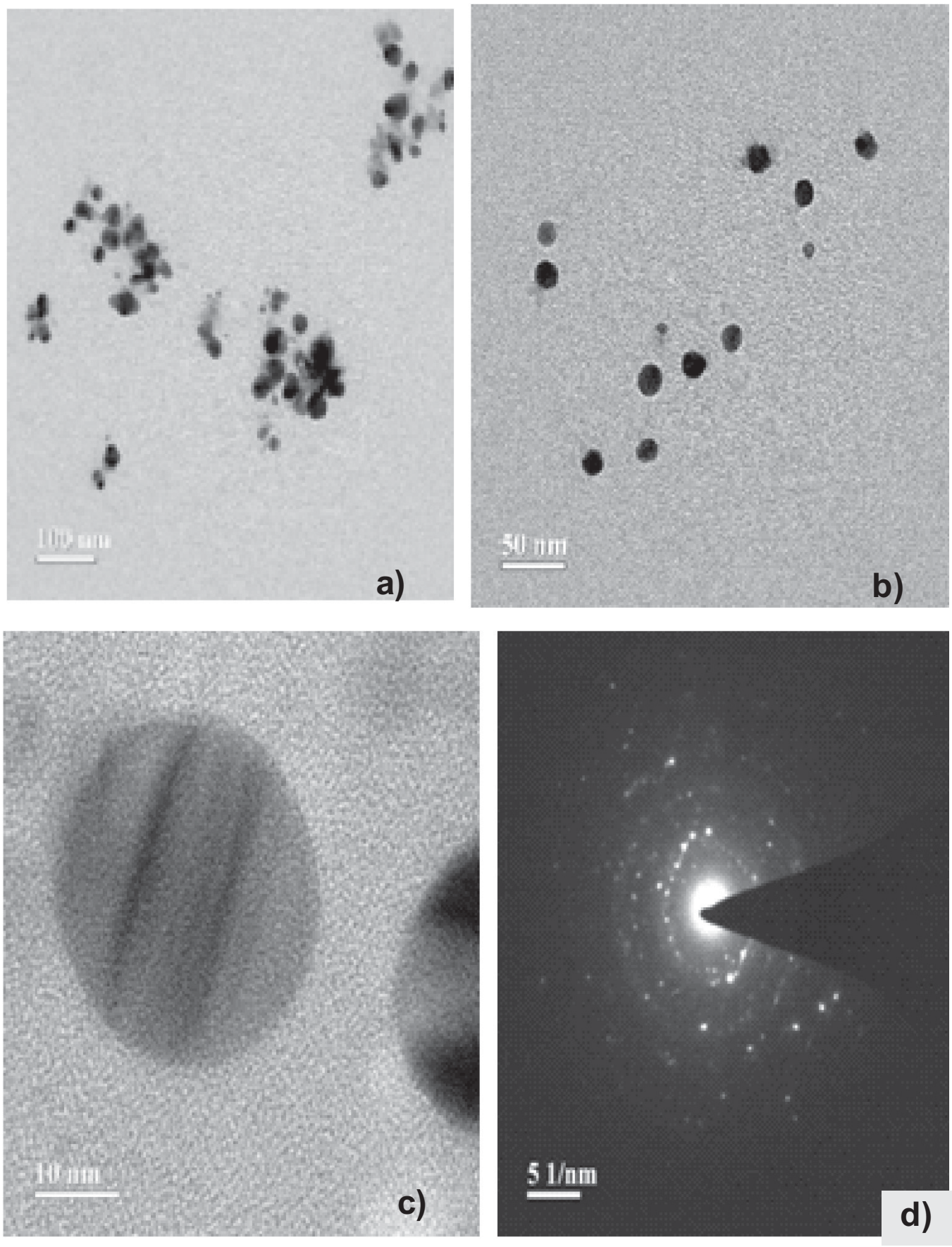

Fig. 1: TEM images of Silver nanoparticles a) 100nm scale image b) 50nm scale image analysis c) 10nm scale image d) SAED pattern

and a reasonable method useful in preventing filariasis, malaria, chikungunya and dengue vectors of public health importance. Further, studies for the screening of toxicity of these nanoparticles are in progress.

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