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EFFICACY OF LEAVES AQUEOUS EXTRACT AND SYNTHESIZED SILVER NANOPARTICLES USING *LAGENARIA SICERARIA* AGAINST *CULEX PIPIENS* LISTON AND *ANOPHELES PHAROENSIS* THEOBALD (DIPTERA: CULICIDAE)

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Abstract

The control of *Culex pipiens* and *Anopheles pharoensis* considered as important strategy in eliminating diseases transmitted by the two mosquito species. Application of plant-derived products in mosquitos' control reported as much effective, less expensive and safer to human, as well as environment than their synthetic counterparts. The present study investigated the activity of *Lagenaria siceraria* leaves aqueous extract and *L. siceraria*- synthesized silver nanoparticles (AgNPs) against immature stages of *C. pipiens* and *A. pharoensis.* Immature stages of both tested mosquito species were exposed to 50, 100, 200, 300 and 400 ppm of *L. siceraria* leaves aqueous extract and 5, 10, 20, 30 and 40 ppm of *L. siceraria*-synthesized AgNPs for 24 h. It was found that *L. siceraria* leaves aqueous extract can reduce aqueous silver ions to generate silver nanoparticles suspended in water. The results of DLS revealed that, the average hydrodynamic diameter calculated for AgNPs was 33.25 nm, while the TEM images showed the occurrence of individual silver nanoparticles (13.38-27.70 nm) and a number of aggregates in the test suspension. In addition, *L. siceraria*- synthesized AgNPs were more toxic against tested mosquito species than *L. siceraria* leaves aqueous extract. The LC_{50} values recorded 15.2, 18.2, 22.5, 24.7 and 29.4 ppm against *C. pipiens* first to fourth instar larvae and pupae; whereas LC_{50} values were 11.9, 14.5, 17.7, 19.8 & 23.1ppm against *A. pharoensis* first to fourth instar larvae and pupae, respectively.

Keywords: Larvicidal, *Culex pipiens, Anopheles pharoensis, Lagenaria siceraria.*

Introduction

Mosquitoes are vectors of many devastating diseases to human, including lymphatic filariasis, West Nile virus, dengue fever, Rift Valley Fever virus and Japanese encephalitis (Velayutham *et al,* 2016; Morsy, 2018; Hassan *et al,* 2014). Members of *Culex pipiens* L. are distributed throughout Egypt transmitting *Wuchereria bancrofti* causing lymphatic filariasis (Abdel-Shafi *et al,* 2017), the most neglectful tropical disease in Africa (Palumbo, 2008). In, World Health Organization (2000) initiated about seventy programs worldwide, in which control of main transmitting vector considered as important strategy in eliminating filariasis by 2020 (Famakinde, 2018). Also, *Anopheles pharensis* is a well-known malarial vector, which infects about 500 million human per year causing death to 1.2-2.7 million/year (Roni *et al,* 2013). Approximately, 90% of malaria cases occur only in Africa (Saleh *et al,* 2019), and cases were reported in Egypt (Saleh *et al,* 2016) and mosquito bites cause allergy, urticarial and angioedema (Peng *et al,* 2004). Thus, control of *C. pipiens* and *A. pharoensis* is urgently needed.

For many decades, mosquitos' larvae targeted with chemical insecticides, however there are significant challenges, such as increasing mosquito resistance to chemical insecticides and adverse effect of these insecticides on non-target organisms and environment (Priyadarshini *et al,* 2012; Fouda *et al,* 2017). Application of several plant products in mosquitos' control reported as much effective, less expensive and safer to human and environment than their synthetic counterparts (Fouda *et al,* 2017). With the progress of nanotechnology in recent years, many researchers around the world have been engaged with the investigation of the

activity of synthesized nanoparticles with different shapes and sizes with unique and thrilling properties supporting their application in many unrelated fields, such as the nanomidicine, the antimicrobials and larvicidal agents against the different mosquito species (Priyadarshini *et al,* 2012; Sirelkhatim *et al,* 2015; Ahmed *et al,* 2016; Bobo *et al,* 2016; Chen *et al,* 2016). Synthesized nanoparticles offer several benefits of eco-friendly due to absence of deadly chemicals in its synthesis (Bhosale *et al,* 2014). Thus, efforts to use synthesized nanoparticles as new mosquitos' larvicidal agents remain necessary.

Lagenaria siceraria used in the present study belonging to Cucurbitaceae proved to have toxic activity against *Musca domestica*, as well as repellent activity against *Culex pipiens* starved females (Hassan *et al,* 2014; 2018). However, there are no reports concerning with the activity of *L. siceraria* synthesized silver nanoparticles against different mosquito species.

The present study dealt with the activity of *Lagenaria siceraria* and its synthesized silver nanoparticles against the most common mosquito species distributed in Egypt, *Culex pipiens* and *Anopheles pharoensis.*

Materials and Methods

Materials: Fresh leaves of *Lagenaria siceraria* were collected within Sadat City, Monofeiya Governorate (30º21´38.7" N, 30º29´58.3" E, altitude 42m), Egypt during March 2019 and identified in Department of Botany, Faculty of Science, Al-Azhar University. $AgNO_3$ was purchased from El- Gomhouria Co. for Trading Pharmaceuticals, Chemicals & Medical Appliances, Cairo.

Mosquitos' cultures and rearing conditions: Eggs of *Culex pipiens* and *Anopheles pharoensis* were obtained from Medical Entomology Research Center, Dokki, Egypt and returned to Mosquito Insectary, Animal House, Department of Zoology, Faculty of Science, Al-Azhar University. Eggs were transferred to enamel trays containing 1 liter of distilled water for hatching. Larvae were fed on 5 g ground dog biscuit and brewer's yeast daily (3:1). Adults were kept in wooden cages (60×60×60 cm) and provided with 10% sucrose solution for a period of 3-5 days after emergence. Then, females were allowed to get a blood meal from the pigeon. Plastic cups (15×15cm) containing 50 ml distilled water were placed in cages for oviposition. Mosquitos larvae and adults were held at 27±2°C and 75-85% RH in a 12:12 (dark/light) photoperiod (Roni *et al,* 2013).

Preparation of plant extract: Leaves of *L. siceraria* (collected from El-Sharquiya Governorate, Egypt) were washed and dried in shade for five days at room temperature. Dried leaves were separately grounded using electrical stainless steel blender (Philips, HR2058). To prepare extract, 4g of *L. siceraria* leaves were boiled with 100 ml distilled water in water bath for three minutes. Then, the solution was filtered and kept into refrigerator at 4°C until use (Mondal *et al,* 2014).

Preparation of $AgNO_3$ solution: According to Mondal *et al,* (2014), 0.17g $AgNO_3$ was dissolved in 100ml distilled water to prepare $AgNO_3$ stock solution.

Synthesis of silver nanoparticles (AgNPs): The leaves extract of *L. siceraria* was mixed with $AgNO_3$ solution in the ratio of 1:9 and incubated at room temperature for 72 hours for the appearance of reddish brown color proving the formation of AgNPs.

Dynamic Light Scattering (DLS): The synthesized AgNPs was sonicated for 15 min before analysis. The DLS analysis of suspension was carried out using Zetasizer nano (Malvern Panalytical, UK). The Zetasizer operated under 25°C, count rate 428.5 kcps and 60 sec time. Measurements of hydrodynamic diameter and polydispersity index were carried out as a function of time.

Transmission electron microscopy (TEM): The AgNPs suspension was sonicated for 15 min and diluted to a slight turbid suspension. The JEOL 1010 Transmission electron microscope was used for TEM studies at Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University.

Larvicidal/Pupicidal assays: Twenty five larvae (instars I-IV) or pupae of each mosquito species were isolated and transferred into 500ml glass beakers containing 249ml of distilled water and 1ml of tested concentration from *L. siceraria* aqueous extract and/or synthesized AgNPs (Priyadarshini *et al*, 2012). Larvae provided with 0.5mg larval food. Control carried out alongside using 25 larvae or pupae in 250ml distilled water. Larval mortality percentages were recorded at 24 h intervals and calculated using Abbott's formula (Abbott, 1925). Each test carried out three times alongside with control to get the mean value of mortality.

Statistical analysis: Probit analysis applied to calculate LC_{50} and LC_{90} at 95% lower and upper confidence limits using Statistical Package Social Science (SPSS) software version 11.5 (SPSS, 2007). All data calculated as M±SD (N= 3).

Results

Dynamic Light Scattering (DLS) Analysis: For calculating the hydrodynamic diameter of nanoparticles suspension, the DLS analysis is the emerging and vastly described technique. The DLS results revealed that the average hydrodynamic diameter calculated was 33.25 nm (Fig. 1) which is slightly larger than the sizes recorded by TEM.

Transmission Electron Microscopy (TEM) Analysis: *Lagenaria siceraria*- synthesized AgNPs were subjected to TEM to find the information of AgNPs morphology and size. TEM images showed the occurrence of individual silver nanoparticles and a number of aggregates in the test suspension. Also, sizes of AgNPs reported by TEM ranged between 16.67 and 27.70 nm, respectively (Fig. 2).

Larvicidal/Pupicidal Activity: The activity of aqueous leaf extract of *L. siceraria* against immature stages of *Culex pipiens* and *Anopheles pharoensis* is given in Table 1. At the lowest concentration (50 ppm), the mortality was 40.0 and 44.0% in *C. pipiens* and *A. pharoensis* first larval instars whereas at 400ppm, it was increased to record 100% in the same larval instar of both *C. pipiens* and *A. pharoensis*. The highest concentration (400 ppm) recorded complete mortality in *A. pharoensis* second larval instar. Also, *L. siceraria* aqueous extract recorded 9.3 and 17.3% mortality in *C. pipiens* and *A. pharoensis* pupae at 50 ppm, these percentages increased to record 77.3 and 84.0% at 400 ppm, respectively.

On the other hand, the combination of *L. siceraria* extract with silver nanoparticles potentiated its activity against both *C. pipiens* and *A. pharoensis* immature stages (Tab. 2). The highest concentration of *L. siceraria*- synthesized AgNPs (40 ppm) recorded 84.0 and 80.0 % mortality in *C. pipiens* third and fourth larval instars, while it recorded 94.7 and 92.0% mortality in the same larval instars of *A. pharoensis*, respectively. Also, there is no mortality percepts recorded in control groups in the concurrent assay.

LC_{50} values of *L. siceraria* extract against immature stages *C. pipiens* and *A. pharoensis* recorded 101.7 & 75.2 ppm against first instar larvae, 131.6 & 111.2 ppm against second instar, 169.3 &144.6 ppm against third instar, 218.4 & 177.7 ppm against fourth instar, 267.8 & 230.4 ppm against pupae (Tab. 3). LC_{90} values of *L. siceraria* extract recorded 481.6 & 454.7ppm against *C. pipiens* and *A. pharoensis* pupae, respectively. Also, *L. siceraria*- synthesized nanoparticles evoked LC_{50} and LC_{90} values lower than that recorded by *L. siceraria* aqueous extract. The LC_{50} values of AgNPs against *C. pipiens* and *A. pharoensis* first instar larvae were 15.2 & 11.9 ppm, it increased to record 24.7 and 19.8 ppm against *C. pipiens* and *A. pharoensis* fourth instar larvae, respectively. The chi-square values were not significant at $P \leq 0.05$ level (Tab. 3&4).

The detailed results are shown in tables (1, 2, 3 & 4) as well as in figures (1 & 2).

Table 1: Activity of *L. siceraria* leaves aqueous extrct against *C. pipiens* and *A. pharoensis* immature stages.

Species	Target	Concentrations (ppm)					
		50	100	200	300	400	Control
C. pipiens	Larvae I	40.0±4.0	53.3±2.3	62.7±4.6	84.0±4.0	100.0±0.0	0.0±0.0
	Larvae II	34.7±2.3	46.7±2.3	58.7±2.3	82.7±2.3	98.7±2.3	0.0±0.0
	Larvae III	25.3±2.3	38.7±6.1	53.3±2.3	78.7±2.3	93.3±2.3	0.0±0.0
	Larvae IV	14.7±2.3	29.3±2.3	41.3±6.1	69.3±2.3	86.7±2.3	0.0±0.0
	Pupae	9.3±2.3	21.3±2.3	33.3±6.1	54.7±2.3	77.3±6.1	0.0±0.0
A. pharoensis	Larvae I	44.0±4.0	57.3±2.3	66.7±4.6	86.7±2.3	100.0±0.0	0.0±0.0
	Larvae II	38.7±2.3	50.7±2.3	62.7±2.3	82.7±4.6	100.0±0.0	0.0±0.0
	Larvae III	30.7±2.3	45.3±2.3	57.3±2.3	80.0±4.0	97.3±2.3	0.0±0.0
	Larvae IV	24.0±4.0	38.7±6.1	52.0±4.0	74.7±2.3	90.7±2.3	0.0±0.0
	Pupae	17.3±2.3	32.0±4.0	38.7±4.6	60.0±4.0	84.0±4.0	0.0±0.0

Table 2: Activity of silver nanoparticles synthesized using *L. siceraria* leaves against *C. pipiens* & *A. pharoensis* immature stages.

Species	Target	Concentrations (ppm)					
		5	10	20	30	40	Control
C. pipiens	Larvae I	29.3±2.3	42.7±2.3	57.3±2.3	80.0±4.0	94.7±2.3	0.0±0.0
	Larvae II	25.3±2.3	34.7±2.3	54.7±2.3	72.0±4.0	89.3±4.6	0.0±0.0
	Larvae III	17.3±2.3	29.3±2.3	42.7±6.1	64.0±4.0	84.0±6.9	0.0±0.0
	Larvae IV	14.7±2.3	22.7±2.3	41.3±2.3	57.3±2.3	80.0±4.0	0.0±0.0
	Pupae	6.7±2.3	13.3±2.3	28.0±4.0	49.3±2.3	73.3±2.3	0.0±0.0
A. pharoensis	Larvae I	32.0±4.0	49.3±2.3	68.0±4.0	86.7±2.3	100.0±0.0	0.0±0.0
	Larvae II	29.3±2.3	41.3±2.3	64.0±4.0	81.3±6.1	97.3±2.3	0.0±0.0
	Larvae III	22.7±2.3	36.0±4.0	56.0±4.0	73.3±2.3	94.7±2.3	0.0±0.0
	Larvae IV	16.0±4.0	32.0±4.0	54.7±4.6	68.0±4.0	92.0±4.0	0.0±0.0
	Pupae	14.7±2.3	21.3±2.3	48.0±4.0	61.3±4.6	84.0±4.0	0.0±0.0

Table 3: Propit values (ppm) of *L. siceraria* leaves aqueous extrct against *C. pipiens* & *A. pharoensis* different stages.

Species	Target	LC_{50}	95% Confidence limits (LCL-UCL)	LC_{90}	95% Confidence limits (LCL-UCL)	χ^2
C. pipiens	Larvae I	101.7	(95.9-143.5)	341.9	(313.3-370.4)	2.28^{Ns}
	Larvae II	131.6	(115.6-147.5)	351.5	(331.2-317.7)	1.11^{Ns}
	Larvae III	169.3	(144.4-194.2)	374.9	(367.4-382.5)	0.57^{Ns}
	Larvae IV	218.4	(196.1-240.7)	414.7	(395.9-433.6)	2.71^{Ns}
	Pupae	267.8	(235.5-300.1)	481.6	(409.4-553.9)	0.47^{Ns}
A. pharoensis	Larvae I	75.2	(35.0-115.7)	332.4	(306.5-358.4)	0.47^{Ns}
	Larvae II	111.2	(89.0-133.4)	344.6	(317.9-371.3)	2.60^{Ns}
	Larvae III	144.6	(122.7-166.6)	360.3	(329.1-391.5)	0.79^{Ns}
	Larvae IV	177.7	(156.1-199.3)	391.8	(385.3-398.3)	2.49^{Ns}
	Pupae	230.4	(209.0-252.0)	454.7	(394.6-514.8)	0.57^{Ns}

Table 4: Propit values (ppm) of *L. siceraria* synthesized silver nanoparticles against *C. pipiens* and *A. pharoensis* different stages.

Species	Target	LC_{50}	95% Confidence limits (LCL-UCL)	LC_{90}	95% Confidence limits (LCL-UCL)	χ^2
C. pipiens	Larvae I	15.2	(13.7-16.6)	36.8	(32.7-40.8)	1.32^{Ns}
	Larvae II	18.2	(15.1-21.3)	40.0	(34.6-45.5)	1.00^{Ns}
	Larvae III	22.5	(16.9-28.1)	44.0	(33.8-54.3)	0.47^{Ns}
	Larvae IV	24.7	(22.9-26.6)	46.5	(40.2-52.9)	0.79^{Ns}
	Pupae	29.4	(26.8-31.9)	50.5	(46.8-54.1)	0.25^{Ns}
A. pharoensis	Larvae I	11.9	(7.8-15.9)	33.0	(31.6-34.4)	2.17^{Ns}
	Larvae II	14.5	(12.3-16.7)	35.1	(31.0-39.3)	0.68^{Ns}
	Larvae III	17.7	(15.8-19.6)	37.7	(36.6-38.7)	0.79^{Ns}
	Larvae IV	19.8	(17.6-21.9)	39.1	(37.8-40.4)	1.96^{Ns}
	Pupae	23.1	(21.8-24.4)	43.2	(40.4-46.0)	0.15^{Ns}

Discussion

In the present study, the average hydrodynamic diameter of *L. siceraria*- synthesized AgNPs calculated by DLS was 33.25 nm which is smaller than those reported by Jamdagni *et al,* (2016) for zinc oxide nanoparticles synthesized using flower extract of *Nyctanthes arbortristis*. Also, AgNPs sizes recorded by TEM were smaller than those reported by DLS due to the presence of nanoparticles aggregates in the test suspension. Variation in the activity of *L. siceraria* aqueous leaves extract and synthesized AgNPs against *C. pipiens* and *A. pharoensis* different immature stages is noted. Mortality increased as the concentration increased. Generally, *A. pharoensis* different immature stages were more affected by *L. siceraria* aqueous extract and synthesized AgNPs than those of *C. pipiens*. In this context, the LC_{50} values of *L. siceraria* aqueous extract against both *C. pipiens* and *A. pharoensis* immature stages is similar to those reported by many authors for several plant extracts against different mosquito species. However, the most interesting aspect in the present study is the large potentiation in the lethal effect when *L. siceraria* aqueous extract used to synthesize AgNPs and this is consistent with earlier report of Benelli, (2016). Arjunan *et al,* (2012) and Subramaniam *et al,* (2015) reported that, the high efficacy of plant synthesized AgNPs in mosquito larval control is due to its ability in permeating the exoskeleton, penetrating into insects' cells, where they restrict macromolecules like proteins and DNA, changing their structure and therefore their function. Also, LC_{50} values of AgNPs recorded in the present study are very low values and may have a little effect on non-target organisms; Haldar *et al,* (2013) and Rawani *et al,* (2013) reported that at least several mosquito species are specially susptible to the mortal effect of AgNPs.

Also, the results of our study confirm the previously reported by Sap-Iam *et al,* (2010) where, 1 ppm of UV irradiation- induced AgNPs decrease the survival of *Aedes aegypti* fourth larval instar by 88%, Jayaseelan *et al,* (2011) where, LC_{50} of AgNPs synthesized using *Tinospora cordifolia* against *Cx. quinquefasciatus* was 6.96 mg/l, Roni *et al,* (2013) where, *Nerium oleander* aqueous extract showed LC_{50} values of 232.90, 273.71, 318.94, 369.96 & 426.01ppm against *A. stephensi* first to fourth instar larvae and pupae; whereas the synthesized AgNPs recorded LC_{50} values of 20.60, 24.90, 28.22, 33.99 & 39.55ppm against same immature stages of *A. stephensi*, Velayutham *et al.* (2016) where, 1mM silver solution synthesized AgNPs recorded LC_{50} and LC_{90} of 3.08, 3.21 & 9.84, 11.24 mg/ml against *Aedes aegypti* and *C. quinquefasciatus* larvae and Morej´on *et al.* (2018) where, toxic activity of AgNPs synthesized using *Ambrosia arborescens* against *Aedes aegypti* larvae (LC_{50} = 0.28ppm; LC_{90} = 0.43 ppm) was higher than those of plant extract (LC_{50} = 1844.61ppm; LC_{90} = 6043.95ppm), respectively.

Conclusion

The present study evaluated the efficacy of *Lagenaria siceraria*- synthesized AgNPs against filarial vector, *Culex pipiens* and malarial vector, *Anopheles pharoensis*. The *L. siceraria*- synthesized AgNPs dispersed uniformly in water and having a significant efficacy against *C. pipiens* and *A. pharoensis* different immature stages. More studies on activity of green-synthesized AgNPs against other mosquito species are ongoing.

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Explanation of figures

Fig. 1: DLS results of AgNPs.

Fig. 2: TEM images of AgNPs.