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Evaluation of larvicidal potential of *Azadirachta indica* (Neem) plant extract and synthesized AgNPs against *Aedes aegypti* L. (Diptera: Culicidae)

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Abstract

Aedes mosquitoes are the most important group of vectors having ability to cause diseases like Chikungunya fever, Dengue fever and Zika virus in human. These vectors can efficiently and ecofriendly be controlled by using green silver nanoparticles (AgNPs). In the present study, leaves extract of *Azadirachta indica* (Neem) and green silver nanoparticles (AgNPs) synthesized from this extract were evaluated as larvicidal agent for 2nd and 3rd instar larvae of the *Aedes aegypti* (main mosquito vectors). UV-Vis spectroscopy, X-ray spectroscopy (XRD), Fourier transform infrared spectroscopy (FT-IR) and scanning electron microscopy (SEM) were used to study the characteristics of green synthesized AgNPs.2nd and 3rd instar larvae of *Aedes aegypti* were kept in the different concentrations (50-250ppm) of plant extract and green AgNPs to calculate the percentage mortality at time intervals of 12, 24, 36 and 48h of exposure. The nanoparticles proved significant toxic for the *Ae. aegypti* larvae (dengue vector) having (LC_{50} =15.94 and LC_{90} =111.82 ppm) compared to the plant extract (102.46 ppm; 251.80 ppm) respectively. Our results suggest the extract of *A. indica* and synthesized nanoparticles as excellent controlling agents for vector mosquitoes instead of pollution causing existing chemical pesticides.

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Introduction

Mosquitoes caused a serious threat to public health (Service, 2004). These vectors are busy in spreading of very dangerous diseases such as dengue, malaria, chikungunya, Zika virus, Japanese encephalitis, filariasis, and leishmaniasis (Remia and Logaswamy, 2010). These diseases cause millions of deaths annually in all countries of the world (Ravikumar and Rahuman, 2011). *Aedes aegypti* is not only the major dengue virus vector but itis also busy in spreading of Zika virus and chikungunya fever.

Pakistan is at the great risk of vector-borne diseases especially dengue because of poor vaccination, packed cities and insufficient sanitation. In Pakistan, dengue cases are reported throughout the year but situation, usually, become worst in the post monsoon period (Jahan, 2011). Pakistan had the worst dengue epidemic in 2011, during which more than 20,000 cases and 300 deaths were reported officially. Chikungunya virus was detected in 1983 (Darwish, 1983) and more than 4000 cases have been confirmed through qualitative RT- PCR. Zika virus has reached near border areas in neighboring countries like China and India, so outbreak of the disease may occur in Pakistan (Rauf *et al.*, 2017). Large numbers of plant extracts have been studied to evaluate the larvicidal efficacy against different species of vector mosquitoes.

These plants extracts, not only, act as effective larvicidal agents but also greatly reduce the risk of environmental pollution without inducing resistance in mosquitoes (Isman, 2006). Larvicidal phytochemicals such as saponins, isoflavonoids, tannins, terpenes and steroids are found in the medicinal plants. These plants have played a key role in the protection of human health because these phytochemicals are target specific, rapidly biodegradable, less toxic to human health (Zhu *et al.*, 2008; Ghosh *et al.*, 2012) but can kill mosquito larvae with high mortality rate (KhairulBariyah *et al.*, 2012; Mdoe *et al.*, 2014; Pavela, 2016) by bringing changes in development, midgut epithelium (Al- Mekhlafi, 2018), mutation in DNA and production of reactive oxygen species (Arjunan *et al.*, 2012; Subramaniam *et al.*, 2015) Biologists have started the use of these phytochemicals to control the mosquitoes (Eliman *et al.*, 2012). Green silver nanoparticles (AgNPs) synthesized from these phytochemicals are proved more toxic than Phytochemical as larvicides ((Bilal and Hassan, 2012). Because of small size ranging from 1–100 nm and large surface area of AgNPs made them unique larvicidal agents at very low concentrations (Borase *et al.*, 2013; Muthukumaran *et al.*, 2015). *Azadirachta indica* (Neem) plant belongs to a big family Meliaceae. Medicinal products of neem tree have been used as antibacterial, contraceptive, antifungal, antiviral, sedative, antidiabetic and skin diseases like eczema and psoriasis (Sergio *et al.*, 2007). This study evaluated the larvicidal activity of the acetone extract of *A.indica* (Neem) and green AgNPs of these plantagainst 2nd and 3rd larval stages of *Ae. aegypti*.

Materials and methods

Extraction of Acetone plant extract

Healthy and fresh leaves of the *A. indica* (Neem) plant were collected (hand plucked) from the Government College University, Faisalabad, Pakistan, during the month of May, 2017 and identified by a taxonomist. Leaves were washed several times to remove dust. Leaves were kept in shady place at room temperature to make dry. Dried leaves were grinded in an electric grinder (Anex Germany). Leaf extract was made by adopting the standard simplex centroid experiment design method adopted by Satyavani *et al.* (2011) with slight changes. Fifty grams powder of leaves along with acetoneloaded in the inner tube of the Soxhlet apparatus boiled gently at boiling point range 55.5-56.50C for 8hrs. The collected plant extract was kept at 4°C for further use (Vogel, 1978).

Preparation of Green AgNPs

Silver nitrate ($AgNO_3$) purchased from Pakistan scientific store in Faisalabad Pakistan. 1mM solution of silver nitrate ($AgNO_3$) was prepared in 250 ml Erlenmeyer flask in the darkness to avoid action of light. 10 ml acetone plant extracts of *A. indica* (Neem) was put in 250 ml conical flask having 90 ml of 1mM

silver nitrate solution. Two to three drops of 1% NaOH were added for the adjustment of pH at 8 and mixed continuously by magnetic stirrer (Sathyavathi *et al.*, 2010). UV-Vis spectroscopy, X-ray spectroscopy (XRD), Fourier transform infrared spectroscopy (FT-IR) and scanning electron microscopy (SEM) were used to study the characteristics of green synthesized AgNPs.

Collection and Rearing of Mosquitoes

Larvae and pupae were collected from both indoor and outdoor breeding sites by using aquatic net and dipper from Faisalabad district Punjab, Pakistan (31° 25' 7.3740" N and 73° 4' 44.7924" E, 192 meters above the sea level) during 2017. Specimens were brought back to the Zoology Lab, Department of Zoology, Government College University, Faisalabad, inside beakers closed with muslin cloth. Identification was made with the help of identification keys (Qasim *et al.*, 2014) and reared to adults in 1000 ml beakers containing water under ideal conditions at 27±2˚C and 75±3% relative humidity (Ahmed *et al.*, 2017). Adults further reared in separate glass cages. Male adult were fed with 10% sugar solution and females with blood on live white rats in separate glass cages for egg laying. Larvae emerged from the eggs were reared in batches of 300 each, in 1200 ml deionized water in stainless steel trays (35x30x5 cm) for the bioassays.

Two drops of 10% sugar and a yeast suspension of 0.02% was given to each batch daily for first instars and then with finely ground fish food up to the development of 3rd instars larvae (Arivoli *et al.*, 2012).

Bioassay

Groups of 20 actively swimming 2nd and 3rd instars larvae of *Ae. aegypti* were released in 250ml beakers containing 200ml distilled water separately (for both species and larval stage). Five concentrations viz., 50, 100, 150, 200 and 250 ppm of larvicidal solution of *A.indica* extract and green AgNPs synthesized from the extract were tested for their larvicidal activity separately.

The distilled water was used as control. Five replications were done for each treatment. Mortality rates were calculated using the WHO (2006) bioassay protocol with slight modifications.

Statistical analysis

The percentage mortality data was subjected to probit analysis using Minitab -17 statistical software (2017) for calculating lethal concentration of 50% (LC_{50}) and 90% (LC_{90}) of larvae and for getting dose and time mortality regression lines.

Results

UV-Vis spectrum of silver nanoparticles

Synthesis of AgNPs due to reduction of Ag ions by the phytochemicals of *A.indica* (Neem) turned the colour of mixture reddish brown. The visible colour change indicated the formation of green AgNPs and was confirmed by UV-Visible absorption spectroscopy.

Table 1. Larvicidal activity of AgNPs synthesized from *A. indica* against *Aedes aegypti* larvae.

Exposure Time	Larval Instars	Lethal Concentration		95%Confidance limits		P value	Regression equation
				LFL	UFL		
12H	2nd	LC_{50}	113.29	211.68	253.25	0.410	Y= -1.80+0.072X
		LC_{90}	280.62	339.71	443.70		
	3rd	LC_{50}	170.96	244.79	312.09	0.342	Y= -1.99+0.073X
		LC_{90}	245.03	385.68	545.74		
24H	2nd	LC_{50}	78.75	155.98	176.02	0.001	Y=-2.163+0.013X
		LC_{90}	163.94	246.82	286.33		
	3rd	LC_{50}	166.58	153.47	180.68	0.042	Y= -1.50+0.090X
		LC_{90}	108.46	280.41	348.89		
		LC_{50}	12.578	25.828	35.886	0.438	Y=-0.913+0.014X

36H	2nd	LC_{90}	129.24	114.07	149.40		
	3rd	LC_{50}	84.956	71.661	96.057	0.043	Y= -1.06+0.012X
		LC_{90}	184.23	169.85	203.48		
48H	2nd	LC_{50}	11.184	17.33	72.698	0.097	Y= -0.153+0.133X
		LC_{90}	89.530	73.192	107.53		
	3rd	LC_{50}	27.267	0.0632	44.583	0.05	Y= -0.384+0.122X
		LC_{90}	118.43	105.52	136.03		
Control		0	0	0	0	0	-

LC_{50}=Lethal concentration 50 at which 50% of target population died.

LC_{90}= Lethal concentration 90at which 90% of target population died.

LFL = Lower fiducial limit UFC = Upper fiducial limit. P value = Level of significance p≤ 0.05.

Fourier Transform Infrared Radiation Spectroscopy (FTIR) Analysis

The FTIR spectra of AgNPs synthesized from the *A. indica* (Neem) leaf extract (Fig. 3) showed peaks at 1264.3, 977.1, 850.8, 709.7, 662.6, 503.5, and 436.8/cm.The carbonyl groups and amino acids which capped the silver nanoparticles indicated by these peaks.

Table 2. Larvicidal activity of Leaf extracts of *A. indica* (neem) against *Aedes aegypti* larvae.

Exposure Time	Larval	Lethal conc.(ppm)		95% Confidance limits		P value	Regression equation
				LFL	UFL		
12H	2nd	LC_{50}	432.30	340.72	697.30	0.090	Y= -1.86+ 0.043X
		LC_{90}	729.06	541.03	1286.5		
	3rd	LC_{50}	503.88	377.33	1004.9	0.030	Y= -2.45+ 0.030X
		LC_{90}	784.41	553.99	1711.7		
24H	2nd	LC_{50}	420.44	333.01	667.01	0.089	Y= -1.76+ 0.042X
		LC_{90}	725.17	540.01	1261.4		
	3rd	LC_{50}	420.59	334.05	662.87	0.066	Y= -1.87+ 0.043X
		LC_{90}	714.51	534.09	1232.8		
36H	2nd	LC_{50}	249.47	226.11	284.86	0.051	Y= -1.76+ 0.070X
		LC_{90}	430.90	374.12	526.21		
	3rd	LC_{50}	283.12	250.17	340.32	0.092	Y= -1.71+ 0.060X
		LC_{90}	494.65	415.46	642.40		
48H	2nd	LC_{50}	110.36	96.349	122.58	0.001	Y= -1.130+ 0.010X
		LC_{90}	235.45	216.21	261.88		
	3rd	LC_{50}	172.05	159.77	185.43	0.048	Y= -1.68+ 0.1982X
		LC_{90}	302.66	277.18	338.48		
Control			0	0	0	0	-

LC_{50}=Lethal concentration 50 at which 50% of target population died.

LC_{90}= Lethal concentration 90at which 90% of target population died.

LFL = Lower fiducial limit UFC = Upper fiducial limit. P value = Level of significance p≤ 0.05.

These residues prevent from agglomeration of AgNPs, and made the medium stable. Result of FTIR clearly indicated the role of proteins and other compounds of leaf extract in the formation and stabilization of AgNPs. SEM image of AgNPs synthesized from *Azadirachta indica* confirmed the spherical shape with ovoid tendency and 25 nm approximately size (Fig. 1).

Larvicidal Activity of Leaf Extracts and Synthesized Silver Nanoparticles

Results of 2the larvicidal activity of leaf extracts of *A.*

indica (Neem) and AgNPs of this plant with varying concentrations of 50-250 ppm against 2nd and 3rd instar larvae of *Ae.aegypti* after 12, 24, 36 and 48 hrs exposure times are presented in Tables 1-2. Both the aqueous extract and the plant synthesized AgNPs showed a dose and time-dependent toxic effect against the larvae. No mortality was observed in the control groups.

AgNPs synthesized from *A.indica* (Neem) showed 100% mortality for all the exposed larvae within 48 hours at the concentration of 250ppm (Fig. 3).

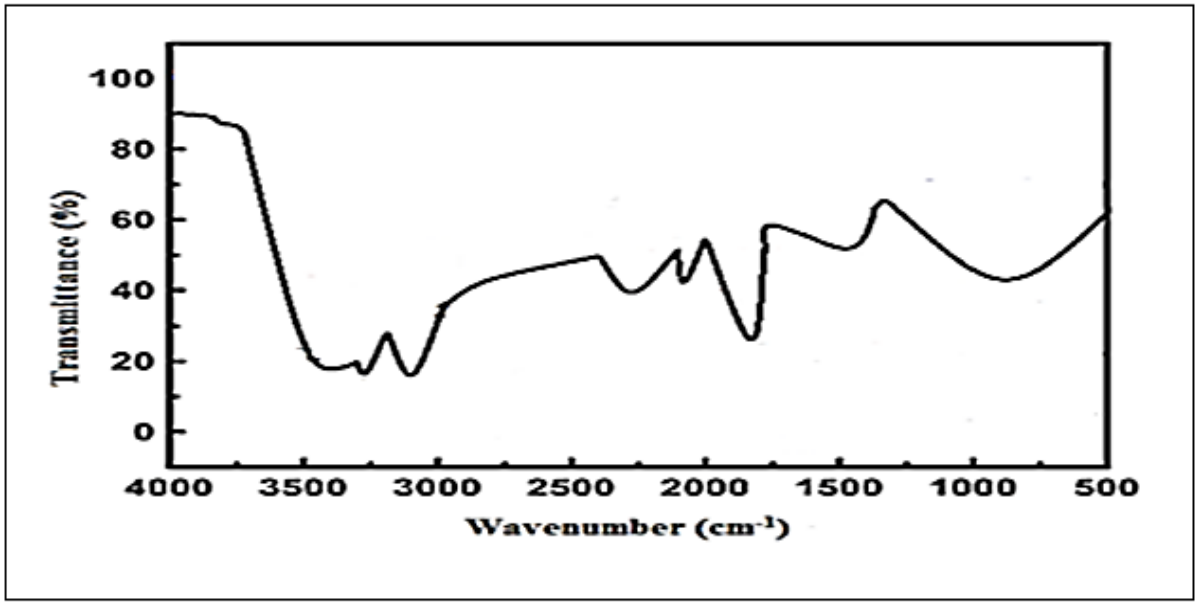

Fig. 1. FTIR spectra of AgNPs synthesized from leaf extract of *A.indica* (Neem).

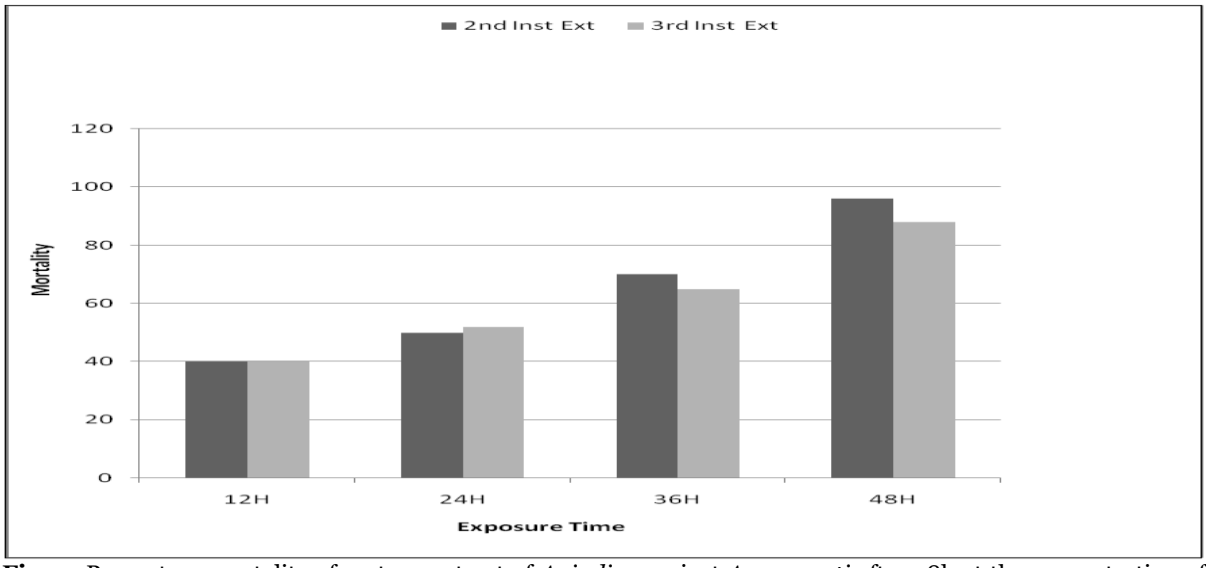

Fig. 2. Percentage mortality of acetone extract of *A. indica* against *Ae. aegypti* after 48h at the concentration of 250 ppm.

At the same conditions LC_{50}, LC_{90} values, 95% lower and upper confidence limit of LC_{50}, LC_{90} (LCL- UCL) and regression equation of synthesized AgNPs were 11.18,89.53 ppm, (17.33 – 72.69) and (73.19 – 107.53) ppm and Y= -0.153+ 0.133X for 2nd instar *Ae. aegypti* larvae while for the 3rd instar larvae these values were calculated as: 27.26, 118.43 ppm, and (0.63 - 44.58), (105.52 – 136.03) ppm and Y= -0.384+ 0.122X (Table 1). At the concentration of 250ppm of leaves extract of *A.indica*, mortality rate for *Ae.aegypti* was 96% for

2nd and 88% for 3rdinstar larvae respectively (Fig 2). The values of LC_{50} and $LC_{90}$of leaf extract at the same conditions of concentration and time were, 110.36, 235.45 ppm for 2ndinstar while 172.05, 302.66 ppm for 3rdinstar larvae respectively. The 95% lower and upper confidence limit of LC_{50} and LC_{90} (LCL-UCL) against 2ndinstar larvae were (96.34 – 122.58) and (216.21 – 261.88) ppm respectively and regression equation was Y= -01.130+ 0.010X. While for the 3rdinstar larvae the values of 95% lower and upper confidence limit of LC_{50} and LC_{90} (LCL-UCL) were (159.77-185.43) and (277.18- 338.48) ppm respectively with regression equation Y= -1.685+ 0.1989X. (Table 2) The liquid extract of *A. indicus* exhibited prominent larvicidal potential against the 2nd instar larvae of the *Ae. Aegypti* than3rdinstar larvae.

Discussion

Reduction of silver ions by the phytochemicals found in the leaf extract involved in the formation of silver nanoparticles (AgNPs). Results of FTIR confirmed that biofabrication occurred by different functional groups found in the leaf extract to form green AgNPs. In our study, the AgNPs of *A.indica* showed 100% mortality for *Ae.aegypti* after 36h, while LC_{50} value of the leaf extract of *A. indica* and AgNPs synthesized from this extract after 48h exposure to *Ae. aegypti* was calculated as: 110.36 ppm and 11.18 ppm respectively.

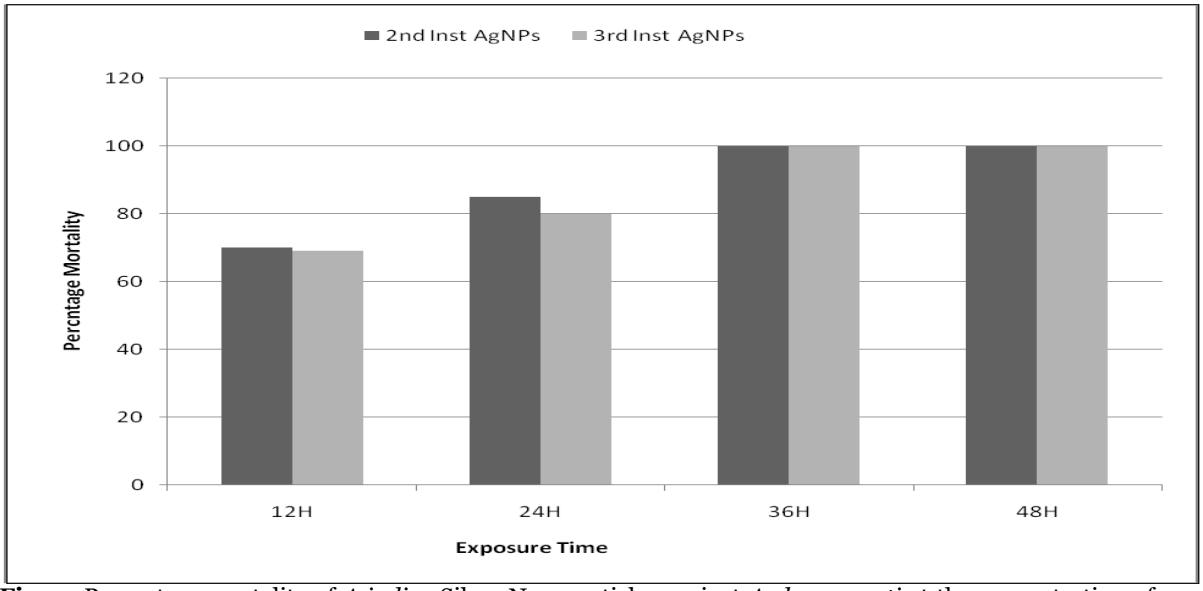

Fig. 3. Percentage mortality of *A.indica* Silver Nanoparticles against *Aedes aegypti* at the concentration of 250 ppm.

The results of present study are comparable with previous reports in the literature. Azhari *et al.*, 2012 investigated the larvicidal potential of different solvent *viz.*, acetone, chloroform and ethanol extracts from different parts such as bark, leaf, root, and seed of*A. indica* (Family: Meliaceae) against *Ae. aegypti* larvae reported the LC_{50} value from 50 to 837.5 ppm and LC_{90} value 94 to 950 ppm respectively.Virendra (2009) evaluated LC_{50} of oil *A. indica* formulation against *Anopheles stephensi, Culex quinquefasciatus* and *Aedes aegypti* as: 1.6, 1.8 and 1.7 ppm respectively. In another report the LC_{50}= 3.99 and LC_{90}=10.92 of AgNPs of leaf extract of *A.indica*against 3rd larval stage of *Aedes aegypti*, while LC_{50} (4.35) and LC_{90} (11.88) mg/L was calculated for the 4th instar larvae (Busi *et al.*, 2015). AgNPs of *Helitropium indicum* showed the showed the larvicidal potential with LC_{50} value of 20.10 µg/ml 3rd instar larvae of *Ae. aegypti* (Veerakumar, 2016). AgNPs synthesized from the neem leaves extract having varying concentrations (0.07-25 mg/l) for 24hrs showed the LC_{50} and LC_{90} values of 0.006 and 0.04 mg/l for *Ae. aegypti*(Poopathi, 2014). Cited results are close to our finding but not exactly same

due to using different plant, mosquito species, larval stage, concentration and different types of solvent for plant extraction.Results of our study strongly suggested that the leaf extract of *A. indica* is toxic to*Ae.aegypti* larvae and toxicity increased when extract combined with AgNPs. Both extract and synthesized AgNPs of *A.indica* showed more toxicity for 2nd instar larvae of*Ae. aegypti* than 3rd larval stage. Our results clearly proved the excellent larvicidal efficacy of *A.indica* against *A.aegypti* (dengue vector).

Conclusion

It is concluded from this study that, both the leaf extract and silver nanoparticles (AgNPs) of *A.indica* poses significant larvicidal potential for 2nd and 3rd larval stages of *Ae. aegypti*. These green synthesized nanoparticles (AgNPs) have high larvicidal activity and can be used in the formation of bionanopesticides. So, the population of vector mosquito eco-friendly be controlled by applying this plant extract along with synthesized green silver nanoparticle on mosquito breeding places.

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