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Mosquito larvicidal and antimicrobial activities of synthesized silver nanoparticles (AgNP) using mature fruit extract of *Cestrum diurnum* L.

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Silver nanoparticles (AgNP) were prepared using mature fruit extract of *Cestrum diurnum* L. (family: Solanaceae) as a reducing agent. The stabilized AgNPs were characterized by time-dependent UV–Vis Spectrophotometric analysis. The spherical/oval shape of the nanoparticle was confirmed by Transmission Electron Microscopy analysis with an average particle size of about 50 nm. The crystalline nature of the AgNPs was confirmed in the XRD spectrum by the characteristic Bragg peaks. Fourier Transform Infrared Spectroscopic analysis of AgNPs confirms the presence of several functional groups. AgNPs showed effective larvicidal activities against larval instars of *Anopheles stephensi* and *Culex quinquefasciatus* mosquitoes in the laboratory bioassay. Synthesized AgNPs also showed good antibacterial activity against some fish pathogenic and human pathogenic bacteria which is evident from the inhibition zone diameter in the antibacterial bioassay experiment.

Keywords: Antimicrobial activity, *Cestrum diurnum* L., Larvicidal activity, Silver nanoparticles.
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Introduction

Mosquito-borne diseases are a serious threat to modern civilization in terms of mortality, morbidity and economic loss. Mosquitoes are now regarded as "Public enemy No 1" according to World Health Organization. Among various types of mosquito-borne diseases, Filariasis, Malaria and Dengue fever are most common. Filariasis is caused by *Wuchereria* species and is mainly transmitted by *Culex quinquefasciatus* in tropical countries. It is estimated that about 120 million people in 83 countries of the world are infected with Lymphatic Filariasis parasites. In India, it is found that approximately about 21 million people have symptomatic filariasis and 27 million are microfilaria carriers[1]. The principal vector of malaria in Tropics is *Anopheles stephensi* carrying *Plasmodium* genus (protozoa) as a causative organism. According to WHO's World Malaria Report (2019), approximately 219 million cases of malaria occur in the world and the disease killed about 4,35,000 people in the year 2017. According to National Health Report, in India, the total number of malarial cases reported was 842095 with 104 deaths in 2017.

Microbial diseases create a threat to humans and other organisms. *Staphylococcus aureus* is a Gram-positive coccal bacterium, generally found in our respiratory tract and skin and it causes skin infections, respiratory disease, and food poisoning[2-3]. *Bacillus subtilis* is a Gram-positive, rod-shaped bacteria, commonly found in soil. But it is also found in the human body, mostly on the skin or in the intestinal tract[4], and can cause allergic reactions[5]. *Escherichia coli* is the common intestinal bacterium that can cause diarrhoea[6]. *Pseudomonas aeruginosa* infections of the blood, pneumonia and infections following surgery in hospitalized people can lead to severe illness and death[7]. People may also develop mild illnesses with ear infections, especially in children, and skin rashes after exposure to inadequately chlorinated swimming pools having *P. aeruginosa* contamination[8]. Among the fish bacteria, *Aeromonas salmonicida* can cause furunculosis, which can produce septicemia, haemorrhages, muscle lesions, inflammation of the lower intestine, spleen enlargement, and may cause death in freshwater fish populations[9]. In a study, *Pseudomonas putida* was observed to cause ulceration on the dorsal side of fish[10].

The field of nanotechnology has emerged as a very prospective area of research in pharmaceutical, industrial, and biotechnological science[11-15]. Green

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synthesis is advantageous over the chemical and physical methods of synthesis of nanoparticle as it is cost-effective, eco-friendly and do not involve any toxic material. The plant extract used during green synthesis act as both reducing and stabilizing agent[16].

Cestrum diurnum L. (family: Solanaceae), commonly called Day Jasmine, is a shrub having cosmopolitan distribution. The fruit of the plant is an oval berry, greenish and ripening through violet to shiny blue-black in colour. There are several traditional applications of this plant in folk remedies[17]. The antimicrobial[18] and the larvicidal property[19] of this plant have been recorded earlier.

At present in mosquito control programs, synthetic chemical insecticides are generally used. But over and injudicious application of chemical insecticides for a long time leads to environmental pollution, affects human health, and results in the appearance of physiological resistance in vector species against commonly used chemical insecticides. The present situation demands the application of a novel eco-friendly strategy to manage the vector population. The use of plant-mediated fabrication of metallic nanoparticles may be a suitable alternative approach to reduce the vector population and resurgence of vector-borne diseases. The plant-based nanoparticles are non-toxic, target-specific, ecofriendly, biodegradable, easy to produce, and effective in low doses. Plant-based nanoparticles combine the microbicide activities of silver particles and the mosquitocidal properties of plant extract and a remarkable biocontrol potentiality are achieved due to the favourable surface area to volume ratio as the nanoparticles are very small in size. The objective of this study was the green synthesis of silver nanoparticles by mature fruit extract of *C. diurnum*, physical and chemical characterization of the synthesized nanoparticles, and analyze the larvicidal activity and antimicrobial efficacies of the AgNPs in laboratory conditions.

Materials and Methods

Plant collection and identification

Mature green fruits of *C. diurnum* were harvested randomly from plants growing at the outskirts of Burdwan University campus, Golapbag (23°16'N, 87°54'E) in the months of August and September 2019. The fruits were identified in the Department of Botany [Ref No of Letter: BU/DMC/2019/01 (03)b], Burdwan University and a voucher specimen (GC-CESD/2019/21) is kept in the Department of Zoology, Burdwan University.

Collection of larvae

The raft of *Cx. quinquefasciatus* eggs was collected from cemented drains surrounding the Burdwan University campus. After hatching, the 1st instar larvae were fed with a small amount of flour. *An. stephensi* larvae were collected from underground and overhead tanks of the Kolkata metropolis and carried to the laboratory. The 2nd and 3rd instar larvae of both the species were properly identified with available keys and were used in the experiment.

Synthesis of silver nanoparticles

After collection from the field, fruits of *C. diurnum* were thoroughly washed in the laboratory by distilled water to remove any impurities or dust particles present on them. After washing and drying, 10 g of fruits were taken in sterile filter paper and air-dried for a week. The dried fruits were then taken in a grinder and crushed. The crushed fruits were then taken into a 500 mL beaker containing 100 mL of double distilled water and boiled for 10 minutes on a hot plate to prepare the aqueous extract. The extract was then filtered with sterile Whatman filter paper No. 42. The filtrate was used for the green synthesis of silver nanoparticles that can reduce any silver-containing salt from Ag+ to Ag^0. The prepared fruit extract was taken in a 500 mL beaker and treated with aqueous 10^{-3} M $AgNO_3$ solution (10: 1, v/v), boiled at 60 °C for 20 minutes and then kept undisturbed. The change of colour takes place within a few hours (from colourless to reddish-brown). The final nano-colloidal solution was subjected to centrifugation (twice) at 10,000 rpm for 20 minutes in a Remi Research Centrifuge instrument to get rid of any un-interacted biological molecules. Then the pellet was collected, dried in a rotary evaporator, and stored in desiccators for future use. Different test concentrations (1.25, 2.5, 5, 7.5, and 10 ppm) of synthesized NPs were prepared by mixing distilled water according to a suitable methodology[15].

Mosquito larvicidal bioassay

The toxicity of biologically synthesized nanoparticles on mosquito larvae was evaluated according to WHO methodologies[20]. With each tested concentration prepared earlier, larvicidal activity was measured as a mean value of four trials having one control set up where only distilled water was used. Randomly, twenty 2nd and 3rd instar larvae of *An. stephensi* and *Cx. quinquefasciatus* were placed into 200 mL of each tested concentration along with control set up and kept in an environmental chamber

at 27 °C with a photoperiod of 16:8-h light/dark cycle. The larval mortalities were recorded at 24 hours of exposure. The numbers of dead larvae were counted and the percentage of mortality was also recorded. The data of larval mortality were corrected according to mortality in control as recommended in Abbott's formula[21].

Test microorganisms

During the laboratory bioassay, to test the antibacterial potentiality of the silver nanoparticles, four human pathogenic bacterial strains, viz. *Staphylococcus aureus* MTCC 2940, *Escherichia coli* MTCC 739 *Bacillus subtilis* MTCC 441and *Pseudomonas aeruginosa* MTCC 2453 and five fish pathogenic bacterial strains namely *Bacillus licheniformis* MTCC 530, *Pseudomonas fluorescens* MTCC 103, *Aeromonas salmonicida* MTCC 1945, *Pseudomonas putida* MTCC 1654, and *Bacillus mycoides* were used. All the tested strains were reference strains and were collected from the Microbiology Laboratory of Burdwan Medical College. The bacterial cultures were maintained in nutrient broth (Himedia, M002) at 37 °C and maintained on nutrient agar (Himedia, MM012) slants at 4 °C.

Antibacterial assay by Disc diffusion method

Antibiogram was done by Disc diffusion method[22] using dry silver nanoparticles. Depending upon the solubility of the silver nanoparticles, the test quantity of nanoparticles were dissolved in distilled water/dimethylsulphoxide (DMSO). The dissolution of the organic extracts was aided by 1% (v/v) DMSO and that of the aqueous extracts with distilled water, similar to the control experiments carried out in the laboratory. The surfaces of the culture media of each petriplate were inoculated with bacteria. After 24 h of incubation, respective concentrations of the silver nanoparticles were transferred to each of the petriplates creating small holes (6 mm in diameter) within the plates. The plates were examined after 24 h and the diameters of the inhibition zones (IZD) were measured to the nearest millimeter[23].

Toxicity test on non-target organism

To study the effect of dry nanoparticles synthesized by *C. diurnum* different non-target organisms like *Toxorhynchites* larvae (mosquito predator), *Diplonychus annulatum* (predatory water-bug), and *Chironomus circumdatus* larvae (chironomid) were selected. All the non-target organisms share the common habitats of mosquito larvae in aquatic bodies. The non-target organisms were exposed to appropriate lethal concentrations (similar to mosquito larvae at 24 h) up to 72 h to observe any mortality or abnormalities like sluggishness and reduced swimming activity.

Characterization of AgNPs

The formation of silver nanoparticles was characterized through UV-Vis spectrophotometer study. The instrument was operated at 1 nm resolution having an optical length of 10 mm within 190–700 nm wavelength range for the time duration of 300 sec.

The crystalline nature of the synthesized nanoparticles, x-ray-diffraction (XRD) studies were analyzed through Siemens X-Ray diffractometer (Japan), operated at 30 kV and 20 mA current with CuKα (I=1.54A°). Films of colloidal form AgNP were tested by drop coating on Si (III) substrates and data were recorded at a scanning range between 10^o to 80^o with a scan rate of 1.5°/min.

Technai-20 Philips instrument operated at 200 kV and beam current of 104.1 μA was used for transmission electron microscopy (TEM) images of nanoparticles. The dried AgNPs were placed on a carbon-coated copper grid (300 mesh size) by slow evaporation and then dried in a vacuum at 25 °C for overnight[14].

For FTIR studies, the plant extract containing AgNP was prepared by centrifuging the phytosynthesized AgNP solution at 10,000 rpm for 20 min. The solid residue obtained was thoroughly washed with deionized water to remove any unattached biological moieties to the surface of the synthesized nanoparticles. The residue was then dried completely in a vacuum evaporator and properly lyophilized through a rotary evaporator. The powder obtained was used for FTIR measurements by FTIR spectrometer (Perkin Elmer Lx10 – 8873). The scanning range was 40 to 4000 cm^{-1} at a resolution of 4 cm^{-1}.

Statistical analysis

The data on the larvicidal efficacy were subjected to probit analysis[24]. By using the computer software "STAT PLUS 2009 (Trial version)" and MS EXCEL 2003; the corresponding LC_{50} and LC_{90} values, regression equations (Y = mortality; X = concentrations) and regression coefficient values were calculated.

Results

Characterization of nanoparticles

The change in colour of the reaction mixture from colourless to reddish-brown within a few hours indicates the formation of AgNP. The characteristic surface plasmon absorption spectral band showed at

(λ_{max}) 450 nm indicates the presence of Ag particle. The absorbance of AgNP solution was noted after 1,2, 3, 4, 5, 6, 7, and 8 h (Fig. 1).

The TEM study reveals that most of the nano-crystals formed are spherical (or oval) in shape. They maintain their individual identity. The average size of the nanoparticles was about 50 nm in diameter. Fig. 2a,b shows a representative TEM photograph of the synthesized AgNP from dried fruits of *C. diurnum*.

The XRD pattern of the phyto reduced Ag NPs showed similarity with the literature values of face-centered-cubic (fcc) crystal structure of Silver. Fig. 3 showed the XRD pattern observed from the synthesized nanoparticles of dried fruits of *C. diurnum*. The peaks at respective 2θ values could be assigned to the four main facets known for zero-valent fcc silver crystal planes due to Bragg's reflections. A few unassigned peaks were also noticed. From the XRD results, it was also found that crystallization of the bio-organic phase occurs on the surface of the AgNPs[25].

Fourier transform infrared spectroscopy (FTIR) was used to verify that silver nanoparticles were coated with *C. diurnum* fruit extract. The fruit extract during synthesis of AgNP acted as a reducing as well as a stabilizing agent. FTIR absorption spectra of the AgNPs of the fruit extract is shown in Fig. 4. The interpretation of the data and the presence of some active functional groups are presented in Table 1.

Mosquito larvicidal bioassay study

The pellet of AgNPs prepared by using dried fruits of *C. diurnum* was subjected to mosquito larvicidal bioassay on 2nd and 3rd instars larvae of *Cx. quinquefasciatus* and *An. stephensi*. The rate of mortality of *Cx. quinquefasciatus* larvae and *An. Stephensi* larvae are expressed in Table 2. A 100% mortality was observed in 10 ppm concentration in 2nd and 3rd larval instar of *An. stephensi* while 95 and 100% mortality were recorded in 2nd and 3rd instar larvae of *Cx. quinquefasciatus* after 24 hours of exposure.

In Table 3, the corresponding LC_{50} and LC_{90} values and associated regression equations are given. The results of regression analysis showed clear dose-dependent mortality, which is evident from the positive correlation between the rate of mortality (Y) and the concentration (X) of the AgNP.

Antimicrobial study

The AgNPs were also subjected to antibacterial assay and the corresponding results are shown in Table 4. A concentration of 10 mg/disc was used during the bacterial assay because in the selected concentration promising bacteriocidal activity of

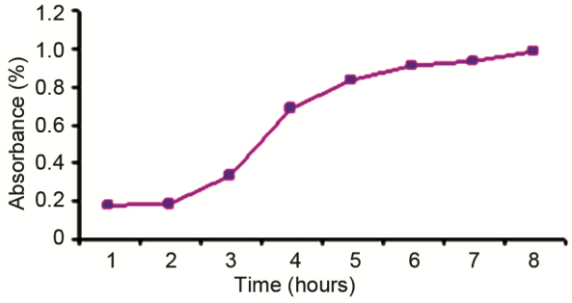

Fig. 1 — Time dependent absorption spectrum of synthesized AgNP.

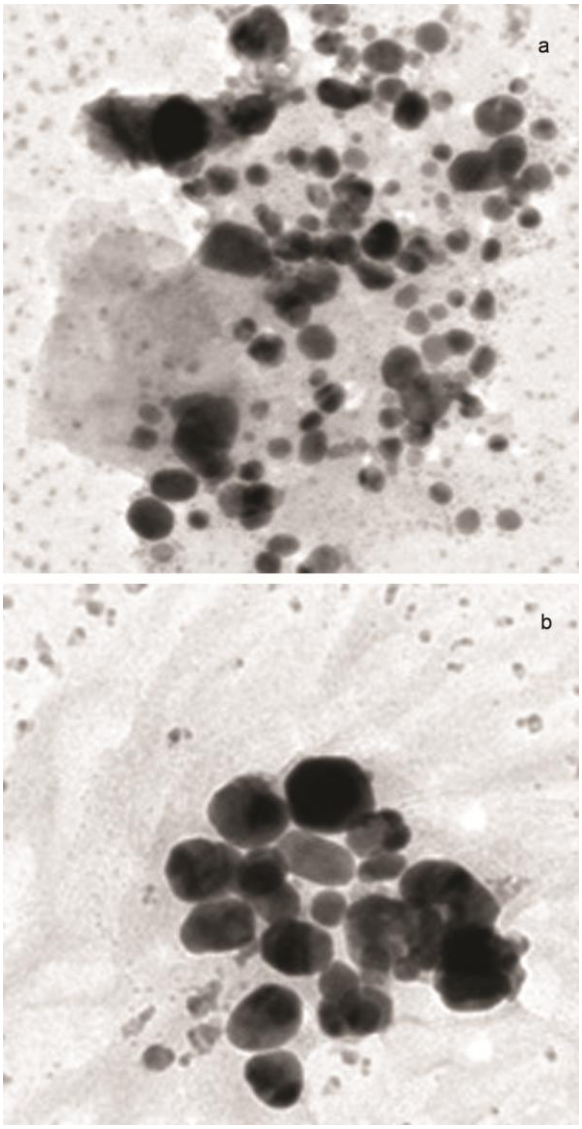

Fig. 2a,b — TEM image of synthesized AgNP.

AgNPs were recorded. In presence of the human pathogenic bacteria, AgNPs showed better antibacterial activity (with reference to IZD) against *S. aureus* and *B. subtilis*. However, bacterial strains such as *E. coli* and *P. aeruginosa* were unaffected. The AgNPs were effective against all the fish pathogenic bacteria used during antibacterial bioassay. The highest zone of inhibition appeared in *P. putida* bacterial strain.

Effect on non-target organisms

The AgNPs synthesized by *C. diurnum* showed no toxicity against non-target organisms viz. *Toxorhynchites* larvae, *Diplonychus annulatum*, and *Chironomus circumdatus* in the selected concentration.

Discussion

The colour changed from colourless to reddish-brown when *C. diurnum* dried fruit extract was added to $AgNO_3$, showing that Ag^+ was reduced to Ag^o and AgNPs were formed. A time-dependent absorption spectrum of synthesized AgNP showed that the formation of AgNPs began within 3 hours of addition of *C. diurnum* fruit extract to $AgNO_3$ solution. TEM analysis reported the spherical or oval shape of the synthesized AgNPs and the size of the nanoparticles were about 50 nm in diameters. In some earlier studies, similar observations are recorded[12-13]. XRD analysis of the AgNPS suggests that crystallization of the bio-organic phase occurs on the surface of the

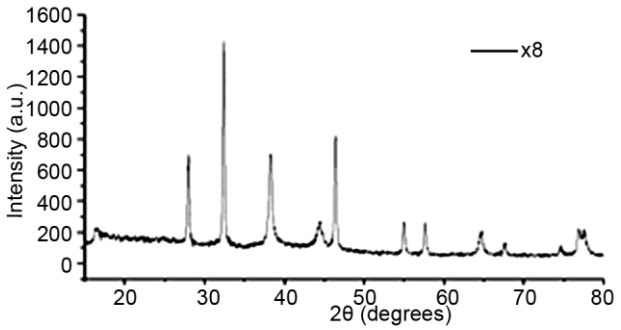

Fig. 3 — XRD image of synthesized AgNP.

Table 1 — FTIR absorption spectra and probable functional group of AgNPs of *C. diurnum* fruit extract

Frequency (cm^{-1})	Probable functional group
3439	Amides/Phenolics
2386	Phosphate compounds (Phosphine)
1718	Ketones/Carboxylic acids/Esters
1621	Alkenes/Amides
1518	Nitro compounds
1439	Aromatic compounds
1385	Alkanes/Nitro compounds
592	Alkyl compounds

Table 2 — Larvicidal activity of AgNP against 2nd and 3rd instars larvae of *Cx. quinquefasciatus* and *An. stephensi.*

Species of mosquito	Dose (ppm)	Percent mortality (mean±SD)	
		2nd instar	3rd instar
Cx. quiquefasciatus	1.25	41.65±0.33	50±0.58
	2.5	55±0.58	65±0.58
	5	71.65±1.20	78.35±0.88
	7.5	75±0.58	85±0.58
	10	95±0.58	100±0.00
An. stephensi	1.25	45±0.58	51.65±0.88
	2.5	76.65±0.67	83.35±0.33
	5	90±0.58	93.35±0.33
	7.5	98.35±0.33	95±0.58
	10	100±0.00	100±0.00

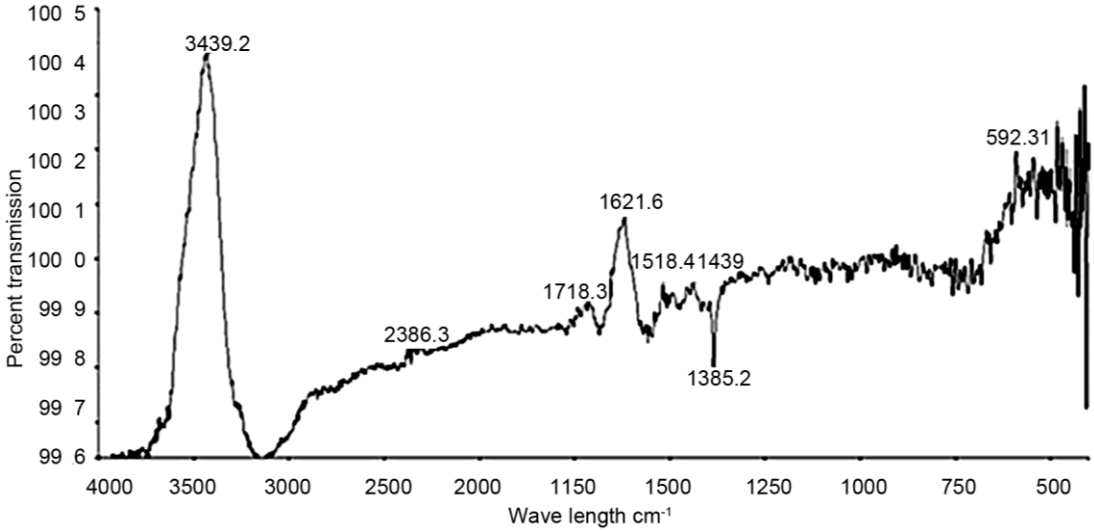

Fig. 4 — FTIR absorption spectra of AgNPs synthesized by mature fruit extract of *C. diurnum*.

Table 3 — Lethal concentrations and Regression analysis of the larvicidal activity of AgNP against 2nd and 3rd instars larvae of *Cx. quinquefasciatus* and *An. stephensi*

Species of mosquito	Larval instars	LC_{50} (ppm)	LC_{90} (ppm)	Regression equations	R value
Cx. quiquefasciatus	2nd	1.93	12.63	Y= 1.19x + 7.71	0.94
	3rd	1.41	7.69	Y= 1.05x + 9.64	0.95
An. stephensi	2nd	1.40	4.13	Y= 1.10x + 10.60	0.85
	3rd	1.13	3.92	Y= 0.89x + 12.23	0.81

Table 4 — Antibacterial activity of specific concentration (10 mg/disc) of synthesized AgNP in disc diffusion assay

Bacterial strain	Diameter of the inhibitory zones (mm)
Human pathogen	
S. aureus MTCC 2940	16±0.58
B. subtilis MTCC 441	11±0.58
E. coli MTCC 739	—
P. aeruginosa MTCC 2453	—
Fish pathogen	
P. fluorescens MTCC 103	15.33±0.33
B. licheniformis MTCC 530	16±0.58
A. salmonicida MTCC 1945	13±0.58
P. putida MTCC 1654	23±0.58
B. mycoides (Clinical isolates)	14±0.58

silver nanoparticles which is also evidenced from FTIR analysis. The FTIR spectra of AgNPs showed peak characteristics of the available functionalities that facilitated the reduction of the silver ions to form silver nanoparticles. The characteristic functional group includes amides, carbonyl, aliphatic and aromatic compounds, and nitro compounds that are capable of reducing the silver ions as well as stabilization of the silver nanoparticles.

The larvicidal activity of the phyto-synthesized metallic nanoparticles is well established. Aqueous leaf extract of *Hibiscus rosasinensis* is reported against the larvae of *Aedes albopictus* mosquito[26], nanoparticles from fungus are reported to have larvicidal activity against *An. stephensi*[27]. Larvicidal activities of the synthesized AgNPs from fresh leaves, dry leaves, and green berries of *S. nigrum* against larvae of *Cu. quinquefasciatus* and *An. stephensi* was also established[15]. AgNPs were also synthesized by dried green fruits of *Drypetes roxburghii* and it showed promising larvicidal activities against *Culex quinquefasciatus* and *Anopheles stephensi*[14]. The results of the present study indicated the larvicidal activity of synthesized AgNPs of *C. diurnum* against the larval forms of *Cx. quinquefasciatus* and *An. stephensi*. The rate of mortality is higher in the 3rd instar larvae than the 2nd instar larvae. It may be caused due to an increased feeding rate of the 3rd instar larva compared to the 2nd instar larva. Although the exact mechanism behind the larvicidal potentiality of AgNPs is still unknown, it can be assumed that the AgNPs penetrate through the larval surface membrane and cause an interaction with cell molecules resulting in the death of larvae[28]. Choi *et al.*, also reported penetration of metallic nanoparticles through the skin as a result of an attraction of positive silver ions and the cell membrane[29].

In recent years, traditional antimicrobial agents have become increasingly less effective, and many of them are highly toxic and unsuitable for application in food and medicine[30]. The appearance of bacterial resistance against the commonly used antibiotics is also common. So an alternative approach is required to minimize/control the incidence of microbial diseases.

The disinfectant properties of certain metals such as silver are well documented from the ancient past and are widely used in traditional medicines[30-31]. Silver has been commercially employed as an antimicrobial agent[32,33]. With the continuous improvement in the field of nanotechnology, silver has become the metal of choice in the food industry in recent years[34] and are widely recommended to have antimicrobial activities. A perusal of literature revealed that in contrast to the common modern antibiotics, no such report of bacterial resistance has been recorded against AgNPs[35-37]. However, an extensive and long term use of AgNPs may result in the development of metal resistance in the future[38].

The antibacterial efficiency of the nanoparticles was also investigated and it was found that they exhibited antibacterial effect at low concentrations. It is believed that the high affinity of Ag towards sulfur or phosphorous which are found in abundance throughout the cell membrane is the key element for displaying antibacterial properties[39]. Previously, silver nanoparticles from *Ocimum tenuiflorum*, *Solanum tricobatum*, *Syzygium cumini*, *Centella asiatica*, and *Citrus sinensis* have been tested against *Staphylococcus aureus*, *P. aeruginosa*, *E. coli*, and *Klebsiella pneumonia*. *O. tenuiflorum* extract showed significant activity against *S. aureus* and *E. coli*[40]. In the present study also, a promising bacteriocidal activity of Ag

NPs was recorded against some human and fish pathogenic bacteria.

Conclusion

Synthesized AgNPs were characterized through UV–visible spectroscopy and the surface plasmon absorption spectral band showed at (λ_{max}) 450 nm indicates the presence of Ag particle. FT-IR studies showed that AgNPs produced from the extracts are surrounded by different functional groups such as amide, phosphates, nitrates, aromatic and aliphatic groups. X-ray diffraction experiments proved the shape, size, and crystalline character of AgNPs. The energy dispersive analysis of x-rays revealed significant signals in the silver area and verified silver nanoparticles production. In conclusion, the approach of green synthesis of AgNPs using *C. diurnum* fruit extract showed that dried fruit of *C. diurnum* can be used as an effective reducing and stabilizing agent for the synthesis of silver nanoparticles and the formed silver nanoparticles are stable and have significant mosquito larvicides and antimicrobial properties both against human pathogenic and fish pathogenic bacteria. Further studies are required to know the exact mechanism behind the larvicidal and bacteriocidal potentiality of AgNPs.

Conflict of interest

The authors declare no conflict of interest.

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