Green synthesis of silver nanoparticles using *Cadaba indica* lam leaf extract and its larvicidal and pupicidal activity against *Anopheles stephensi* and *Culex quinquefasciatus*

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Abstract

Green nanoparticle synthesis was achieved using environmentally acceptable plant extracts and eco-friendly reducing and capping agents. In the present study, activity of silver nanoparticles (AgNPs) synthesized using *Cadaba indica* lam plant against *Anopheles stephensi* and *Culex quinquefasciatus* was determined. A range of concentrations of synthesized AgNPs (3.125, 6.25, 12.5, 25, 50 ppm) and crude extract (50, 100, 150, 200, 250 ppm) were tested against *A. stephensi* and *C. quinquefasciatus*. The synthesized AgNPs from *C. indica* lam were much more toxic than crude extract in both mosquito species. The cured extract high mortality values were 50% lethal concentration (LC_{50})=88.22, 90.84 ppm; 90% lethal concentration (LC_{90})=172.94, 178.55 ppm, and the AgNPs high mortality values were LC_{50}=3.90, 4.39 ppm; LC_{90}=19.04, 17.35 ppm against *A. stephensi* and *C. quinquefasciatus,* respectively. The results recorded from ultraviolet-visible spectrophotometer, scanning electron microscopy, energy dispersive X-ray and Fourier transformed infrared support the biosynthesis and characterization of silver nanoparticles. These results suggest that the leaf cured extracts of *C. indica* lam and green synthesis of silver nanoparticles have the potential to be used as an ideal eco-friendly approach for the control of *A. stephensi* and *C. quinquefasciatus*.

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Introduction

Vector-borne diseases transmitted by blood-feeding mosquitoes, such as dengue hemorrhagic fever, Japanese encephalitis, malaria, and filariasis, are increasing in prevalence worldwide, particularly in tropical and subtropical zones. Malaria now is responsible for illness in more than an estimated 300 million people, resulting in one million deaths per year (WHO, 2007). *Culex quinquefasciatus* is a vector of lymphatic filariasis, which affects 120 million people worldwide, and approximately 400 million people are at risk of contracting filariasis, resulting in an annual economic loss of 1.5 billion dollars (WHO, 2002). Lymphatic filariasis is a serious public health problem in India, constituting one third of the infected population in the world (WHO *et al*., 1997). Mosquito-borne diseases are endemic to India due to favorable ecological conditions for the vectors, their close contact with humans, and their reproductive biology. In rubber plantations, the rich organic content, stagnant water, low light levels and protected conditions in the coconut shells used in rubber production favors intense breeding (Sumodan, 2003). Mosquito control is improving in many areas, but there are significant challenges, including increasing resistance to insecticides and a lack of alternative, cost-effective, and safe insecticides. This increase in insecticide resistance requires the development of strategies for prolonging the use of highly effective vector control compounds. The use of combinations of multiple insecticides and phytochemicals is one such strategy that may be suitable for mosquito control. Attempts to develop novel materials as mosquito larvicides are still necessary. With the progress of nanotechnology research, many laboratories around the world have investigated silver nanoparticles (AgNPs) production.

The development of green processes for the synthesis of nanoparticales is evolving into an important brach of nanotechnology. Nanoparticles play an indispensable role in drug delivery, diagnostics, imaging, sensing, gene delivery, artificial implants, and tissue engineering (Morones & Elechigerra, 2005). The development of a reliable green process for the synthesis of silver nanoparticles is an important aspect of current nanotechnology research. Biological methods for nanoparticle synthesis using microorganisms, enzymes, and plants or plant extracts have been suggested as possible eco-friendly alternatives to chemical and physical methods (Mohanpuria *et al.,* 2008). Recently, green silver nanoparticles have been synthesized using various natural products such as *Nelumbo nucifera* (Santhoshkumar *et*

al., 2011), *Pongamia pinnata* (Rajesh *et al.,* 2010), *Azadirachta indica* (Tripathi *et al.,* 2009), *Glycine max* (Vivekanandhan *et al.,* 2009), *Cinnamon zeylanicum* (Sathishkumar *et al.,* 2009), and *Camellia sinensis* (Begum *et al.,* 2009). In recent studies, potential mosquito larvicidal activity of synthesized AgNPs from plant extracts as well as physical methods is well documented (Marimuthu *et al.,* 2011; Thirunavukkarasu *et al.,* 2010; Sap-Iam *et al.,* 2010).

Plants are rich sources of bioactive organic chemicals and offer an advantage over synthetic pesticides, as these are less toxic, less prone to development of resistance, and easily biodegradable. India can utilize its rich supply of herbs for such purposes, as plant extracts are not only potentially insecticides, but also can act as effective antimicrobial, antifungal, anti- parasitic and anti-malarial agents. Plant materials not only offer effective mosquito control agents, but also promise to be environmentally safer. Therefore, an alternative approach for mosquito control is the use of natural products of plant origin. The botanical insecticides are generally pest-specific, readily biodegradable, and usually lack toxicity to higher animals (Bowers, 1992). In traditional medicine systems, different parts of the plant have been described to be useful against a variety of diseases. The leaves of *Cadaba indica* lam plant are rich in lactones, steroids, flavonoids, alkaloids, reducing sugars and tannins (Peach & Tracy 1955; Rastogi & Mehrotra, 1991). *C. indica* lam leaf extract is used on boils; its leaf juice is used as eye drops. Against cattle fever, a decoction of fresh leaves, pepper and garlic is administered orally (Reddy *et al.,* 2007). However, the activity of the ethanol extract of the leaves was found to be most effective against bacteria and fungi (Selvamani & Latha, 2005). The leaf and flower liquid extract mixed with castor oil and turmeric is taken as a remedy for menorrhagia, syphilis, and as a purgative (Alagesaboopathi, 2009). Pathak *et al.* (2000) reported that the steam-distilled whole plant oil extract of *Tagetes minuta* gave 100% mortality against larvae of *Anopheles stephensi*, *Culex quinquefasciatus* and *Aedes aegypti* at doses lower than 100 ppm. Volatile oil extracted from the peel of citrus fruits has also shown toxic effects on mosquito larvae as well as adults (Ezeonu *et al.,* 2001).

In the present study, we report on the synthesis of silver nanoparticles, reducing the silver ions present in the solution of silver nitrate by *C. indica* lam leaf extract, and its efficacy against *A. stephensi* and *C. quinquefasciatus.*

Materials and methods

Materials

The *Cadaba indica* lam plants were collected in and around Kaveri river bank, Namakkal District, in Tamilnadu, India, and identified by the taxonomist, Department of Botany, Bharathiar University, Coimbatore, India. The voucher specimen was numbered and kept in the authors' research laboratory for further reference. Silver nitrate ($AgNO_3$) was purchased from Precision Scientific Co., Coimbatore, India.

Mosquito rearing

The eggs of *A. stephensi* and *C. quinquefasciatus* were collected from the National Centre for Disease Control field station of Mettupalayam, Tamil Nadu, India. These were brought to the laboratory and transferred (in approximately the same aliquot numbers of eggs) into 18 cm L×13 cm W×4 cm D enamel trays containing 500 mL of water, where they were allowed to hatch. Mosquito larvae were reared (and adult mosquitoes held) at 27±2°C and 75-85% relative humidity in a 14:10 (L/D) photoperiod. The larvae were fed 5-g ground dog biscuit and brewer's yeast daily in a 3:1 ratio. The pupae were collected and transferred into plastic containers with 500 mL of water. The container was placed inside a screened cage (90 cm L×90 cm H×90 W) to retain emerging adults, for which 10% sucrose in water solution (v/v) was made available. On day 5 post-emergence, the mosquitoes were provided access to a rabbit host for blood feeding. The shaved dorsal side of the rabbit was positioned on the top of the mosquito cage in contact with the cage screen (using a cloth sling to hold the rabbit) and held in this position overnight. Glass Petri dishes lined with filter paper and containing 50 mL of water were subsequently placed inside the cage for oviposition by female mosquitoes.

Synthesis of silver nanoparticles

Leaves were washed with distilled water and dried for 2 days at room temperature. A plant leaf broth was prepared by placing 10 g of the leaves (finely cut) in a 300-mL flask with 100 mL of sterile distilled water. This mixture was boiled for 5 min, decanted, stored at –4°C, and used in our tests within 1 week. The filtrate was treated with aqueous 1 mM $AgNO_3$ solution in an Erlenmeyer flask and incubated at room temperature. As a result, in a brown-yellow solution indicating the formation of AgNPs, it was found that aqueous silver ions can be reduced by aqueous extract of the plant parts to generate extremely stable silver nanoparticles in water.

Characterization of silver nanoparticles

The presence of synthesized silver nanoparticles was confirmed by sampling the reaction mixture at regular intervals and the absorption maxima was scanned by ultraviolet-visible (UV-vis) spectra at the wavelengths of 350-600 nm in a UV-3600 Shimadzu spectrophotometer at 1 nm resolution. Further, the reaction mixture was subjected to centrifugation at 15,000 rpm for 20 min; the resulting pellet was dissolved in deionized water and filtered through a millipore filter (0.45 μm). An aliquot of this filtrate containing silver nanoparticles was used for scanning electron microscopy (SEM) and energy dispersive X-ray (EDX) studies. Thin films of the sample were prepared on a carbon-coated copper grid by dropping a small amount of the sample on the grid; extra solution was removed using a blotting paper, and the films on the SEM grid were allowed to dry by placing it under a mercury lamp for 5 min. The surface groups of the nanoparticles were qualitatively confirmed by using Fourier transformed infrared spectroscopy (FTIR) (Stuart, 2002), with spectra recorded by a Perkin-Elmer Spectrum 2000 FTIR spectrophotometer.

Larval/pupal toxicity test

Twenty-five larvae (instars I-IV) or pupae were placed in 249 mL of dechlorinated water in a 500-mL glass beaker, and 1 mL of the desired concentration of silver nanoparticles was added; 0.5 mg of larval food was provided for each test concentration. Tests of each concentration against each instar and the pupae were replicated three times. In each case, the control comprised 25 larvae or pupae in 250 mL of distilled water. Control mortality was corrected by using Abbott's formula (Abbott, 1925), and percent mortality was calculated as follows:

$$\text{Percent mortality} = \frac{\text{Number of dead larvae/pupae}}{\text{Number of larvae/pupae introduced}} \times 100$$

Statistical analysis

Average larval mortality data were subjected to probit analysis for calculating 50% and 90% lethal concentration (LC_{50} and LC_{90}) values, using the method of Finney (1971). SPSS software, ver. 9.0 (StataCorp., College Station, TX, USA), was used. Results were considered to be statistically significant at $P<0.05$.

Results and discussion

Several approaches have been employed to obtain better biosynthesis of nanoparticles, which is preferable to chemical and physical methods, as it is a cost-effective and environmentally friendly method, and does not require the use of high pressure, energy, temperature, or toxic chemicals (Sinha *et al.,* 2009; Goodsell, 2004). In the present study, the larvicidal and pupicidal effects of ethanol leaf extracts and synthesized AgNPs of *C. indica* lam were noted; the highest mortality was found in synthesized AgNps against larvae and pupae of *A. stephensi* (LC_{50}=3.90, 4.67, 10.20, 15.41, 25.27 ppm and LC_{90}=19.04, 27.06, 47.72, 61.07, 78.32 mg/L) and *C. quinquefasciatus* (LC_{50}=4.39, 5.07, 8.21, 15.44, 23.83 mg/L and LC_{90}=17.35, 20, 35.76, 58.37, 75.33 ppm), respectively (Table 1 and Figure 1). The highest mortality was found against the larvae and pupae of *A. stephensi* (LC_{50}=88.22, 107.34, 136.98, 169.04, 270.68 mg/L and LC_{90}=172.94, 209.09, 284.08, 314.46, 474.85 ppm) and *C. quinquefasciatus* (LC_{50}=90.84, 115.37, 145.18, 172.92, 288.86 mg/L and LC_{90}=178.55, 211.37, 277.29, 311.29, 525.13 ppm), respectively (Table 2 and Figure 2). Chi-square values were significant at the P≤0.05 level. Fifty-percent hydroethanolic extracts of *Bonninghausenia albiflora* whole plant, *Calotropis procera* root, *Citrus maxima* flower, *Acorus calamus* rhizome, and *Weidelia chinensis* whole plant showed acaricidal efficacy ranging from 4% to 35% within 24 h of application on *Rhipicephalus (Boophilus) microplus.* Rhizome extract of *A. calamus* revealed that a 79.31% correlation with log concentration in probit mortality could be assigned to the concentration of the extract, and the regression line of the extract showed the LC_{85} as 11.26% (Ghosh *et al.,* 2011). Chandran *et al.* (2006) synthesized silver nanoparticles by using *Aloe vera* extract at 24 h of incubation. Previous authors reported that the methanol extract of *Cassia fistula* exhibited LC_{50} values of 17.97 and 20.57 mg/L for *A. stephensi* and *C. quinquefasciatus,* respectively (Govindarajan *et al.,* 2008). A 23% mortality was noted against first-instar larvae of *A. stephensi* by treatment of *A. ilicifolius* extract at 20 ppm; this increased to 89% at 100 ppm (Kovendan & Murugan, 2011). Mosquitocidal properties of *Calotropis gigantea* leaf extract and bacterial insecticidal properties of *Bacillus thuringiensis* against these mosquito vectors have been reported by Kovendan *et al.* (2012).

Reduction of silver ions in the aqueous solution during reaction with the ingredients present in plant leaf extract was observed by UV-visible spectroscopy. The color change was noted by visual observation in the *C. indica* lam leaf extracts when incubated with $AgNO_3$ solution. *C. indica lam* leaf extract without $AgNO_3$ did not show any change in color (Figure 3). The color of the extract changed to light brown within an hour, and later changed to dark brown during a 1 h incubation period, after which no significant change occurred. Appearance of the yellowish brown color was an indication of formation of colloidal silver nanoparticles in the medium. The brown color could be due to the exci-

Table 1. Larvicidal activity of *C. indica* lam crude leaf ethnolic extract against larva and pupa of *A. stephensi* and *C. quinquefasciatus*.

Species	Life stages (instars)	Percentage of larval and pupal mortality Concentration of EEC (ppm) 50	100	150	200	250	Slope	$LC_{50}(LC_{90})$	95% confidence limit LCL $LC_{50}(LC_{90})$	UCL $LC_{50}(LC_{90})$	X^2 (df=3)
A. stephensi	I	30.0±2.4	56.2±2.3	80.0±2.0	95.8±2.2	100±1.2	0.359	88.22 (172.94)	77.06 (160.36)	97.89 (189.33)	1.383
	II	25.6±3.6	42.2±2.4	71.8±2.6	88.4±3.0	96.2±1.0	0.374	107.34 (209.09)	95.61 (194.30)	117.80 (228.84)	1.051
	III	21.2±3.3	33.8±3.0	62.8±2.2	70.4±2.2	81.2±1.6	0.313	136.98 (284.08)	122.60 (258.08)	150.50 (321.54)	3.890
	IV	13.8±2.2	24.2±3.6	49.6±2.8	61.6±3.8	73.4±1.2	0.313	169.04 (314.46)	155.65 (285.28)	183.56 (356.63)	2.571
	Pupa	8.0±1.0	15.2±3.4	22.8±2.0	30.2±3.0	46.4±2.8	0.183	270.68 (474.85)	241.07 (402.53)	320.08 (605.86)	0.522
C. quinquefasciatus	I	28.2±2.2	54.8±2.0	81.2±2.0	92.6±2.2	100±1.0	0.362	90.84 (178.55)	79.50 (165.69)	100.69 (195.26)	1.731
	II	21.0±2.4	40.8±2.2	66.4±1.6	85.6±2.8	97.8±1.2	0.396	115.37 (211.37)	104.70 (197.44)	125.20 (230.68)	1.107
	III	16.8±3.0	30.2±2.8	58.6±2.2	70.0±3.0	82.40±1.4	0.342	145.18 (277.29)	132.51 (254.27)	157.58 (309.39)	2.607
	IV	11.8±2.8	22.6±3.4	45.2±2.8	64.6±2.2	71.8±1.0	0.324	172.92 (311.29)	160.05 (283.70)	187.01 (350.82)	2.921
	Pupa	8.8±2.1	15.4±2.2	24.6±3.2	31.4±3.2	40.6±2.6	0.159	288.86 (525.13)	251.76 (432.37)	357.53 (709.61)	0.387

LC_{50}, 50% lethal concentration; LC_{90}, 90% lethal concentration; EEC, ethanolic extract of *Cadaba indica* lam; Control nil mortality; LCL, lower confidence limit; UCL, upper confidence limit; df, degrees of freedom. Each value is the mean±SD of five replicates.

Table 2. Larvicidal activity of synthesized silver nanoparticles using *C. indica* lam leaf extract against larvae and pupa of *A. stephensi* and *C. quinquefasciatus*.

Species	Life stages (instars)	Percentage of larval and pupal mortality Concentration of AgNPs (ppm) 3.125	6.25	12.5	25	50	Slope	$LC_{50}(LC_{90})$	95% confidence limit LCL $LC_{50}(LC_{90})$	UCL $LC_{50}(LC_{90})$	X^2 (df=3)
A. stephensi	I	42.0±2.8	61.2±3.0	80.8±1.0	94.6±1.1	100±1.2	1.068	3.90 (19.04)	1.42 (16.48)	5.72 (23.00)	3.378
	II	40.8±2.4	55.6±3.8	71.4±2.4	88.4±1.4	98.8±1.0	1.128	4.67 (27.06)	1.43 (23.32)	7.13 (32.80)	3.376
	III	35.2±2.0	42.6±3.5	58.2±2.5	75.0±1.8	88.4±1.4	1.099	10.20 (47.72)	6.04 (40.95)	13.68 (58.18)	4.909
	IV	29.8±3.4	40.0±2.6	51.2±2.2	66.4±2.4	80.2±1.0	1.010	15.41 (61.07)	10.97 (51.50)	19.51 (76.74)	4.895
	Pupa	21.8±3.6	33.4±2.8	42.6±2.6	55.8±2.6	68.8±1.3	0.918	25.27 (78.32)	13.80 (53.53)	45.48 (190.03)	6.095
C. quinquefasciatus	I	44.2±3.0	53.2±2.4	85.8±1.0	96.2±1.0	100±1.0	1.099	4.39 (17.35)	2.37 (15.12)	5.93 (20.74)	5.051
	II	40.0±2.4	55.2±2.1	77.8±2.1	94.2±1.0	100±1.0	1.158	5.07 (20.00)	2.89 (17.40)	6.76 (23.96)	1.843
	III	36.8±1.6	43.2±2.0	63.4±2.7	82.4±2.0	95.4±1.4	1.227	8.21 (35.76)	4.99 (31.11)	10.92 (42.62)	4.851
	IV	30.4±3.4	36.4±2.0	51.6±3.5	68.4±1.8	81.2±1.2	1.063	15.44 (58.37)	3.93 (42.23)	25.07 (110.23)	5.801
	Pupa	27.2±3.0	30.4±2.4	42.6±3.3	56.4±2.7	71.4±1.0	0.942	23.83 (75.33)	19.26 (62.54)	29.18 (97.33)	2.888

LC_{50}, 50% lethal concentration; LC_{90}, 90% lethal concentration; AgNPs, silver nanoparticles; Control nil mortality; LCL, lower confidence limit; UCL, upper confidence limit; df, degrees of freedom. Each value is the mean±SD of five replicates.

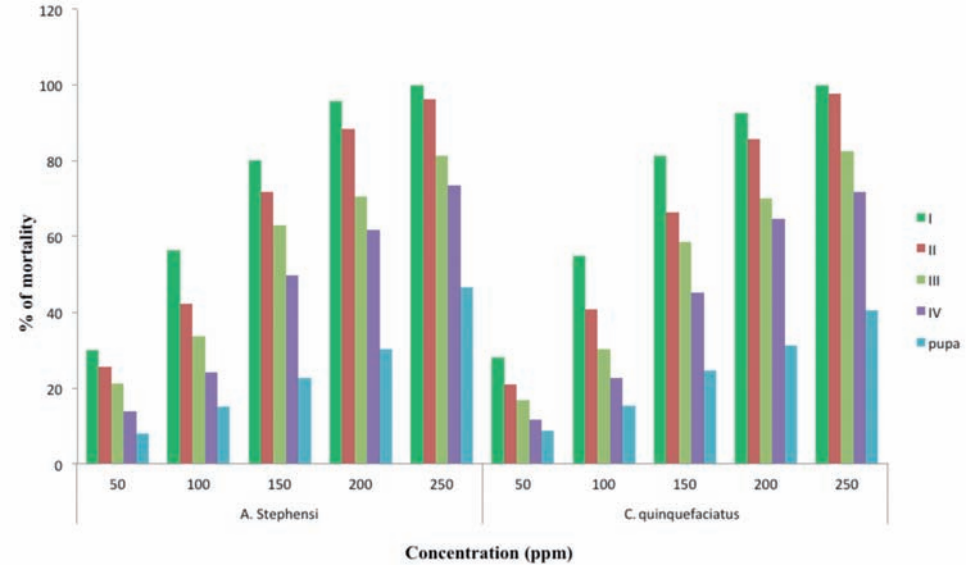

Figure 1. Larvicidal activity of *C. indica* lam crude leaf ethnolic extract against larva and pupa of *A. stephensi* and *C. quinquefasciatus*.

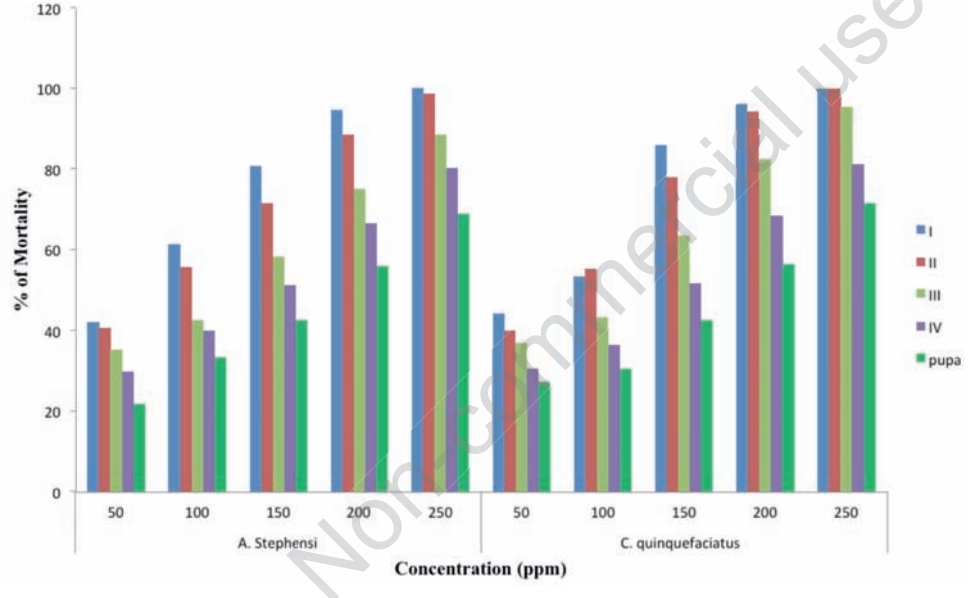

Figure 2. Larvicidal activity of synthesized silver nanoparticles using *C. indica* lam leaf extract against larvae and pupa of *A. stephensi* and *C. quinquefasciatus*.

tation of surface plasmon vibrations, typical of silver nanoparticles (Ahmad *et al.,* 2003; Krishnaraj *et al.,* 2010). The dark brown color of the silver colloid is attributable to surface plasmon resonance arising from the group of free conduction electrons induced by an interacting electromagnetic field (Song & Kim, 2008). The strong surface plasmon resonance band appears at the range of 420-480 nm and the broadening peak indicates that the particles are monodispersed (Figure 4). These color changes arise because of the excitation of surface plasmon vibrations in the silver nanoparticles (Mulvaney, 1996).

SEM (JEOL-MODEL 6390) image showing high density Ag nanoparticles synthesized by *C. indica* lam plant extracts further confirmed the presence of Ag nanoparticles (Figure 5). It was shown that relatively spherical and uniform Ag nanoparticles were formed with a diameter of 30-60 nm. The SEM image of silver nanoparticles synthesized by plant extracts were assembled on the surface due to interactions such as hydrogen bonding and electrostatic interactions between the bio-organic capping molecules bound to the Ag nanoparticles. It was found that relatively spherical and uniform silver nanoparticles were formed. The nanoparticles were not in direct contact, even within the aggregates, indicating stabilization of the nanoparticles by a capping agent (Song & Kim, 2008). Silver nanoparticles have been characterized using SEM by various investigators (Durán *et al.,* 2005; Balaji *et al.,* 2009). Silver nanoparticles were synthesized using leaf extracts of *Acalypha indica*; from the SEM image, the size of the control silver nitrate obtained was more than 1000 nm, whereas synthesized silver nanoparticles measured 20-30 nm in size (Krishnaraj *et al.,* 2010). Tian *et al.* (2007) reported that numerous flavonoids, including quercetin or quercetin 3-O-glycosides, were isolated from lotus leaves that were used for silver nanoparticle synthesis. The element analysis of the silver nanoparticles was performed using EDX on the SEM. Figure 6

shows the EDX spectrum of AgNPs synthesized at 25ºC and 80ºC; strong signals from the silver atoms in the nanoparticles were observed, and signals from calcium, potassium, oxygen, sodium, magnesium, sulphur, Ag and chloro were also recorded. The results indicate that the reaction product was composed of higher level Ag nanoparticles.

The AgNPs produced by *C. indica* lam leaf extract were distinct and scattered in distribution. The Fourier transformed infrared spectra of AgNPs exhibited prominent peaks at 3453; 3288; 1790; 1638; 1384; 1114; 1077; 371; 360 cm^{-1} (Figure 7). The sharp absorption peak at 1638 cm^{-1} was assigned to C=O stretching vibration in the carbonyl compounds, which may be characterized by the presence of a high content of terpenoids and flavonoids. The peaks at 1077 cm^{-1} correspond to C–N stretching vibration of aliphatic amines or alcohols/phenols, representing the presence of polyphenols. The absorption bands at 1088 cm^{-1} in the fingerprint region indicate several modes such as C–H deformation or C–O or C–C stretching, pertaining to carbohydrates. The bands at 1383 to 1431 cm^{-1} were assigned to scissoring modes of methylene tails, CH3 R. A broad intense band at 3402 cm^{-1} in both the spectra can be assigned to the N–H stretching frequency arising from the peptide linkages present in the proteins of the extract (Mukherjee *et al.,* 2008).

Conclusions

The present study of green synthesis shows that the environmentally benign and renewable source of *C. indica* lam is used as an effective reducing agent for the synthesis of AgNPs. This biological reduction of silver nanoparticles would be a boon for the development of a clean, nontoxic, and environmentally acceptable green approach to production of AgNPs, involving organisms extending even to higher plants. The AgNPs did not exhibit any noticeable effects on *C. indica* lam exposure at their LC_{50} and LC_{90} values against larvae of *A. stephensi* and *C.*

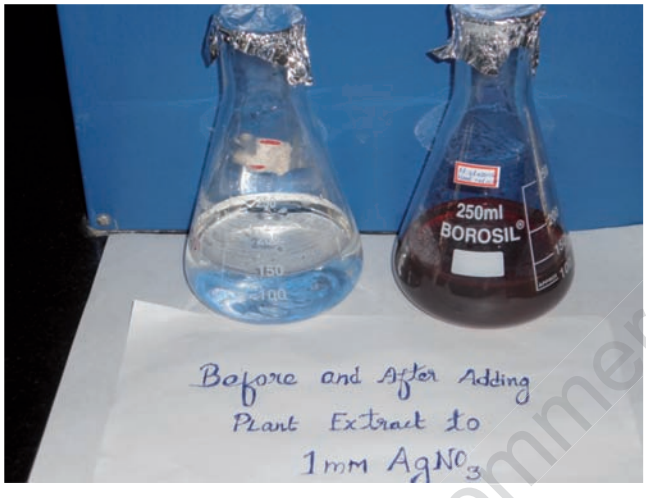

Figure 3. Photographs showing change in color after adding silver nitrate ($AgNO_3$) before reaction and after reaction time of 30 min.

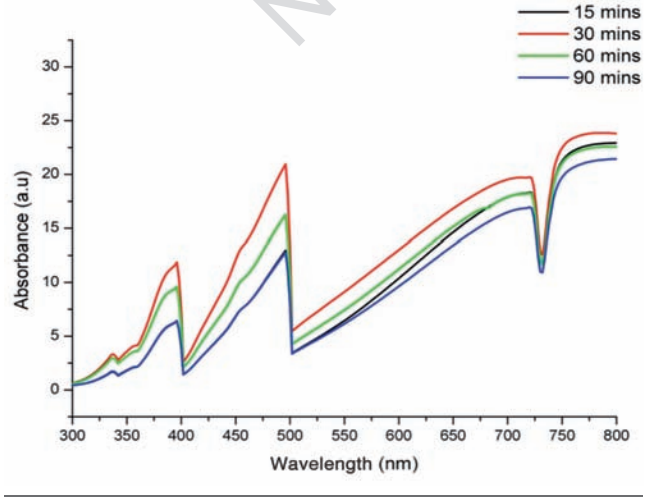

Figure 4. Ultraviolet-visible spectra of aqueous silver nitrate with *C. indica* lam leaf extract at different time intervals.

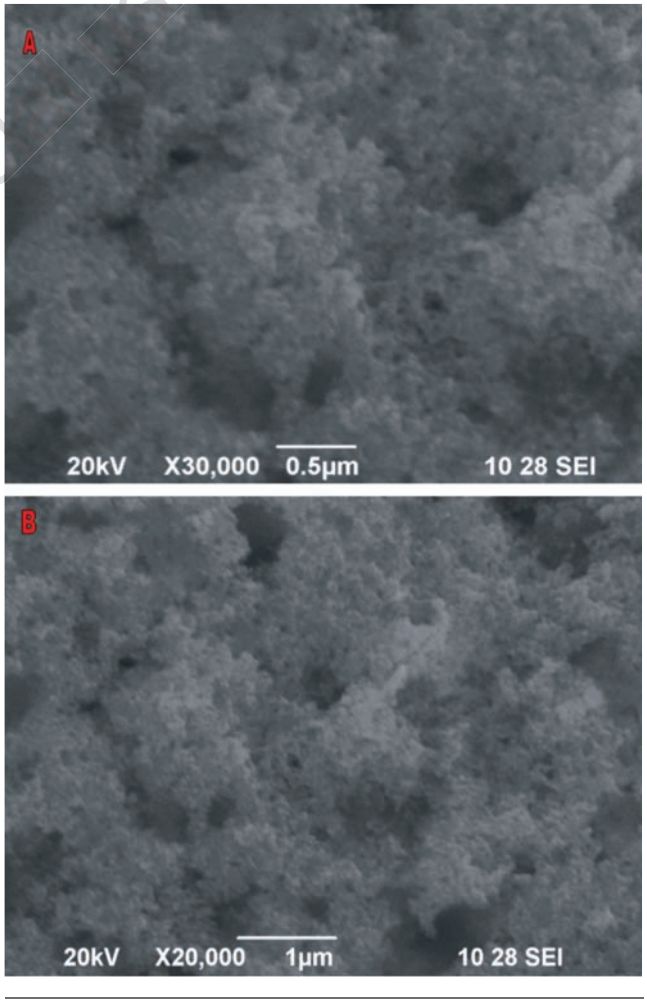

Figure 5. Image of scanning electron microscopic observation of synthesized silver nanoparticles. A) Lower magnification (0.5 µm); B) Higher magnification (1 µm).

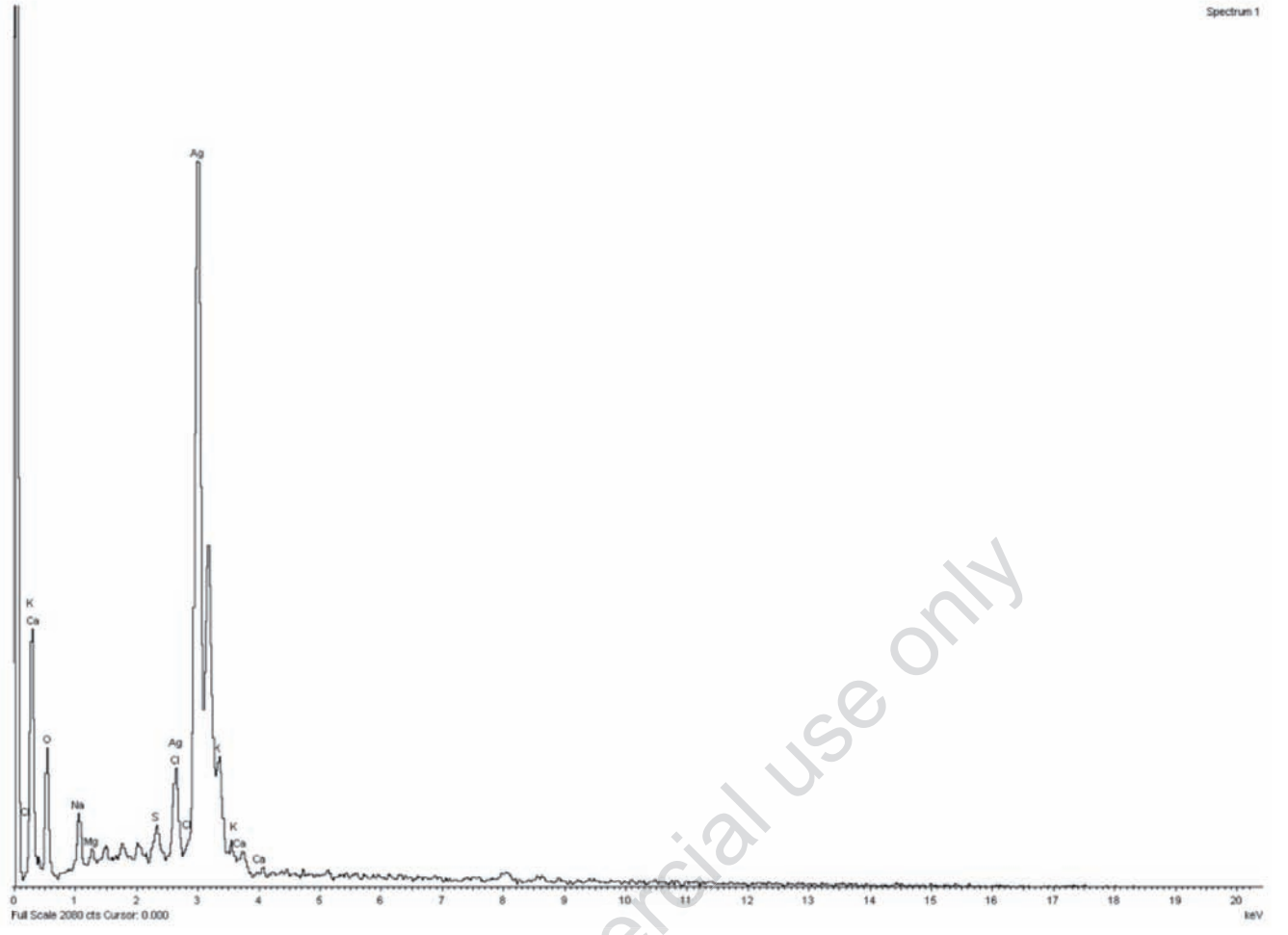

Figure 6. Energy dispersive X-ray spectra recorded form a film, after formation of silver nanoparticles with different X-ray emission peaks labeled. Cl, chloro; K, potassium; Ca, calcium; O, oxygen; Na, sodium; Mg, magnesium; S, sulphur; Ag, silver.

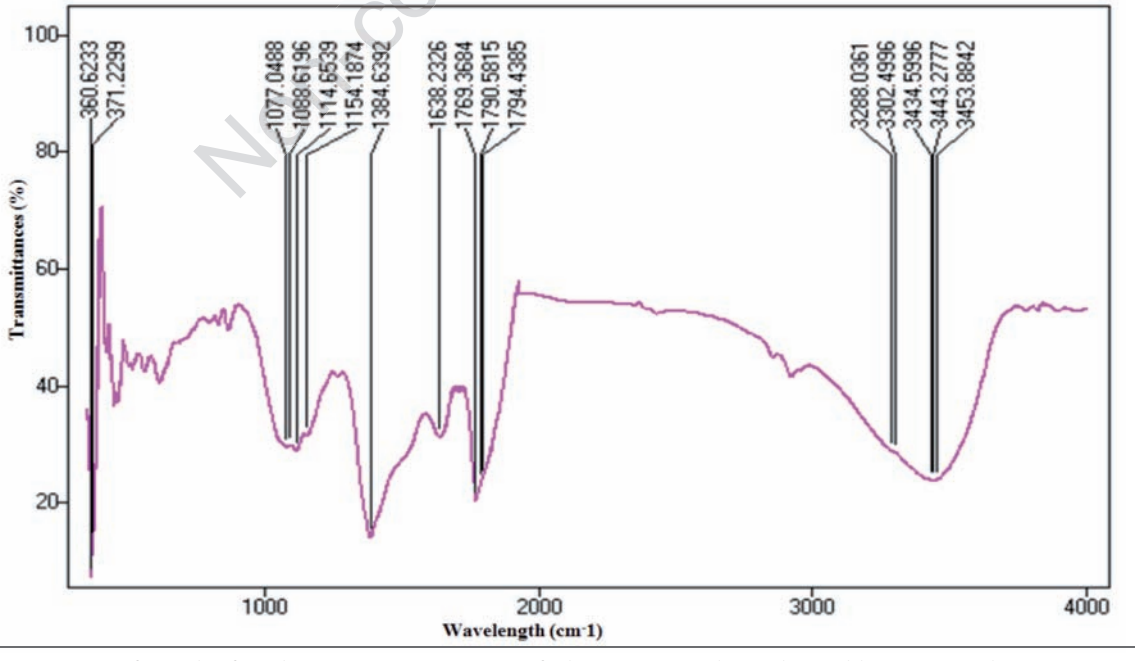

Figure 7. Fourier transformed infrared spectroscopy spectrum of silver nanoparticle synthesized by reacting silver nitrate with *C. indica* lam leaf extract.

quinquefasciatus. The AgNPs formed are highly stable and significant mosquito larvicides. The synthesized AgNPs in a methanol extract and the isolation and purification of crude methanol extracts of *C. indica* lam are in progress. We also seek to develop methods and techniques necessary for green synthesis of silver nanoparticles by using microorganisms, such as bacteria and fungi, for mosquito control.

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