Biochemistry and Biophysics Reports 10 (2017) 76–81

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

Antioxidant, antibacterial and cytotoxic potential of silver nanoparticles synthesized using terpenes rich extract of *Lantana camara* L. leaves

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ARTICLE INFO

Keywords:
Lantana camara L.
Terpenes-rich extract
Silver nanoparticles
Antioxidant
Antibacterial
Brine shrimp cytotoxicity

ABSTRACT

Several attempts have been made for green synthesis of silver nanoparticles (AgNPs) using different plant extracts. Present study revealed that, antioxidant, antibacterial and cytotoxic AgNPs were synthesized using terpenes-rich extract (TRE) of environmentally notorious Lantana camara L. leaves. AgNPs were characterized by advanced techniques like UV–Visible and Infra red spectroscopy; XRD, SEM techniques as terpenes coated sphere shaped NPs with average diameter 425 nm. Further, on evaluation, AgNPs were found to exhibit dose – dependent antioxidant potential, good to moderate antibacterial activity against Staphylococcus aureus, Escherichia coli and Pseudomonas aeruginosa; and toxicity on Brine shrimp (A. salinanauplii) with LD_{50} value 514.50 µg/ml.

1. Introduction

Development of Nanoparticles (NPs) nowadays has become an attribute of development of Richards Feynman laid down concept of nanotechnology. NPs of different metals like Cu, Pb, Ca, Pt, Ag, Au etc. have been synthesized and evaluated for their applications in different domains. NPs have variety of applications in various fields like energy, medicine, agriculture, environment sciences etc. Several methods have been developed for synthesis of NPs. Physical and chemical methods use radiations and/or reductants, harmful to environment and thereby human health; and expensive too. However biological methods use eco-friendly natural resources likes plant extracts, microbial cultures and enzymes [16] on expenditure of less energy. Right from Prokaryotic cells to eukaryotic fungi and even higher plants have ability to synthesize NPs (Table 1). Among all biological systems, plant extract mediated synthesis is faster and NPs so synthesized are more stable as compared to those synthesized using microbes [16]. However, some plant extracts have successfully been employed in synthesis of bimetallic NPs; for example, *C. platycladi* leaf extract reduces both Au(II) and Pd(II) to synthesize AuPd NPs [57]. Silver Nanoparticles (AgNP) are gaining attention because of wide range of applications in various domains especially in pharmaceutical sciences which includes treatment of skin diseases like acne, dermatitis and ulcerative colitis; cell labelling; coating of surgicals and medical devices; molecular imaging of cancer cells [17]. Various antibacterial formulations and devices like household antiseptic sprays and antimicrobial bandages have also been designed and developed from most common man-made nanomaterial, AgNPs [37].

Intrinsic ability of plant material contributes in amalgamation of metal ions to NPs [35]. This intrinsic ability is because of plant metabolites which could be oppressed as reducing and capping agents; and are available ubiquitously [42]. Plant metabolites can be primary metabolites like monosaccharides [33]; proteins [36]; enzymes [27] and lipids or secondary metabolites like polyphenolics [55], flavonoids [19,23], alkaloids and terpenes [45].

Lantana camara L. (Verbanaceae) is a notorious and ornamental herb found in tropical and sub-tropical countries. It has wide traditional claims for treatment of various illness [8]. Its leaves are rich in essential oil [20]. Essential oils are composed of hydrocarbons, Terpenes and their oxygenated derivatives, Terpenoids. Chemically, hydrocarbons constitute in the form of Isoprene (2-Methyl-1,3-butadiene) units with molecular formula (C_5H_8); biosynthesized via Mevalonic acid pathway, using acetyl coA as precursor. The 'head' of an isoprene unit attaches to 'tail' of another isoprene unit, forming higher terpenes, viz. hemiterpene, monoterpene, sesquiterpene, diterpene, triterpene with increasing molecular weight. Then, these terpenes may form oxygenated derivatives with different functional groups like alcohol, ester, aldehyde, ether, ketones etc. Similarly, terpenoids can be hemiterpnoid, monoterpnoid, sesqiterpenoid, diterpenoid, triterpenoids where, building blocks are Prenol (3-Methyl-2-buten-1-ol) or Isovaleric acid (3-Methyl butanoic acid) units not Isoprene units [11]. Several researchers have found these essential oils and thereby terpenes and terpneoids

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http://dx.doi.org/10.1016/j.bbrep.2017.03.002
Received 29 October 2016; Accepted 2 March 2017
Available online 10 March 2017

Table 1
List of organisms used in synthesis of AgNPs.

Organism	Example	Reference No.
Bacteria	***Bacillus licheniformis***	[50]
	***Pseudomonas stutzeri* AG259**	[25]
	Klebsiella pneumonia	[41]
	Escherichia coli	[41]
	Enterobacter cloacea	[41]
	Lactobacillus spp.	[31]
Fungi	***Fusarium oxyporum***	[1]
	Aspergillus flavus	[56]
	Penicillium fellutanum	[22]
	Coriolus versicolor	[39]
Algae	***Chaetoceros calcitrans***	[34]
	Chlorella salina	
	Isochrysis galbana	
	Tetraselmis gracilis	
Plants	***Andrographis paniculata***	[52]
	Dalbergia spinosa	[30]
	Iresine herbstii	[10]
	Melia azedarach	[51]
	Tinospora cordifolia	[18]

involved in AgNPs synthesis [49].

As per literature survey, so far, not a single attempt has been made for synthesis of AgNPs using extract of *Lantana camara* L. leaves; however, once NPs had been synthesized using extract *L. camara* L. berries and evaluated for antibacterial potential [26]. With this prior art, present study aimed towards terpenes –rich extraction of *Lantana camara* L. leaves; green synthesis and characterization of AgNPs; and screening of their antioxidant potential; antibacterial activity against few microorganisms and cyto-toxicity on Brine shrimps.

2. Materials and methods

2.1. Plant collection and preparation of terpenes rich extract

Lantana camara L. plant was collected from local area around institute and identified by morphology and microscopy. Leaves were rinsed with purified water to remove dust particles and dried. Powder (10 gm) of dried leaves of *Lantana camara* L. was extracted with petroleum ether (30 ml) at room temperature for 6 hrs with frequent shaking. It was then treated with 30 ml of warm 10% aqueous KOH, shaken and polarity-based two layers were separated. Petroleum ether layer was then concentrated to dryness under reduced pressure to obtain sticky mass (0.3 gm). This unsaponified matter of petroleum ether extract was considered as Terpenes-rich extract (TRE). TRE was tested for presence of the phyto chemicals like alkaloids, flavonoids, polyphenols, terpenes etc. present in it. Tests were based on simple chemical reactions determined by change in colour or formation of precipitate. Further, TRE was standardized using β-caryophyllene as marker by GLC.

2.2. Green synthesis of AgNP using TRE and their characterization

For synthesis of silver nanoparticles (AgNP), 1 ml of TRE was mixed with 6 ml of 1 mM $AgNO_3$ solution in Erlenmeyer flask at room temperature and was kept in dark for 24 hrs. After specific time; greenish colour of solution gradually turned into reddish colour indicating synthesis of AgNP. These NPs were then purified by centrifugation and repeated washings. Supernatant was discarded and concentrated slurry was collected. It was then dried under vacuum. Further AgNP were characterized by spectroscopic and microscopic studies.

2.3. Spectroscopic study

Synthesis of AgNP were confirmed by UV–Visible spectra; determined by dissolving 0.02 g in 2 ml deionized water on Shimadzu UV–Vis Spectrophotometer 1800, while strong adsorption of *Lantana camara*L. metabolites on the surface of NPs and functional groups were identified by FTIR spectra; recorded on Bruker Alpha by KBr pellet technique.

2.4. Microscopic study for particle size and surface

XRD spectra of AgNP coated on XRD grid was recorded using Phillips PW 1830 with specifications of process: Voltage of 40kv; Current of 30 mA; CuK α radiations; energy of KeV wavelengths was 1.54 A. Scherer equation was applied for estimation of size of nanoparticles. Morphological study was executed on Scanning electron microscopy, carried out on JSM-6360 (JEOL)at voltage 7.50 kV; sample for which was prepared by vacuum drying a drop on NP solution on graphite grid.

2.5. Zeta potential determination

The zeta potential of AgNP was evaluated using a Zetasizer Nano ZS (Malvern Instruments Inc., USA), which measures electrophoretic mobility of nanoparticle using phase analysis light scattering.

2.6. Evaluation of antioxidant potential

Antioxidant activities of *Lantana camara* L. leave's TRE and AgNPs synthesized using TRE were evaluated by Dot-blot rapid screening method described by Shirmila and Radhamany [44], with minor modification. Aliquots of 10 µL of ascorbic acid (0.1 M), TRE and AgNPs in different concentrations of 0.5, 1, 2 mg/ml were spotted on the TLC plate; allowed to air dry and placed in methanolic solution of DPPH (0.1 mM/L) for 10 s. Then, intensities of bright yellowish spots against purple background were recorded manually for each spot. Ascorbic acid was used as standard.

2.7. Screening of antibacterial activity

2.7.1. Test microorganisms

Antibacterial activity of TRE and AgNPs was tested on three microorganisms namely, *Staphylococcus aureus*(MTCC 87), *Escherichia coli*(MTCC 443) and *Pseudomonas aeruginosa*(MTCC 741); procured from the Microbial Type Culture Collection (MTCC, Chandigarh, India). The strains were maintained on nutrient agar slants at 4 °C. A loopful of each bacterial strain was added to a 50 ml sterile nutrient broth in a 100 ml conical flask. The flasks were further incubated for 24 hrs for activation.

2.7.2. Antibacterial activity

Agar-well diffusion method was used to evaluate antibacterial activity of TRE and AgNPs. To prepare Nutrient agar, about 2.3 gm Nutrient agar was added to 100 ml of distilled water; pH was adjusted at 7.0 and was autoclaved. It was allowed to cool up to 45 °C. About 75 ml of seeded nutrient agar seeded with microorganisms was poured in each of 9 petri plates and allowed to solidify. Wells were bored into the agar using a sterile 6 mm diameter cork borer. Approximately,10 µL of the TRE and AgNPs at concentrations of 1 mg/ml were added into the wells, allowed to stand at room temperature for about 2 h and incubated at 37 °C. Standards were set in parallel. Zones of inhibition was determined after 24 h. The effects were compared with that of standard, Ciprofloxacin.

2.8. Brine shrimp cytotoxicity assay

Test organisms used in brine shrimp cytotoxicity assay were *A. salina* and procedure followed was described by McLaughlin in 1998 [29]. Approximately 100 mg of *A. salina* cysts were hatched. Cysts were incubated inartificial sea water prepared by dissolving 38 g of NaCl in 1 l of distilled water at room temperature. Exactly ten *A. salina* nauplii were transferred to each of test tube using Pasteur pipette and volume was make up to 9 ml with saturated solution of NaCl in distilled water. For cytotoxicity measurement, AgNPs were suspended in dimethyl sulfoxide, DMSO (2 mg/2 ml) and diluted to get final concentrations of 10 µg/ml, 100 µg/ml, and 1000 µg/ml. Test tubes containing 9 ml of NaCl solution and 30 brine shrimp were added with 1 ml of AgNPs dilutions. For each concentration, a set of three test tubes were prepared (thereby 90 shrimp per concentration). Mixtures were then kept for hatching in incubator at 30º C. After 24 h living and dead *A. salina* nauplii were counted manually and LC_{50} was determined.

3. Result and discussion

The results of present investigation point toward the emerging role of leaves of notorious *Lantana camara* L. for synthesis of NPs having wide range of applications. Phytochemical prospection of TRE showed presence of only terpenes; no phytoconstituents of other class were found in it. On GLC analysis, β –caryophyllene content of TRE was found to be ranging between 31.01 to 31.8%, higher than what found by Sonibare and Effiong [48] (8.9%,) and Alitonou et al., [2] (18.5%) from essential oil and Unnithan and Unnikrishnan [54] (0.06%) in petroleum ether extract of *Lantana camara* L. leaves. This revealed that saponification removed fixed oil and wax content from leaves and its unsaponified matter is now rich in terpenes (hence it is Terpenes rich extract, TRE).

3.1. Green synthesis and characterization of AgNPs

This attempt of AgNPs synthesis was found to be successful; primary indication was change in green colour of TRE to reddish (Fig. 1) after 24 h of mixing with $AgNO_3$ solution. Subsequently conducted spectroscopic studies confirmed this finding. Nanoparticulate silver showed a well-defined absorption peak in visible region at 439 nm(Fig. 2),

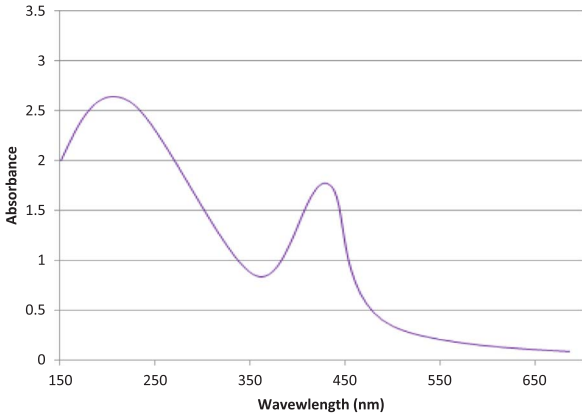

Fig. 2. UUV–visible spectrum of AgNPs synthesized using TRE of *L. camara*L. leaves.

corresponding to the surface plasmon resonance of AgNPs. The interaction of AgNPs with terpenes of *L. camara* L leaves validated the reduction of Ag^+ ions to Ag^0 by the terpenes that may get in turn oxidized to other species. The FTIR spectra of biosynthesized AgNPs, (Fig. 3) showing transmission peaks at 821, 1039, 1104, 1415, 1613, 3415 cm^{-1}, corresponding to bending vibrations, CH_3–C–CH_3 skeletal vibrations,– OH and G- H deformations of germinal methyls, C=C bonds of aromatic rings indicating the presence of carboxylic, hydroxyl, carbonyl and phenyl groups responsible for reduction of Ag^+ ions to $Ag^0$and for capping of AgNPs biosynthesized using *L. camara* L. leave's TRE. According to [13] and Si and Mandal [46], nanoparticles synthesis involves three phases, 1) reduction of metals to metal ions and their nucleation; 2) growth phase involving coalesce of small nanoparticles into larger size nanoparticles with increased thermodynamic stability (Ostwald Ripening); 3) termination of nanoparticle growth.

XRD configurations of AgNPs indicated that AgNP has spherical structure of metallic silver (Fig. 4). In addition, the diffraction peaks at 2θ values of 31.8°,44.9°, 74.9° and 96.1° could be credited to (111), (200), (220), (311) respectively, can be correlated to standard metallic silver XRD pattern JCPDS No. 89–3722. On solving Scherrer equation, the average crystallite size, in term diameter of AgNPs was found to be 425 nm. These peaks are owed to reduction of the silver ions and stabilization of their nanoparticulate forms [4]. SEM study gave an idea

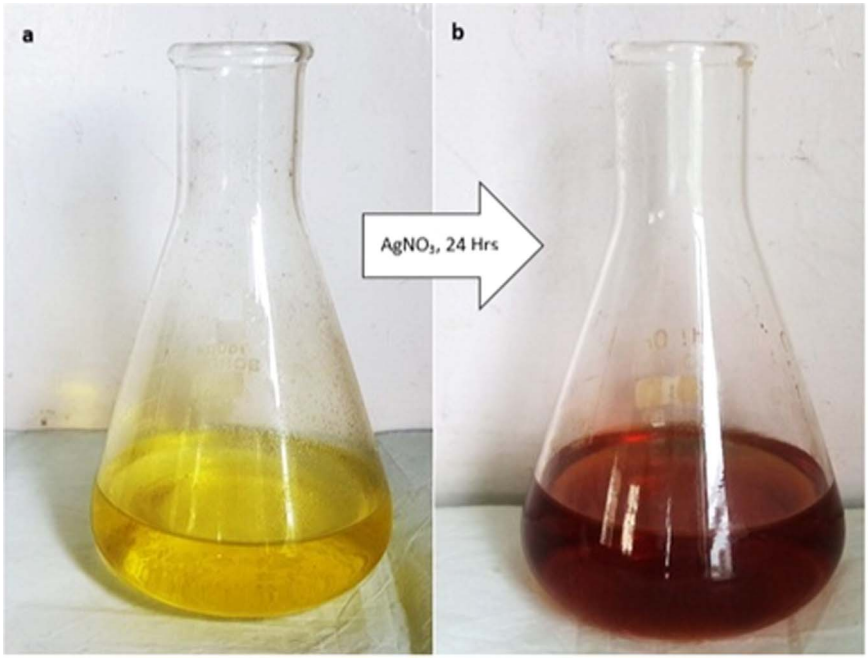

Fig. 1. Change in colour of mixture containing TRE of *L. camara* L. leaves and $AgNO_3$ solution; a) just after mixing; b) after 24 h.

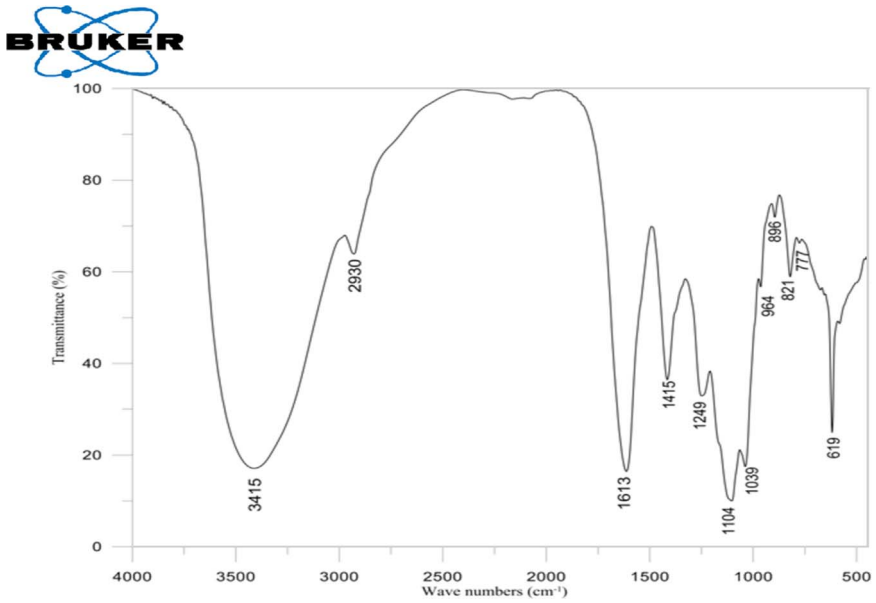

Fig. 3. FT-IR spectrum of AgNPs synthesized using TRE of *L. camara*L. leaves.

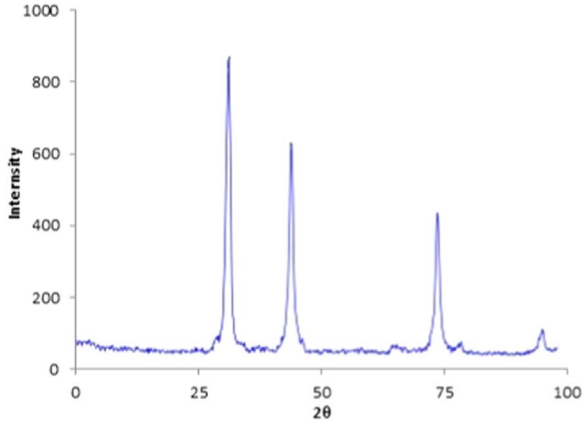

Fig. 4. XRD image.

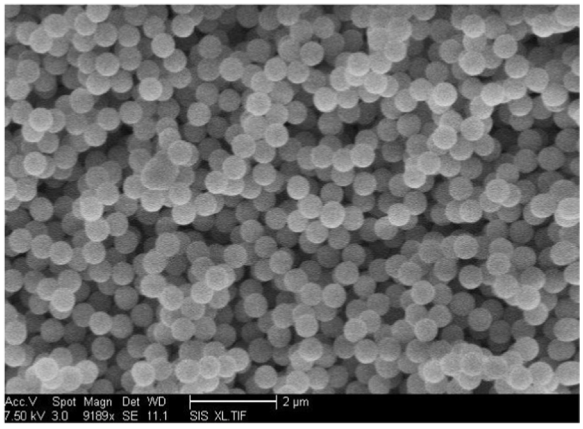

Fig. 5. SEM image obtained for AgNPs synthesized using TRE of *L. camara*L. leaves.

about topography of AgNPs. SEM image (Fig. 5) showed that individual AgNP has nearly spherical geometry with a mean size of 410–450 nm and no agglomeration.

Zeta potential on the surface of AgNPs was found to be −15.2 mV and thereby this can be anticipated that AgNPs showed good stability in water due to the electrostatic repulsive forces. This stability and zeta potential clues for an electrosteric mechanism due to adsorption of terpenes from TRE to NPs. These terpenes act as spacers and inhibit close contact between AgNPs.

Table 2
Intensities of spots of different concentrations of test materials.

Test Material	Concentrations (mg/ml)	Intensity[a]
TRE	2	+ + +
	1	+ +
	0.5	+
AgNPs	2	+ + + +
	1	+ + + +
	0.5	+ + +
Ascorbic acid	2	+ + + + +
	1	+ + + + +
	0.5	+ + + +

[a] Manual measurement of intensity, +, poor; + +, moderate; + + +, good; + + + +, excellent; + + + + +, highest intensity.

3.2. Antioxidant potential

The antioxidant efficacy of the AgNPs was depicted in Table 2, which showed AgNPs have antioxidant potential comparable with standard ascorbic acid. For quantity of 10 µL of AgNP (2 mg/ml), intensity of spot was found to be comparable with that of ascorbic acid. TRE spot (2 mg/ml) had also shown good intensity but as compared to high intensities of ascorbic acid spots. Antioxidant activity in plant extract is because of redox potential of phytoconstituents [58], which could play an important role in satiating singlet and triplet oxygen, rotting the peroxides or nullifying the free radicals. Therefore, it is anticipated that higher antioxidant activity of nanoparticles is might be due to the preferential adsorption of the antioxidant material from the extract onto the surface of the nanoparticles.

3.3. Antibacterial activity

Considering zone of inhibition, among the tested materials, AgNPs showed more significant antimicrobial activity against Gram positive *Staphylococcus aureus*(28.1 mm) than Gram negative *Pseudomonas aeruginosa*(21.3 mm) and *Escherichia coli*(22.1 mm); comparable with standard, Ciprofloxacin. TRE showed 26.5 mm wide zone of inhibition against*S. aureus*, while zones of inhibition against *E. coli* and *P.*

Table 3
Zones of inhibition (mm) for different test materials against different microorganisms.

Microorganism	Zone of inhibition (mm)		
	TRE	AgNPs	Ciprofloxacin
Staphylococcus aureus	26	28	29
Escherichia coli	21	22	27
Pseudomonas aeruginosa	22	21	28

aeruginosa were found to be 21.2 and 22.4 mm, respectively (Table 3). These results were also compared with antibacterial activity of simple petroleum ether extract and essential oil isolated from *Lantana camara* L. leaves, investigated by other researchers. Saikia and Sahoo [38], found highest zone of inhibition (12.2 mm) when essential oil is tested on *S. aureus* than that on *E. coli* (10.9 mm) and *P. aeruginosa* (8.5 mm); however, [40], found 24 and 20 mm wide zones of inhibition against *E. coli* for essential oil and petroleum ether extract of *L. camara* L. leaves, respectively; it also showed 18 mm wide zone of inhibition against *P. aeruginosa* and 10 mm wide for *S. aureus*. Reason for these outcomes may be the high peptidoglycans content of gram positive bacterial cell wall than that of gram-negative bacteria. Rather than permeation, there may be bond formation between positively charged silver and negatively charged thick layer of peptidoglycan in gram-positive bacteria as compared to that in gram-negative bacteria [3]. Based on these findings, it could be noted that, antibacterial activities exhibited by AgNPs and TRE are more potent than petroleum ether extract and essential oil of L. *camara* L. leaves. [21] revealed that nanoparticles have dose dependent membrane permeation with respect to rate. After being permeated, according to Sondi and Salopek-Sondi [47] nanoparticles disrupt the polymer subunits of cell membrane and disturb the bacterial protein synthesis.

3.4. Brine shrimp cytotoxicity

AgNPs synthesized using L. *camara* L. TRE showed dose-dependent cytotoxicity on *A. salina* nauplii as depicted in Table 4; indicating count of living and dead *A. salina* nauplii and average number of dead nauplii. Then, plot of concentration of AgNPs versus average no. (Fig. 6) gives curved graph and best fit straight line equation with R^2 value. LC_{50}value of AgNPs on *A. salina* nauplii was found to be 514.50 µg/ml. This value is found comparable to that of cytotoxicity studies conducted by other researchers.

Many researchers found AgNPs they synthesized using different plant extracts toxic to different tumour cell lines [5–7]. Cytotoxicity may be the result of entry of AgNPs inside cell and its damage by one of the two mechanisms, forming stable S-Ag bond with thiol group of enzymes in cell membrane and its deactivation; or breaking Hydrogen bonds between Nitrogen bases of DNA and thereby denaturing it [15].

Table 4
Concentrations and corresponding numbers of living and dead *A.salina* nauplii.

Concentration	No. of living *A. salina* nauplii	No. of dead *A. salina* nauplii	Average no. of dead nauplii
10 µg/ml	29	1	1.6
	28	2	
	28	2	
100 µg/ml	24	6	5.6
	26	6	
	25	5	
1000 µg/ml	02	28	27.0
	04	26	
	03	27	

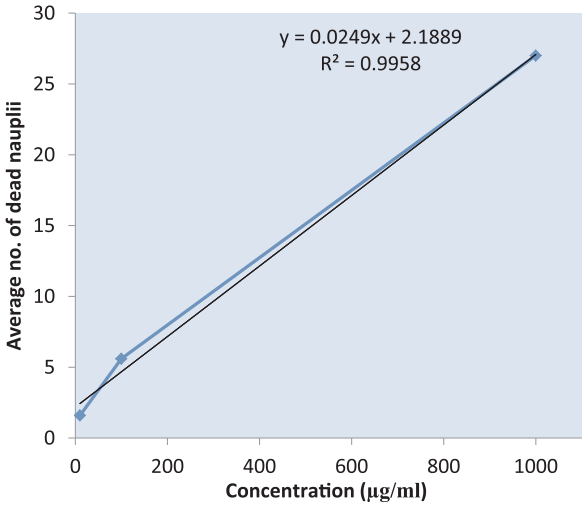

Fig. 6. Plot showing average number of dead *A. salina*nauplii for each concentration.

However some researchers have proposed that Silver may interfere with Electron Transfer Chain [43]; produce free radicals [3]or causes ATP leaking [32]. The extent of Brine shrimp cytotoxicity can be co-related to their smaller size. Smaller the AgNP size, stronger is the cytotoxicity [14,24], because AgNPs size has an effect on its uptake by cells; its penetration through biological membranes and immunological reactions initiated against it [28,32,53]. Electrostatic attraction between NPs and cells also plays important role [9]. Cytotoxicity of nanoparticles towards *A. salina* nauplii maybe in connection with anticancer activity and thereby nanoparticles can be developed to anticancer drugs [12].

4. Conclusion

Plant extract mediated synthesis promises eco-friendly approach for AgNPs synthesis having wide applications in various domains of science and thereby life. In present study, AgNPs synthesized via one of the 'Green' technique i.e. by using plant extract were tested for their antioxidant, antibacterial properties and cytotoxicity on Brine shrimp (*A. salina* nauplii). For green synthesis, we prepared *Lantana camara* L. leave's petroleum ether extract rich in terpenes, TRE and mixed with $AgNO_3$ solution for 24 h. AgNPs formation was justified by simple visual detection of colour change in solution and wavelength vs. absorbance spectrum generated in visible region; capping of certain compounds with functional groups on AgNPs surface were determined by FTIR spectrum; morphology were studied by advance techniques like XRD and SEM. AgNPs so synthesized showed antioxidant potential screened through modified dot-blot method, antibacterial activity evaluated via agar-well diffusion assay with zone of inhibition comparable to standard and cytotoxicity on Brine shrimp (*A. salina* cysts) hatched in artificial sea water with LD_{50} value 514.50 µg/ml.

Conflict of interest

We, authors of this research article declare no conflict of interest.

Acknowledgement

Authors are thankful to Dr. (Mrs.). Anagha M. Joshi, Principal, SCES's Indira College of Pharmacy, Tathawade, Pune for providing laboratory facility for carrying out experimental work involved in conducting this research. Authors are grateful to Dr. Deepawali Thanekar, Assistant Professor, Institute of Science, Mumbai, India for helping us in literature survey and Mr. Prasad P. Devarshi, Department of Nutrition Science, Purdue University, IN, USA for helping us in techno-scientific writing.

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