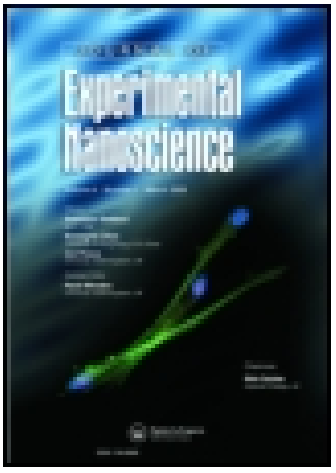

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A novel approach for the biosynthesis of silver oxide nanoparticles using aqueous leaf extract of *Callistemon lanceolatus* (Myrtaceae) and their therapeutic potential

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This study describes a novel biological route for the biosynthesis of silver oxide nanoparticles utilising the aqueous extract of *Callistemon lanceolatus* D.C. leaves. Formation of silver oxide nanoparticles was confirmed by UV−visible spectroscopy, Fourier transform infrared spectroscopy, scanning electron microscope−energy dispersive X-ray spectroscopy and X-ray diffraction spectroscopy analysis. The biologically synthesised silver oxide nanoparticles were found to be 3−30 nm in size with spherical and hexagonal shape by high-resolution transmission electron microscope analysis. Furthermore, the biogenic silver oxide nanoparticles demonstrated significant ($p < 0.05$) dose-dependent antioxidant activity in various *in vitro* antioxidant methods. These particles also exhibited significant ($p < 0.05$) dose-dependent and time-dependent cytotoxic activity towards brine shrimp nauplii. Moreover, the reported method is a simple, cost-effective and eco-friendly approach for the synthesis of silver oxide nanoparticles with useful pharmacological properties.

Keywords: nanoparticle; silver oxide nanoparticle; *Callistemon lanceolatus*; antioxidant activity

1. Introduction

Nanotechnology is the buzzword in the current scientific scenario, and has been instrumental in the development of several research areas, from physics to biology and from drug design to mechanics. Nanoparticles are organic or inorganic particles between 1 and 100 nm in size. Their potential has been established for uses in pharmaceutics, drug delivery systems, biomarkers, optoelectronics, catalytic and sensor technology.[1−5] Another application of nanoparticles is in the synthesis of polymeric membranes that can be used for waste treatment, filtration and gas separation.[6] In a recent review, several industrial applications have been highlighted, such as inert additives, biomimetic materials, antimicrobial compounds and pigments.[7] Nanoparticles have gained popularity for their application in molecular biology, especially as scaffolds for a variety

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of biological molecules, such as antibodies, DNA, RNA and proteins.[8–10] Protein-shelled nanoparticles and dual layered nanoparticles have also been created for a variety of applications in electronics and medicine.[11–13]

Until recently, nanoparticle synthesis could only be carried out using techniques, such as polymerisation, ionic gelation, supercritical fluid extraction and solvent dispersion methods.[1] These techniques involve the use of several chemicals and specialised instrumentation, which add to the cost of production. Also, most of the chemicals used in the aforementioned techniques are non-biodegradable, which causes an adverse impact on the environment. The rise in demand for environment-friendly methods of nanoparticle synthesis has resulted in the exploration of biosynthesis techniques. Plant extract-mediated biosynthesis is shown to be an efficient technique for nanoparticle production.[14–16] Besides being cost-effective, these nanoparticles have been found to be active against bacteria,[17–21] fungi [22] and larvae.[23] In addition, these nanoparticles have been reported to show antioxidant activities [24] and several other therapeutic potentials.[25] This has further cemented their potential for application in medicine.

Silver oxide is known for its ability to act as a sensor for various chemicals, such as ammonia and carbon monoxide. It is also known to catalyse reactions involving the oxidation of ethylene and methanol.[26,27] Silver oxide nanoparticles exhibit good fluorescence [4] and optical properties,[5] and have the potential to be used in data storage devices of the future. Their property of optical fluorescence memory facilitates their usage in optical media to enhance the readout signal by efficient utilisation of laser energy. Silver oxide nanostructures also have the potential to be used in photovoltaic devices,[28–30] optical storage devices [31] and plasmon photonic devices.[32] They can also be used as active cathode materials for zinc/silver oxide batteries.[33,34] Until now, silver oxide nanoparticles have been prepared using reactive sputtering [31,35] and electrochemical/thermochemical [36–38] means. These have resulted in development of techniques for relatively inexpensive silver oxide nanostructures, but require specialised equipment and involve a variety of chemicals that could have an impact on the environment. A biological route for synthesis of silver oxide nanoparticles using *Lactobacillus* extract has also been explored recently.[39] Synthesis of silver oxide nanoparticles using polyethylene glycol (PEG) has also been discussed.[40] However, plant extract mediated synthesis of silver oxide nanoparticles is still unknown.

Synthesis of nanoparticles using herbal extracts is currently a very popular strategy as this approach is cost effective and produces more stable nanoparticles than other methods. Therefore, in this study, *Callistemon lanceolatus* D.C. is used for the synthesis of silver oxide nanoparticles. *C. lanceolatus* is an ornamental plant that belongs to the family Myrtaceae and commonly known as the bottle-brush tree.[41] This plant is known for several medicinal properties, viz., anti-inflammatory properties [42] and inhibition of anticholinesterase activity.[43] It is known to contain several volatile oils,[44] polyphenols [41,45] and triterpenoids [46,47] which might be responsible for its medicinal properties. Hence, in this study, we have demonstrated the biological synthesis of silver oxide nanoparticles using the aqueous extract of *C. lanceolatus* and reported their significant antioxidant and cytotoxic activity. The physicochemical properties of synthesised silver oxide nanoparticles were characterised by UV–visible spectroscopy, X-ray diffraction spectroscopy (XRD), Fourier Transform infrared (FT-IR) spectroscopy, scanning electron microscope–energy dispersive X-ray spectroscopy (SEM-EDX) and high-resolution transmission electron microscope (HRTEM).

2. Materials and methods

2.1. Collection and processing of plant

Fresh leaves of *C. lanceolatus* were collected from the campus at VIT University, Vellore (Lat. 12°58' N; Long. 79°09' E), Tamil Nadu, India, during August 2011. The identity of the plant was verified and a herbarium specimen was maintained in the laboratory for further reference (CL/VIT/MMRL/4.08.2011-1). The freshly collected leaves were washed with distilled water to get rid of surface impurities and chopped finely with sterilised scissors under aseptic conditions. The leaf extract was prepared by boiling 15 grams of finely cut leaves with 50 ml of distilled water in an Erlenmeyer flask for 30 minutes. The concoction was then filtered using Whatman no.2 filter paper. The filtrate, or the aqueous leaf extract, was allowed to cool and stored in an amber coloured airtight bottle at 4 °C for further use.

2.2. Synthesis of silver oxide nanoparticles using C. lanceolatus leaf extract

For preparation of silver oxide nanoparticles, 100 ml of 1 mM AgNO3 solution was taken in an Erlenmeyer flask and allowed to react with 5 ml of freshly prepared aqueous leaf extract of *C. lanceolatus*. One mg of sodium dodecyl sulfate (SDS) was added to stabilise the nanoparticles.[48] The setup was incubated at 37 °C in a shaking incubator under dark conditions to minimise photoactivation of silver nitrate. A control setup consisting of only 1 mM silver nitrate solution was also maintained under the same conditions. The synthesis was monitored using the UV–visible spectroscopy and the absorption readings were noted at regular intervals to identify the time taken for the reaction to occur. After a certain period of time (1–3 hrs), the resulting solution was centrifuged at 10,000 rpm for 10 mins in a cooling centrifuge. The pellet was washed thrice using deionised water to completely remove unwanted surface impurities from the nanoparticles. The pellets were dried on a Petri dish in a hot air oven at 60 °C.[39,49] After drying completely, the powder was scraped off to yield silver oxide nanoparticles. These were stored in Eppendorf tubes at room temperature for further usage and analysis.

2.3. Characterisation of silver oxide nanoparticles

2.3.1. UV–visible spectral analysis

The change in colour in the silver nitrate solution incubated with aqueous plants extract indicated the formation of silver oxide nanoparticles and was monitored using the UV–visible spectroscopy. Aliquots (3 ml) of the mixture were sampled periodically and their spectrum was measured with a UV–visible spectrophotometer (Shimadzu, UV 2500, Japan) in the range 200–800 nm with a resolution of 1 nm.

2.3.2. Fourier transform infrared spectroscopic (FT-IR) analysis

Analysis of different functional groups involved in the biosynthesis of nanoparticles was carried out using FT-IR spectroscopy. For the analysis, 2 mg of the powdered nanoparticle sample was mixed with 20 mg KBr (FT-IR grade) and pressed into a

pellet.[16] The pellet was placed into the sample holder and FT-IR spectra were recorded in the range 4500−450 cm^{-1} in FT-IR spectroscopy (AVATAR 300 FT-IR, Thermo Nicolet, USA).

2.3.3. X-ray diffraction (XRD) analysis

The powdered sample was placed on a glass slide and subjected to XRD analysis using a Bruker D8 Advance Powder Diffractometer. Analysis was carried out over a 2-theta scale of 10−80 degrees. The resultant data was analysed using Match! Software (Crystal Impact, USA; version 1.3b).

2.3.4. Scanning electron microscope-energy dispersive X-ray (SEM-EDX) analysis

After the colour change of the reaction mixture during the biosynthesis of nanoparticle, a drop of nanoparticle suspension was placed on an aluminium coated SEM grid and analysed under an INCA SEM instrument coupled with EDX.

2.3.5. High-resolution transmission electron microscope (HRTEM) analysis

The morphology, size and shape pattern of the biologically synthesised silver oxide nanoparticles were studied by HRTEM. For the analysis, a drop of suspension containing the nanoparticles was placed on a carbon coated TEM grid in a vacuum desiccator and subsequently analysed using HRTEM (JEOL-JEM-2100 instrument, Japan).

2.4. Therapeutic potential of silver oxide nanoparticles

2.4.1. Antioxidant activity

Antioxidant potential of the silver oxide nanoparticles was determined by 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) radical scavenging activity,[50] total antioxidant activity,[51] reducing power potential [52] and β-carotene bleaching activity [53] assays in a dose-dependent manner. Ascorbic acid was used as a positive control and each experiment was performed in triplicates at each concentration.

2.4.2. Cytotoxic activity (brine shrimp lethality assay)

Brine shrimp (*Artemia saline* Leach) eggs (Ocean Star International, Inc., Snowville, USA) were placed in a hatching tank containing sea water for 48 hrs.[54] After hatching, 4 ml of sea water containing 30 brine shrimp nauplii was mixed with 1 ml of silver oxide nanoparticles (125, 250, 500 and 1000 μg/ml in sea water). Survival of brine shrimp nauplii was recorded at 2, 4, 6 and 8 hrs. Experiment was performed in triplicates at each concentration.

2.5. Statistical analysis

The results of the DPPH radical scavenging activity, total antioxidant activity, reducing power, β-carotene bleaching activity and brine shrimp lethality of biogenic silver oxide

nanoparticles are expressed as means ± standard deviations of the responses of three replicates per sample. Statistical significance between the groups was determined by one-way ANOVA coupled with Tukey's *post hoc* test at $p < 0.05$. Statistical analysis was performed with Microsoft Excel 2007 and GraphPad Prism 5.

3. Results and discussion

3.1. Biosynthesis of silver oxide nanoparticles

After addition of the plant extract to $AgNO_3$ solution, it was observed that the colour of the solution changed gradually from colourless to pale brown. The colour change indicated the formation of nanoparticles (Figure 1). The time taken for this reaction to occur was estimated using a time-dependent UV–visible analysis. After 3.5 hrs, the absorbance spectrum with the peak at 435 nm was obtained matching the surface plasmon resonance of silver, which indicated the presence of silver oxide nanoparticles. The peaks at 350 nm may be a result of quadrupole plasmon resonance due to silver transition. Both these peaks match those obtained in the synthesis of Ag2O nanoparticles through other natural methods.[39]

The formation of Ag_2O nanoparticles could be mediated by the presence of polyphenols present in the leaf extract. These contribute the OH-group to Ag^+ and result in the formation of AgOH, which is then oxidised to Ag_2O. AgOH is highly unstable because of the high electronegativity of Ag^+ and the size difference between Ag^+ and OH^-

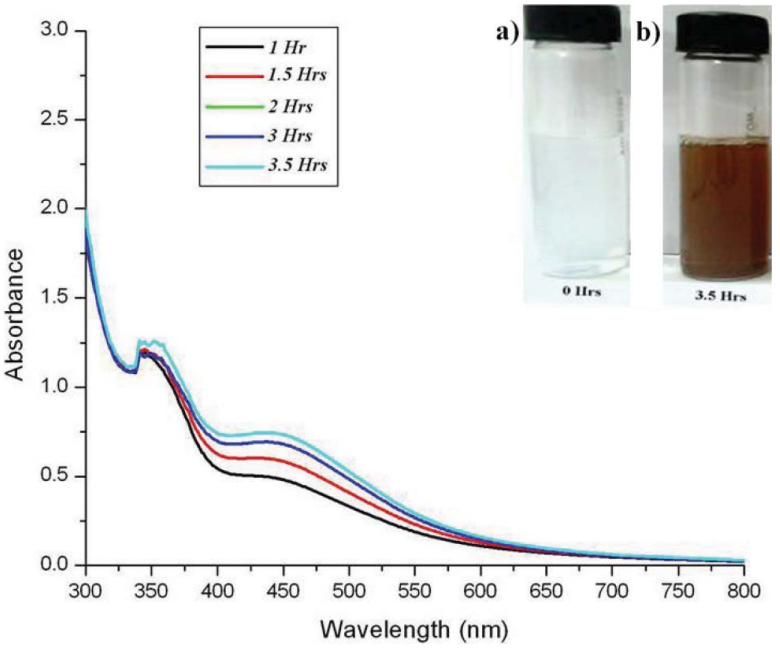

Figure 1. (Colour online) Time-dependant absorbance spectra for Ag_2O nanoparticle synthesis by *C. lanceolatus*; (inset) colour change indicating nanoparticle formation from (a) 0 hrs to (b) 3.5 hrs.

ions. This could account for its conversion into Ag_2O.[55]

$$Ag^+ + OH^- \rightarrow AgOH$$

$$2AgOH \rightarrow Ag_2O + H_2O$$

In the available literature, silver oxide is further reduced to Ag nanoparticles,[5,14–16] but in our experiments we observed that the synthesised Ag_2O nanoparticles were stable and reduction to Ag was not observed.

3.2. Physicochemical characterisation of silver oxide nanoparticles

Physicochemical characterisation of silver oxide nanoparticles was carried out using a combination of spectroscopic and microscopic techniques (XRD, FT-IR, SEM-EDX and HRTEM). The XRD spectra obtained (Figure 2) was found to match with that of the standard spectra of silver oxide (Ag_2O) (JCPDS no. 00-076-1393). Major peaks were obtained at positions that conform to the Miller indices (hkl) of silver oxide (100, 110, 111, 200, 211, 220, 310 and 311).[36,39]

XRD analysis, therefore, confirms the face-centred cubic (FCC) configuration of biosynthesised Ag_2O nanoparticles. The mean particle diameter of silver oxide nanoparticles was calculated from the XRD pattern using the Scherrer equation:

$$D = K\lambda/\beta_{1/2}\cos\theta.$$

Where K is the shape constant, λ is the wavelength of the X-ray, $\beta_{1/2}$ and θ are the half width of the peak and half of the Bragg's angle, respectively. The calculated average crystallite size of the silver oxide nanoparticles was found to be 45 nm.

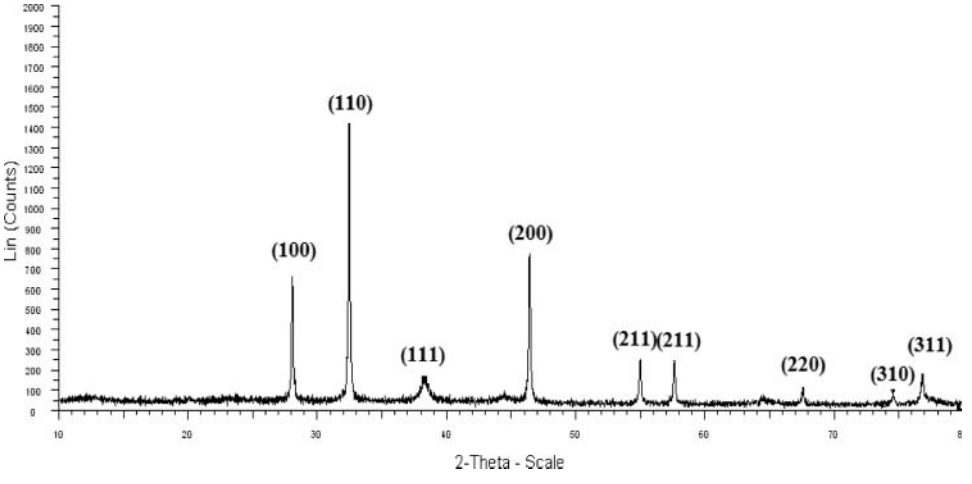

Figure 2. XRD pattern of Ag_2O nanoparticles synthesised using *C. lanceolatus* leaf extract. Data were found to be matching with standard spectra of silver oxide (Ag_2O) (JCPDS no. 00-076-1393).

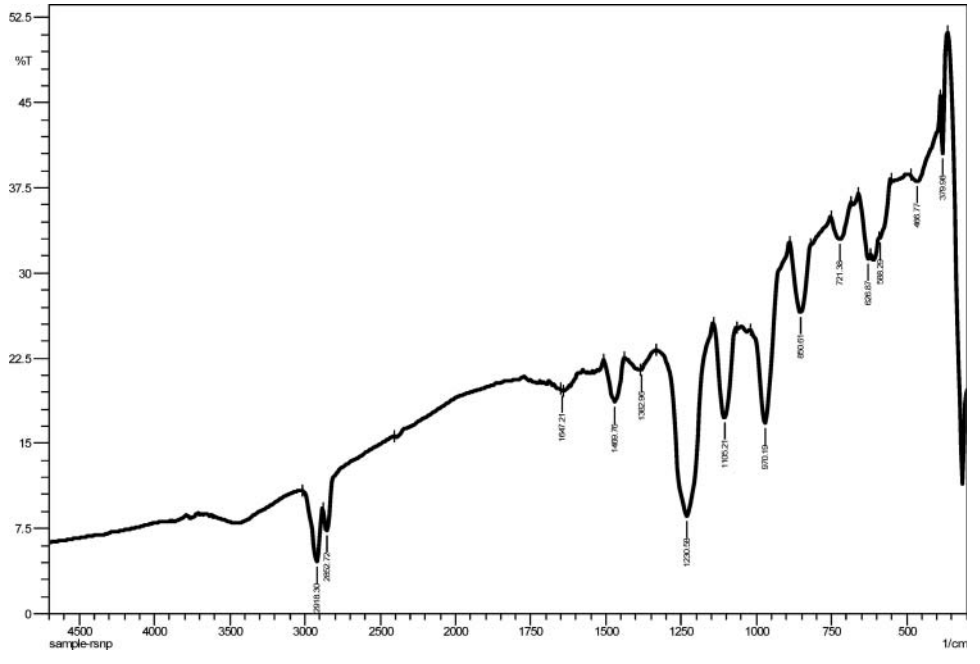

Figure 3. FT-IR spectrum of silver oxide nanoparticles.

FT-IR spectrum of the dried powder of biosynthesised Ag_2O nanoparticles is reported in Figure 3. The band obtained at 588.29 cm^{-1} can be attributed to the Ag−O vibration.[56,57] Other bands can be attributed to silver nitrate and the phytochemical constituents of the leaf extract. Strong bands at 2918.3 and 2882.72 are representatives of alkaline C−H stretch. A faint band at 1647.21 is a representative of alkenyl C−H stretch. Band at 1489.76 is a representative of aromatic C−C stretch. Strong bands at 1382.96, 1230.58 and 1105.21 are representatives of phenolic stretching vibrations. Strong bands at 970.19, 850.61, 721.38 and 626.87 are representatives of aromatic C−H bending.

The results of SEM-EDX analysis of biosynthesised Ag_2O nanoparticles is shown in Figure 4. The results further confirm the presence of silver oxide nanoparticles in the sample. The major components of the sample were found to be Ag and O_2 with a percentage weight ratio of almost 2:1. A peak for Al was seen due to the Al stub used to place the sample in the instrument. Some carbon and sodium signals were seen, which may be attributed to the constituents of the plant extract that still adhered to the nanoparticles after washing.

HRTEM was used to study the size and shape of the synthesised Ag_2O nanoparticles. The results of HRTEM analysis are shown in Figure 5. The results showed that Ag_2O nanoparticles were formed in different sizes, ranging from 3 nm small spherical particles to ~25 nm large hexagonal particles. The HRTEM image suggests that most of the particles are polydispersed and mostly hexagonal in shape (Figure 5(a) and 5(b)). The selected-area electron diffraction (SAED) patterns depicted in Figure 5(c) exhibited concentric rings with intermittent bright dots, indicating that these nanoparticles are highly crystalline in nature. The SAED pattern conforms to the Miller indices of Ag_2O as obtained in the XRD data.

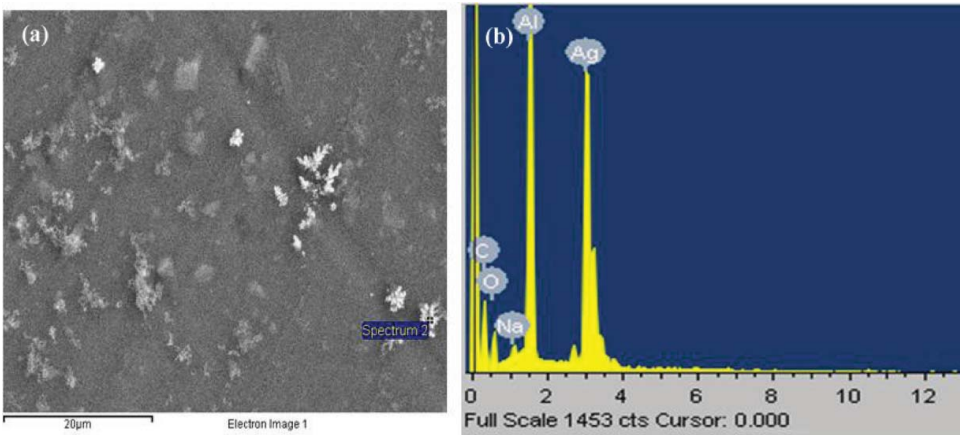

Figure 4. (a) Scanning electron microscopic (SEM) and (b) energy-dispersive **X**-ray (EDX) analysis of silver oxide nanoparticles.

3.3. Antioxidant activity

Free radicals produced in the body are commonly controlled by the antioxidant defence system of the body. Antioxidants are the compounds commonly known to neutralise the free radicals or terminate the chain reaction initiated by the free radicals, therefore, can be used effectively to support the natural antioxidant defence of the body to control the oxidative stress. Lack of antioxidants can lead to accumulation of large quantities of free radicals that can disrupt cells and causes several diseases, or sometimes increase the severity of a disease.[58,59] At present, more than a hundred different types of diseases are reported to associate with free radical-mediated oxidative stress, such as metabolic disorders, renal disorders, gastrointestinal disorders, cardiovascular disorders and neurodegenerative disorders, pulmonary disorders, autoimmune disorders, liver disease, ocular diseases, cancer and ageing.[60]

Usually, fruits, vegetables, plants, algae and microorganisms are considered to be a potent source of natural antioxidants. In addition to them, in recent past, some researchers have reported the antioxidant potential of the biologically synthesised nanoparticles in *in vitro* and

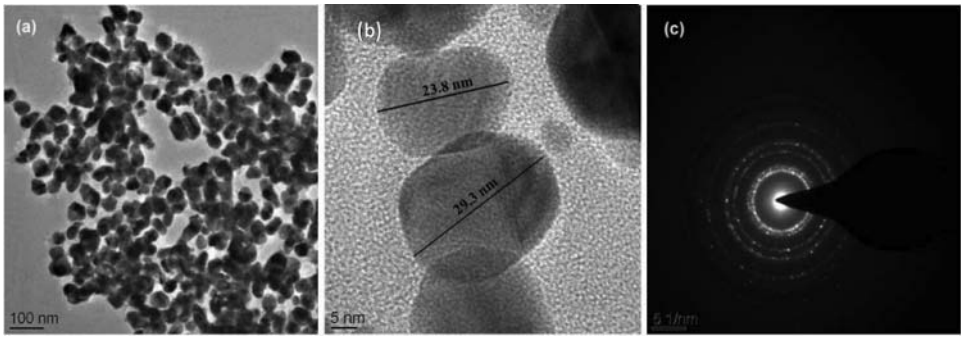

Figure 5. HRTEM image of silver oxide nanoparticles. (a) Nanoparticles as seen through HRTEM, (b) single nanoparticles observed under high resolution and (c) SAED pattern.

in vivo systems.[24,61] However, there is no report on the antioxidant properties of biogenic silver oxide nanoparticles. Therefore, during this study, biogenic silver oxide nanoparticles were evaluated for its antioxidant activity by DPPH radical scavenging activity, total antioxidant activity, reducing power potential and β-carotene bleaching activity test.

3.3.1. *Total antioxidant activity*

During total antioxidant activity test, molybdate (VI) was reduced to molybdate (V) at acidic pH by an antioxidant and forms a green colour compound which can be quantified spectrophotometrically at 695 nm.[51] In this study, aqueous suspension of silver oxide nanoparticles exhibited significant ($p < 0.05$) dose-dependent total antioxidant activity with an optical density (OD) value of 0.433 ± 0.058 (at 200 μg/ml). The results are expressed as mean ± standard deviation ($n = 3$). The total antioxidant activity of the extract was compared with the ascorbic acid standard and reported in Figure 6(a).

3.3.2. *DPPH radical scavenging activity*

DPPH is a purple coloured free radical which reduced to yellow colour after receiving a proton from an antioxidant. This change in the colour can be measured by using a UV−visible spectrophotometer at 517 nm.[62] During this study, aqueous suspension of silver oxide nanoparticles exhibited significant ($p < 0.05$) dose-dependent DPPH radical scavenging activity with IC_{50} value = 62.12 μg/ml. The results are represented as

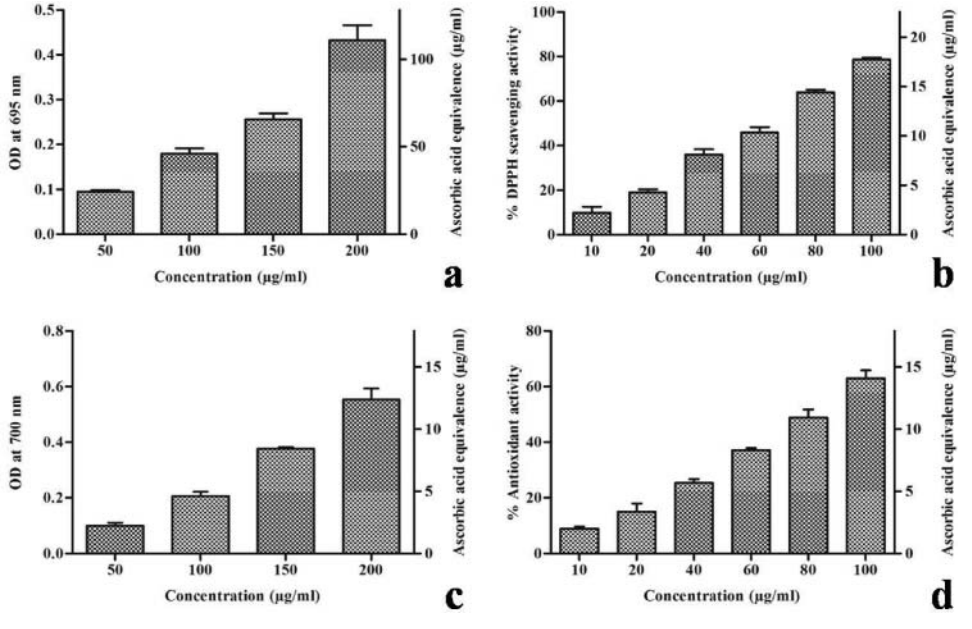

Figure 6. Antioxidant activity of varying concentrations of biologically synthesised silver oxide nanoparticles. Data are given in mean ± SD ($n = 3$ test, $p < 0.05$). Data were compared with the ascorbic acid standard. (a) Total antioxidant activity, (b) percentage DPPH radical scavenging activity, (c) reducing power potential and (d) β-carotene bleaching activity.

percentage inhibition of DPPH and expressed as mean ± standard deviation ($n = 3$). The DPPH radical scavenging activity of the extract was compared with the ascorbic acid standard and reported in Figure 6(b).

3.3.3. Reducing power activity

During reducing power assay, Fe (III) reduced to Fe (II) after receiving an electron from the antioxidant. Reduction of Fe (III) to Fe (II) ions produces a bluish green colour in the reaction mixture, which could be measured spectrophotometrically at 700 nm.[53] In this study, aqueous suspension of silver oxide nanoparticles exhibited significant ($p < 0.05$) dose-dependent reducing power activity with an OD value of 0.553 ± 0.040 (at 200 μg/ml). The results are expressed as mean ± standard deviation ($n = 3$). The total antioxidant activity of the extract was compared with the ascorbic acid and reported in Figure 6(c).

3.3.4. β-carotene bleaching activity

During the β-carotene bleaching assay, linoleic acid free radicals attack the β-carotene molecules and bleach its characteristic orange colour. Presence of an antioxidant can neutralise the linoleate free radical and thus help to retain the colour of β-carotene, which can be monitored at 450 nm using a UV−visible spectrophotometer.[63] During this study, aqueous suspension of silver oxide nanoparticles exhibited significant ($p < 0.05$) dose-dependent β-carotene bleaching activity with IC_{50} value = 80.45 μg/ml. The results are represented as percentage inhibition of β-carotene bleaching and expressed as mean ± standard deviation ($n = 3$). The inhibition of β-carotene bleaching activity of the extract was compared with the ascorbic acid and reported in Figure 6(d).

3.4 . Brine shrimp lethality

The brine shrimp cytoxicity assay was considered as a convenient probe for preliminary assessment of cytotoxicity of the samples.[52] Cytotoxic activity of biologically synthesised

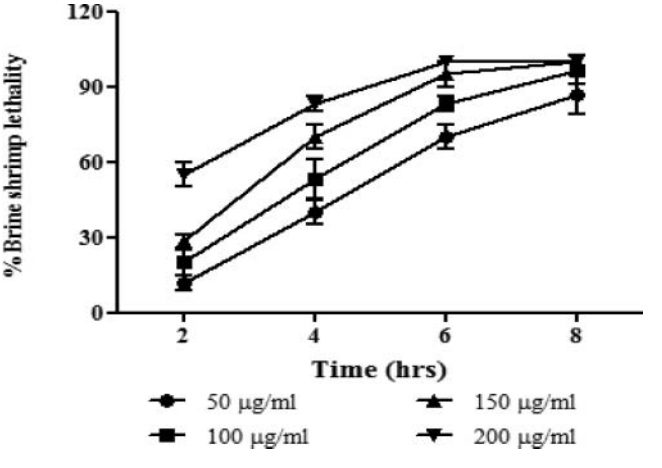

Figure 7. Brine shrimp lethality of varying concentrations of biologically synthesised silver oxide nanoparticles. Data are given in mean ± SD ($n = 3$ test, $p < 0.05$).

silver oxide nanoparticles was evaluated by brine shrimp lethality test in a dose-dependent and time-dependent manner. Silver oxide nanoparticles exhibited high cytotoxic activity against brine shrimp nauplii with an LC_{50} value 85.32 μg/ml and LC_{90} value 221.8 μg/ml (time: 4 hrs). Increase in the treatment time also resulted in an increase in the activity. Results are summarised in Figure 7.

4. Conclusion

As per our literature review, this is the first report of the biosynthetis of Ag_2O nanoparticles from *C. lanceolatus*. This study reports the novel mechanism for the biosynthesis of silver oxide nanoparticles using an aqueous leaf extract of *C. lanceolatus*. Biologically synthesised nanoparticles were 3–30 nm in size and oval or hexagonal in shape. Furthermore, the molecules demonstrated significant antioxidant and cytotoxic potential in various *in vitro* assays, which emphasises on its possible applications in medicine apart from its utilisation in optoelectronics and storage devices. Hence, it could be concluded that this study reports a novel, rapid, economical and environmental friendly method for the production of biologically active silver oxide nanoparticles.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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