Research Article

Synthesis of silver nanoparticles using extract of *Ocimum kilimandscharicum* and its antimicrobial activity against plant pathogens

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Abstract
Biological synthesis of silver nanoparticles using plants is gaining importance as an economically viable and ecofriendly approach. The present study reports synthesis of silver nanoparticles using an important Himalayan medicinal herbal plant *Ocimum kilimandscharicum* and the potential of biosynthesized silver nanoparticles for control of plant pathogens. The reducing effect of the aqueous plant extract for synthesis of silver nanoparticles was investigated at different concentrations and temperatures. UV–visible absorption spectrum recorded for samples prepared with different concentrations (20, 30, 40 and 50% v/v) of plant extract and treated at different temperatures (40, 60, 80 °C and room temperature) confirmed the formation of silver nanoparticles. Antimicrobial activity of the silver nanoparticles was tested against *Fusarium oxysporum*, *Colletotrichum gloeosporioides*, *Bacillus* sp. and *Enterobacter cloacae*. Complete inhibition of *F. oxysporum* was observed at 75 ppm concentration of silver nanoparticles as compared to 67.75 ± 1.15 mm radial growth without silver nanoparticles after 7 days of incubation. No growth of *C. gloeosporioides* was observed in vitro at 100 ppm silver nanoparticles as compared to 44.50 ± 1.14 mm radial growth in the absence of the silver nanoparticles. Inhibitory effect of biosynthesized silver nanoparticles was also observed against bacterial pathogens. The highest inhibition zone of 14.5 ± 1.11 mm was observed against *E. cloacae* with 100 ppm silver nanoparticles. The obtained results confirm strong antimicrobial activity of the silver nanoparticles synthesized using *O. kilimandscharicum* against plant pathogens.

Keywords Silver nanoparticles · *Ocimum kilimandscharicum* · Antifungal activity · Antibacterial activity · Plant pathogens

1 Introduction

Management of plant pests and pathogens for controlling plant diseases and thereby reducing production losses has great economic significance and role in food security [1]. A number of bacterial and fungal pathogens are known to affect vegetable and other food crops. Various natural and artificial methods for control of plant diseases have been applied. Synthetic chemical fungicides and pesticides are most commonly used in agriculture for control of plant diseases. Extensive use of fungicides and pesticides is causing environmental pollution and poses a potential risk to human health [2]. The chemicals used for control of disease are also affecting beneficial non-target species in the environment and impacting biodiversity.

Development of resistance in plant pathogens to commercially available fungicides due to their continuous application is also a increasing concern in recent years [3]. Emerging virulent forms of phytopathogens are also making the pathogen-resistant cultivars ineffective. Biocontrol agents as easy-to-apply and environment-friendly tool for management of plant diseases have been studied.

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The antagonistic potential of several microbes has been reported [4]. However, certain difficulties are associated with biocontrol methods like host specificity and efficacy on environmental parameters. This has resulted in search for new fungicides and alternatives to combat emerging and resistant strains of plant pathogens.

Nanotechnology has immense potential in agriculture for crop protection, controlled release of agrochemicals, transfer of target genes, plant hormone delivery and early detection of plant disease using nanosensors [5]. The use of nanoparticles as safe and effective management method for control of phytopathogens is being explored [6]. Most common nanoparticles that have entered into the arena of plant disease management are nanoforms of silver [7], copper [8], chitosan [9], titanium dioxide [10], magnesium and zinc [11]. Among the metal nanoparticles, efforts have been focused on using silver nanoparticles in agriculture for control of plant pathogens.

Silver nanoparticles are well known to exhibit strong antimicrobial activity against bacteria [12], viruses [13] and fungi [14] due to their unique physical, biological and chemical properties. Antimicrobial property of silver nanoparticles has been exploited to a great extent against a broad range of human pathogens [15]. Many workers have advocated the antibacterial and antifungal efficacy of silver nanoparticles against a diverse and broad range of plant pathogens [16]. However, the beneficial use of silver nanoparticles in agriculture to control and combat plant pathogens is not fully explored.

Chemical and physical methods for synthesis of metallic nanoparticles are often costly and associated with the use of hazardous chemicals. Several biological approaches have been developed as an alternative to obtain non-toxic, inexpensive and ecofriendly nanoparticles [17]. Biogenic synthesis of silver nanoparticles using fungi [18], bacteria [19] and plants [20] are reported. Biological synthesis method using plant is expected to have many advantages over the use of bacteria and fungi. Among these advantages are the easy availability of plants and the presence of a broad range of metabolites that aid in the synthesis of silver nanoparticles.

Ocimum kilimandscharicum Guerke belonging to the family Lamiaceae is an economically important perennial herb. Its occurrence in India was recorded first from the wild habitats of western Himalayan region, Uttarakhand. *O. kilimandscharicum* Guerke, commonly known as camphor basil (Kapur Tulsi), is highly valued for its therapeutic and aromatic properties. In traditional medicine, the plant is used for the treatment of cold, cough, malaria, abdominal pains and diarrhoea [21]. Biologically active constituents from the extracts of whole plant have insecticidal [22], fungistatic and antimicrobial activity [23]. Farmers commonly mix stored grains, pulses and other foodstuff with dry leaves or leaf powder for protection against pests. The presence of ursolic acid imparts leaves of *O. kilimandscharicum* therapeutic potential as an anticancer agent. *O. kilimandscharicum* Guerke is also reported to have analgesic, antiseptic and antioxidant properties.

The use of *O. kilimandscharicum* in pharmaceutical, agriculture, industrial and ornamental sectors has been explored. However, there is no report on the synthesis of silver nanoparticles using *O. kilimandscharicum* Guerke. The present study was designed with an objective of biosynthesis of silver nanoparticles using the medicinal herb *O. kilimandscharicum* and its potential for control of plant pathogenic fungi and bacteria.

2 Materials and methods

2.1 Preparation of plant extract

Fresh leaves from *O. kilimandscharicum* were collected from the herbal garden of Defence Institute of Bio-Energy Research Field Station located in Pithoragarh, Uttarakhand, India (Fig. 1a). Fresh leaves were surface cleaned under running tap water, followed by deionized water. Leaves (25 g) that had been rinsed with deionized water were finely cut into small pieces and transferred to 500-mL beaker. The chopped leaves were boiled in 250 mL of deionized water for 20 min and allowed to cool. The cooled leaf broth was filtered using Whatman No. 1 filter paper. The resulting extract was stored in a refrigerator at 4 °C for further use.

2.2 Biosynthesis of silver nanoparticles

Aqueous solution (10 mM) of silver nitrate ($AgNO_3$) was prepared and transferred to an amber-coloured bottle. The aqueous leaf extract of *O. kilimandscharicum* was added

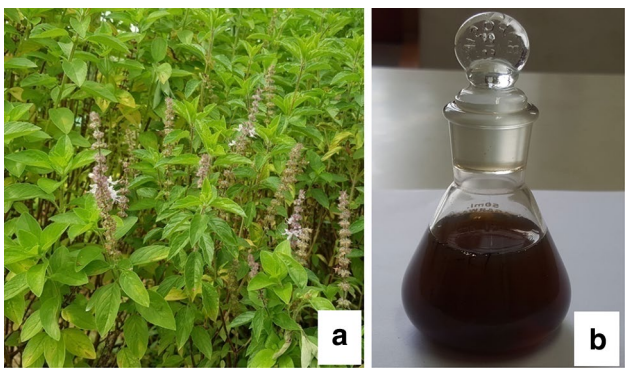

Fig. 1 **a** Plant of *Ocimum kilimandscharicum*, **b** silver nanoparticles synthesized using the leaf extract

to different flasks containing 1 mM $AgNO_3$ for bioreduction. Plant extract at concentrations of 20, 30, 40 and 50% (v/v) was added to 1 mM $AgNO_3$, and the final volume was adjusted to 100 mL by adding the appropriate amount of deionized water. The stoppered flasks were incubated at room temperature for 24 h. The change in colour of the solution from yellowish to dark reddish brown was observed (Fig. 1b). The formation of silver nanoparticles in the presence of extract of *O. kilimandscharicum* was also examined under different temperature conditions. Plant extract (30% v/v) and aqueous solution of $AgNO_3$ (1 mM) were mixed. The contents were heated at different temperatures of 40, 60 and 80 °C for 20 min. The obtained silver nanoparticle suspensions were analysed spectrophotometrically. The suspensions were subsequently examined for their antimicrobial activity.

2.3 Determination of antioxidant constituents of the plant extract

2.3.1 Total phenolic content

The total phenolic content in the plant extract was determined by using Folin–Ciocalteu method [24]. In brief, plant extract was mixed with Folin–Ciocalteu reagent and sodium carbonate (20%). The test solutions were warmed for 1 min, cooled, and absorbance was measured at 650 nm. The total phenol content (mg/g) was measured equivalent to catechol by using the standard curve.

2.3.2 Flavonoid content

The total flavonoid content was determined by aluminium chloride colorimetric method [25]. The plant extract was mixed well with methanol, aluminium chloride solution and potassium acetate solution. Absorbance was measured after incubation for 30 min at 415 nm against the suitable blank (all reagents except aluminium chloride). The concentration of total flavonoid content (mg/g) of the sample was measured by using standard curve of quercetin.

2.3.3 Tannin content

Total tannin content was determined by Folin–Denis method [26]. In brief, the plant sample was boiled for 30 min with double-distilled water. The supernatant after centrifugation was mixed with Folin–Denis reagent and sodium carbonate solution. The absorbance was measured at 700 nm after 30 min. Total tannin content (mg/g) of the sample was measured equivalent to tannic acid by using the standard curve.

2.4 Determination of antioxidant activity of plant extract

2.4.1 DPPH free radical scavenging assay

The total antioxidant activity was determined following the earlier reported method [27] based on free radical scavenging of DPPH (1,1-diphenyl-2-picrylhydrazyl) radicals. In brief, methanolic solution of DPPH (0.1 mM) was mixed with various volumes of plant extract and absorbance was measured after 40 min at 517 nm against methanol as blank. Ascorbic acid was used as standard. Free radical scavenging activity (FRSA) % of test samples was evaluated by comparing with control (DPPH with methanol).

The percentage FRSA of DPPH radicals at different concentrations was determined using the formula:

$$\text{FRSA}\,(\%) = \left[(\text{Ac}-\text{At})/\text{Ac}\right] \times 100, \text{ where,}$$

$$\text{Ac} = \text{absorbance of control,}$$

$$\text{At} = \text{absorbance of test.}$$

Further IC-50 value (mg/g) was calculated using the formula:

$$\text{IC}-50 \text{ value} = (\text{conc. of test/FRSA nearest to the } 50\%) \times 50$$

2.4.2 ABTS free radical scavenging assay

The total antioxidant activity in terms of ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)) free radical scavenging was determined following an earlier reported method [28]. The percentage free radical scavenging activity of $ABTS^{\cdot+}$ radicals at different concentrations and IC-50 values (mg/g) was calculated as described in DPPH free radical scavenging assay.

2.5 UV–Visible spectroscopy analysis

The synthesized silver nanoparticles were characterized by ultraviolet–visible spectroscopy. Double-beam UV–Vis spectrophotometer UV5704SS (ECIL, Hyderabad, India) was employed for the spectrometric analysis of the biosynthesized silver nanoparticles. The bioreduction of silver and the formation of silver nanoparticles by leaf extract of *O. kilimandscharicum* were scanned in the 200–800 nm wavelength range. To determine the optimum conditions for maximum production of silver nanoparticles, the absorption spectra of the samples prepared with different concentrations of plant extract and at different temperatures were taken.

2.6 Evaluation of antifungal activity of silver nanoparticles

Antifungal activity of silver nanoparticles synthesized by *O. kilimandscharicum* was tested in vitro against *Fusarium oxysporum* and *Colletotrichum gloeosporioides*. The test fungal cultures *F. oxysporum, C. gloeosporioides* were grown on potato dextrose agar (PDA) incubated at 25 °C for three days. In vitro assay was performed using silver nanoparticles synthesized in the presence of different concentrations of plant extract and at different temperatures. Further testing was carried out with different concentrations (25, 50, 75 and 100 ppm) of silver nanoparticles synthesized in the presence of optimum concentration of plant extract and heating temperature. Medium containing silver nanoparticles was poured into 90-mm petri dishes. Agar plugs of uniform size (8 mm diameter) containing fungi were inoculated at the centre of each petri dish supplemented with silver nanoparticles. For control treatment, culture medium without silver nanoparticles was inoculated with agar plug containing fungi. The plates were incubated at 25 °C for 7 days. Radial growth of fungal colonies was recorded after 4 and 7 days of incubation. The per cent growth inhibition was calculated using the radial growth of mycelium according to the following formula:

$$\text{Inhibition rate } (\%) = (R-r)/R \times 100$$

where R is radial growth of fungi in control and r is the radial growth of fungi in treated plates.

2.7 Evaluation of antibacterial activity of silver nanoparticles

The silver nanoparticles synthesized from *O. kilimandscharicum* were tested for their antibacterial activity by agar well diffusion method. The antibacterial assay was carried out against both gram-negative bacteria *Enterobacter cloacae* and gram-positive bacteria *Bacillus* species. The test bacterial cultures were inoculated in nutrient broth and incubated overnight at 32 °C. Fresh bacterial suspension 10^6 CFU/ml of each strain was swabbed uniformly on the soyabean casein digest agar (SCDA) plates. Wells of size 6 mm were made in the agar plates using gel puncture. Different concentrations (25, 50, 75 and 100 ppm) of synthesized nanoparticle solution to be tested were poured into wells using micropipette. The plates were incubated at 35 °C for 24 h. After incubation the antibacterial activity was determined by measuring the zone of inhibition (mm) around each well. For each experiment, four replicates were performed.

2.8 Statistical analysis

Experiments were conducted in a completely randomized design with four replicates for all the treatments. The obtained data were analysed by analysis of variance (ANOVA) using Crop Stat (IRRI, Philippines). The mean values were compared using the Duncan's new multiple range test at 0.05 probability level.

3 Results

Biosynthesis of silver nanoparticles was studied using aqueous extract of leaves of *O. kilimandscharicum*. It was observed that the colour of the aqueous silver nitrate solution changed to reddish brown upon the addition of the plant extract. This indicated the formation of silver nanoparticles. No colour change was observed for plant extract solution without silver nitrate or solution with silver nitrate alone. The optical absorbance of silver nanoparticles synthesized using *O. kilimandscharicum* aqueous extract was recorded using a UV–Vis spectrophotometer. The UV–Vis absorption spectra exhibiting an absorption band around 450 nm confirmed the formation of silver nanoparticles using *O. kilimandscharicum*.

Phytochemical analysis of *O. kilimandscharicum* leaf extract was carried out to determine the antioxidant constituents responsible for reduction and stabilization of silver nanoparticles (Fig. 2). *O. kilimandscharicum* leaves were found to be rich in phenolic content. Total phenolic content was 12.48 ± 0.08 mg/g in aqueous extract and 10.57 ± 0.11 mg/g in methanolic extract. Flavonoids varied from 6.41 ± 0.04 to 7.90 ± 0.13 mg/g. Higher tannin content of 20.59 ± 0.38 mg/g in aqueous extract and 21.42 ± 0.08 mg/g in alcoholic extract was observed. Antioxidant property of the leaf extract was determined by DPPH assay and ABTS assay (Fig. 3). The antioxidant activity

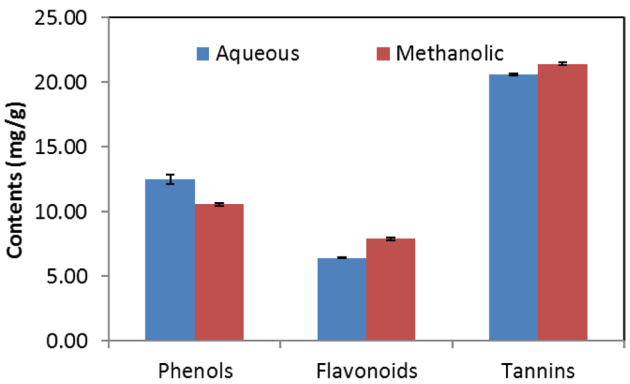

Fig. 2 Antioxidant constituents in extract of *Ocimum kilimandscharicum*

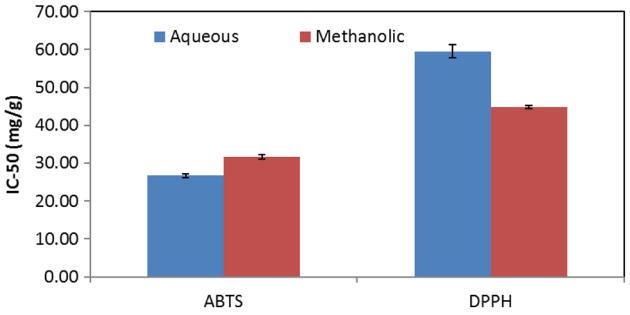

Fig. 3 Free radical inhibition concentration of aqueous and alcoholic extracts of *Ocimum kilimandscharicum*

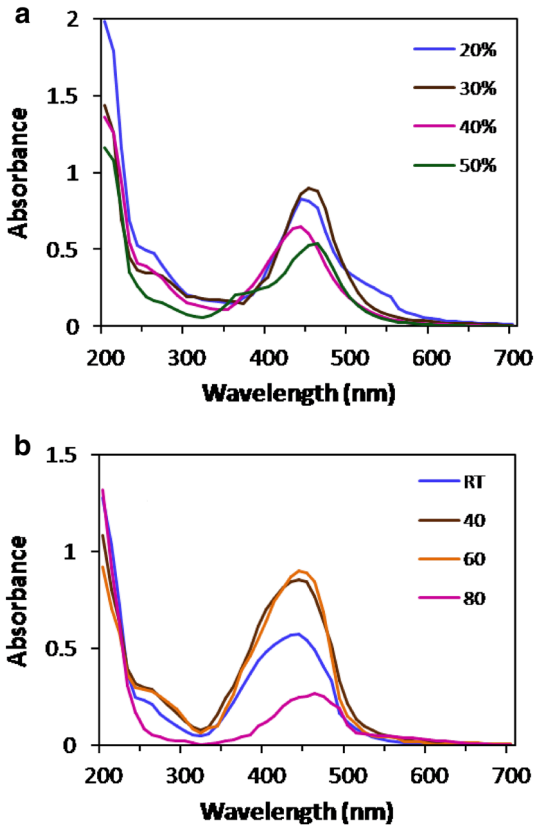

Fig. 4 UV–Vis absorption spectra of silver nanoparticles synthesized **a** in the presence of different concentrations of plant extracts and **b** at different temperatures

of aqueous extract determined as inhibition concentration 50 (IC-50 value) was found to be 59.51 ± 1.71 mg/g in DPPH assay and 26.66 ± 0.51 mg/g for ABTS assay. Free radical scavenging activity was recorded to be 31.61 ± 0.54 to 44.83 ± 0.37 mg/g for methanolic extract. The results confirm the presence of phenolics, flavonoids and tannins in *O. kilimandscharicum* that are responsible for reduction and may act as capping agent. High antioxidant constituents and strong antioxidant activity observed in the leaf extract support the synthesis of silver nanoparticles using *O. kilimandscharicum*. The effect of different concentrations of plant extract (20–50% v/v) on UV–Vis absorption spectra is presented in Fig. 4a. The optical properties of the silver nanoparticles synthesized using *O. kilimandscharicum* were found to be dependent on the initial concentration of the plant extract. With 20% plant extract and 1 mM silver nitrate solution, the absorption peak in the UV–Vis spectrum was exhibited by silver nanoparticles at 450 nm due to the surface plasmon resonance. Shift in wavelength of the plasmon bands was observed with different concentrations of the plant extract to the metal ion. An increase in absorbance with remarkable blue shifts in the plasmon bands was observed with 30% plant extract. With further increase in concentration of the plant extract to 40% and 50%, shift in the plasmon absorption band to higher wavelength was recorded.

Temperature is an important physical parameter in the synthesis of silver nanoparticles. The synthesis of nanoparticles mediated by *O. kilimandscharicum* leaf extract was studied at 40, 60, 80 °C and room temperature (Fig. 4b). An increase in absorbance with temperature for nanoparticles synthesized at 40 and 60 °C was observed. The comparison of UV spectra at different temperatures suggests the low rate of silver nanoparticles at room temperature was enhanced by increasing the temperature of the reaction mixture. An increase in the temperature caused rapid reduction rate of the Ag+ ions and subsequent homogeneous nucleation of silver nuclei, resulting in the formation of silver nanoparticles. Appearance of small broad surface plasmon resonance band at higher temperature of 80 °C indicates the formation of nanoparticles with large size.

Silver nanoparticles synthesized using different concentrations of plant extract of *O. kilimandscharicum* and at different temperatures were tested for their antimicrobial activity against plant pathogens. Antifungal activity of silver nanoparticles against *F. oxysporum* (FO) and *C. gloeosporioides* (CG) synthesized in the presence of 20, 30, 40 and 50% plant extract is presented in Fig. 5a. Mycelial growth of fungi, as measured on the petri dishes, was used to quantify the effect of silver nanoparticles on the phytopathogens. The results show that the inhibition rate differs for silver nanoparticles synthesized with various concentrations of plant extract of *O. kilimandscharicum*. The highest inhibition of 66.5% was observed against *F. oxysporum* at 30% concentration of plant extract, and the lowest inhibition of 28.3% was observed at the highest concentration of 50% plant extract. Inhibition of *C. gloeosporioides* in the presence of silver nanoparticles synthesized using different concentrations of plant extract varied from 18.4 to 48.7%. The result shows that the inhibition rate varies with the concentration of plant extract. Plant extract at 30% concentration was the most favourable for

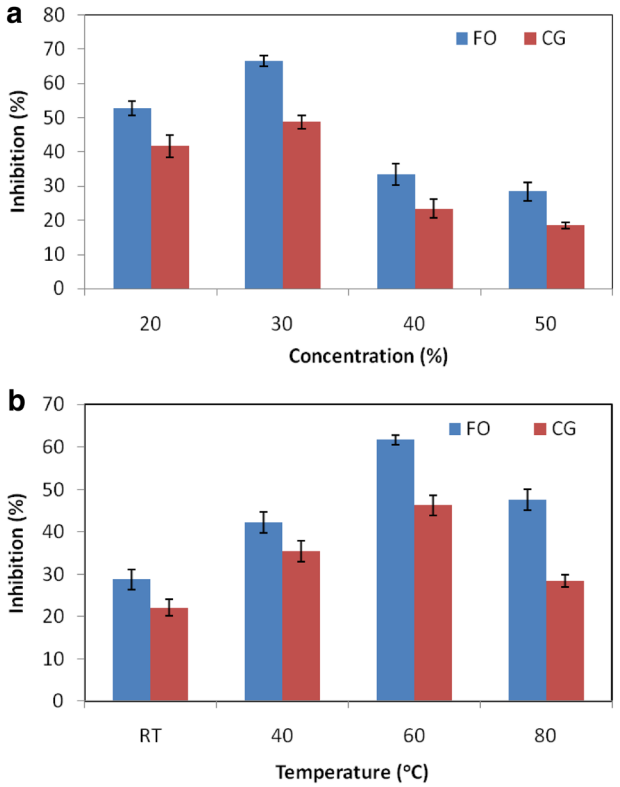

Fig. 5 Antifungal activity of silver nanoparticles synthesized **a** in the presence of different concentrations of plant extracts and **b** at different temperatures

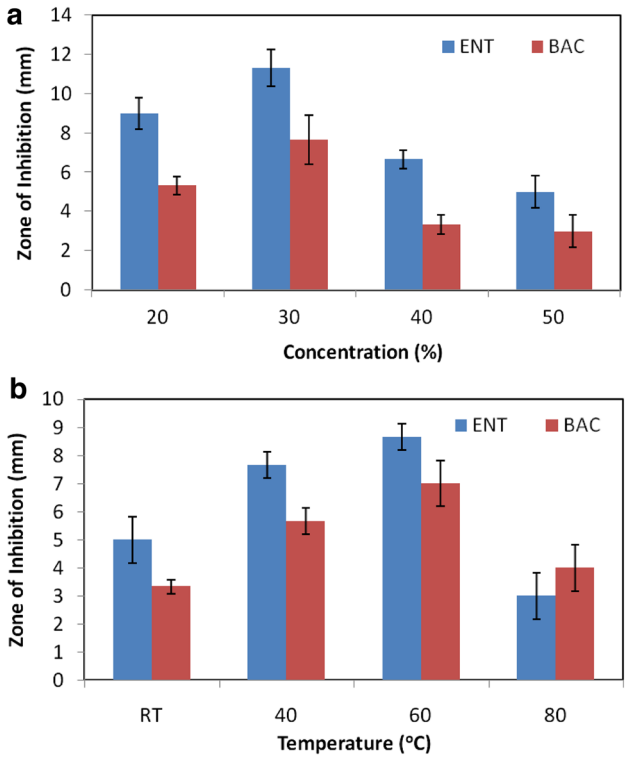

Fig. 6 Antibacterial activity of silver nanoparticles synthesized **a** in the presence of different concentrations of plant extracts and **b** at different temperatures

the synthesis of silver nanoparticles and suppression of pathogens in vitro.

Antifungal activity of silver nanoparticles synthesized using extract of *O. kilimandscharicum* at different temperatures (room temperature, 40, 60 and 80 °C) for 10 min is presented in Fig. 5b. The inhibition of *F. oxysporum* was 28.7% on PDA at 50 ppm concentration with sample prepared at room temperature. Higher inhibition percentage was recorded with heated samples. In total, 42% inhibition for *F. oxysporum* was recorded on PDA at 40 °C, 62% at 60 °C and 47% at 80 °C temperature. The difference in growth of *C. gloeosporioides* was also observed for samples prepared at different temperatures. The inhibition rate observed with sample prepared without heating at room temperature at 50 ppm concentration of nanosilver on PDA media was 22%. The inhibitory effect of heated samples was higher than room temperature. Higher inhibition per cent of 28% to 46% for nanosilver synthesized at 40 to 80 °C was observed. The antagonistic effect of nanosilver against fungi was more notable for the samples prepared by heating as compared to the sample at room temperature. Nanosilver synthesized using extract of *O. kilimandscharicum* had the best inhibitory effect against both the fungi on treatment at 60 °C for 10 min.

Silver nanoparticles synthesized with various concentrations of plant extract and at different temperatures were tested at concentration of 50 ppm against bacterial species. Significant inhibition of both gram +ve *Bacillus* sp. and gram −ve bacteria *E. cloacae* with 50 ppm silver nanoparticles on soyabean casein digest agar plates was evident. Zone of inhibition observed was 7.7 ± 1.2 mm for *Bacillus* sp. (BAC) and 11.3 ± 0.9 mm for *E. cloacae* (ENT) in the presence of silver nanoparticles synthesized using 30% plant extract concentration (Fig. 6a). For both the bacteria, the inhibition zone was higher with silver nanoparticles synthesized using 30% plant extract as compared to 20% plant extract. The bacterial inhibition was found to decrease with further increase in plant extract concentration. The inhibition induced by silver nanoparticles synthesized at different temperatures against bacterial growth is presented in Fig. 6b. The highest inhibition zone of 7.0 ± 0.8 mm for *Bacillus* sp. and 8.6 ± 0.5 mm for *E. cloacae* was observed with nanosilver samples prepared at 60 °C. The inhibitory effect of silver nanoparticles increased at temperature of 40 °C and 60 °C as compared to room temperature. However, the inhibition zone was significantly reduced at higher temperature of 80 °C.

The silver nanoparticles synthesized with 30% plant extract of *O. kilimandscharicum* at room temperature

Table 1 Antifungal activity of silver nanoparticles against *Fusarium oxysporum*

Treatment	Radial growth (mm)	
	4 days	7 days
AgNP-RT25 ppm	23.00b	45.50b
AgNP-RT50 ppm	14.75d	38.25c
AgNP-RT75 ppm	0.00e	0.00e
AgNP-RT100 ppm	0.00e	0.00e
AgNP-H25 ppm	25.00b	46.75b
AgNP-H50 ppm	19.50c	40.25c
AgNP-H75 ppm	12.50d	28.50d
AgNP-H100 ppm	0.00e	0.00e
Control	44.50a	67.75a
SE ($N=4$)	1.14	1.15
5% LSD	3.34	3.37

Mean ($N=4$) values in a column marked with different letters are statistically significant according to least significant difference (LSD) test at $P \leq 0.05$

AgNP-RT, silver nanoparticles synthesized at room temperature; AgNP-H, silver nanoparticles synthesized at 60 °C

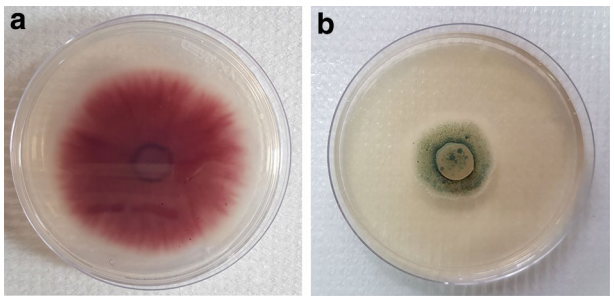

Fig. 7 Growth of **a** *Fusarium oxysporum* and **b** *Colletotrichum gloeosporioides* in the absence of silver nanoparticles

incubation for 24 h and heating at 60 °C for 20 min were tested for activity against the fungal and bacterial plant pathogens. In vitro assay was carried out at different concentrations of 25, 50, 75 and 100 ppm of silver nanoparticles. Antifungal activity of silver nanoparticles was studied against plant pathogenic fungus *F. oxysporum*. Radial growth was measured after 4 and 7 days of incubation on treatment with silver nanoparticles synthesized at room temperature incubation for 24 h or after heating at 60 °C for 20 min (Table 1). The radial growth was significantly ($P \leq 0.05$) higher in control (44.50 and 67.75 mm after 4 and 7 days, respectively) than the treatments with silver nanoparticles (Fig. 7a). Complete inhibition of the fungal growth was observed in silver nanoparticles (75 and 100 ppm) synthesized at room temperature (Fig. 8). In case of silver nanoparticles synthesized after heating at 60 °C, the complete inhibition of the fungal growth was

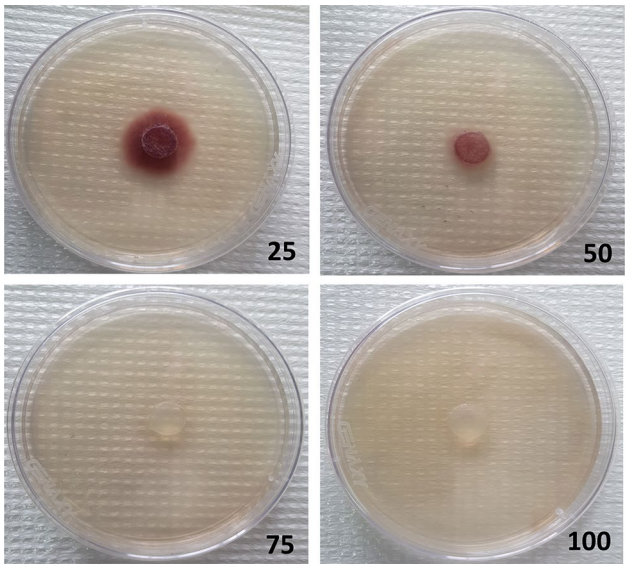

Fig. 8 Antimicrobial activity of different concentrations (25–100 ppm) of silver nanoparticles synthesized at room temperature against *Fusarium oxysporum*

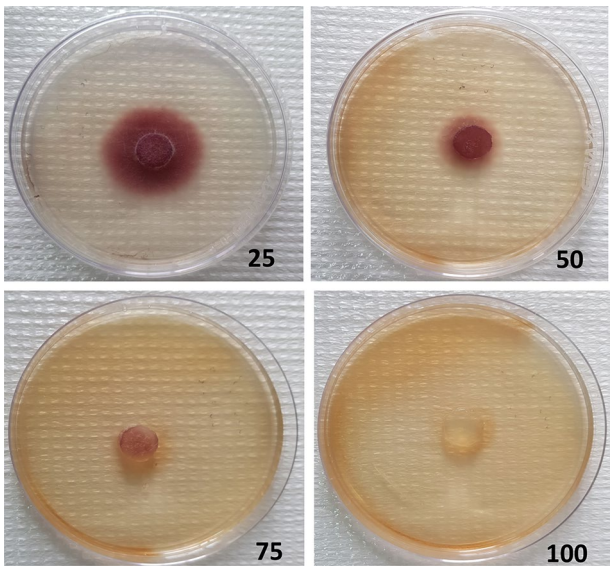

Fig. 9 Antimicrobial activity of different concentrations (25–100 ppm) of silver nanoparticles synthesized by heating at 60 °C against *Fusarium oxysporum*

observed only in 100 ppm treatment (Fig. 9). Thus, the synthesis of the silver nanoparticles at room temperature was found to be more effective than that after heating at 60 °C. The treatment with lower concentrations (25 or 50 ppm) of silver nanoparticles was not found to be effective in controlling the growth of the fungus, irrespective of the method of the synthesis.

Antifungal activity of silver nanoparticles was also studied against another plant pathogenic fungus *C.*

gloeosporioides. The treatments with silver nanoparticles resulted in significantly lower radial growth than the control (Table 2). The radial growth was significantly ($P \leq 0.05$) higher in control (28.00 and 41.00 mm after 4 and 7 days, respectively) than the treatments with silver nanoparticles (Fig. 7b). Complete inhibition of the fungal growth was observed in silver nanoparticles (100 ppm) synthesized at room temperature (Fig. 10). The treatments with the lower concentrations of silver nanoparticles were effective in inhibiting the growth; however, complete elimination was not observed. The complete inhibition of the fungal growth was not observed in any tested concentrations of silver nanoparticles synthesized after heating at 60 °C (Fig. 11). Thus, synthesis of the silver nanoparticles at room temperature for 24 h was found to be more effective against *C. gloeosporioides* than that after heating at 60 °C.

Antimicrobial activity of silver nanoparticles was studied against bacteria *Enterobacter* and *Bacillus*. Treatments with the different concentrations of silver nanoparticles significantly inhibited bacterial growth as measured in terms of zone of inhibition than the control (Table 3). Inhibition of the bacterial growth was not observed in control. Among the treatments, significantly higher zone of inhibition was observed with the higher concentrations (75 or 100 ppm) of silver nanoparticles synthesized at room temperature than the lower concentrations. The extent of inhibition of the bacterial growth was relatively lower on treatments with the silver nanoparticles synthesized at 60 °C than that synthesized at room temperature.

Table 2 Antifungal activity of silver nanoparticles against *Colletotrichum gloeosporioides*

Treatment	Radial growth (mm)	
	4 days	7 days
AgNP-RT25 ppm	19.00b	25.75b,c
AgNP-RT50 ppm	10.25d	21.25d
AgNP-RT75 ppm	4.50f	14.00e
AgNP-RT100 ppm	0.00 g	0.00f
AgNP-H25 ppm	17.25b	26.50b
AgNP-H50 ppm	13.75c	22.75c,d
AgNP-H75 ppm	9.00d	17.00e
AgNP-H100 ppm	6.50e	15.25e
Control	28.00a	41.00a
SE ($N=4$)	0.68	1.27
5% LSD	1.98	3.70

Mean ($N=4$) values in a column marked with different letters are statistically significant according to least significant difference (LSD) test at $P \leq 0.05$

AgNP-RT, silver nanoparticles synthesized at room temperature; AgNP-H, silver nanoparticles synthesized at 60 °C

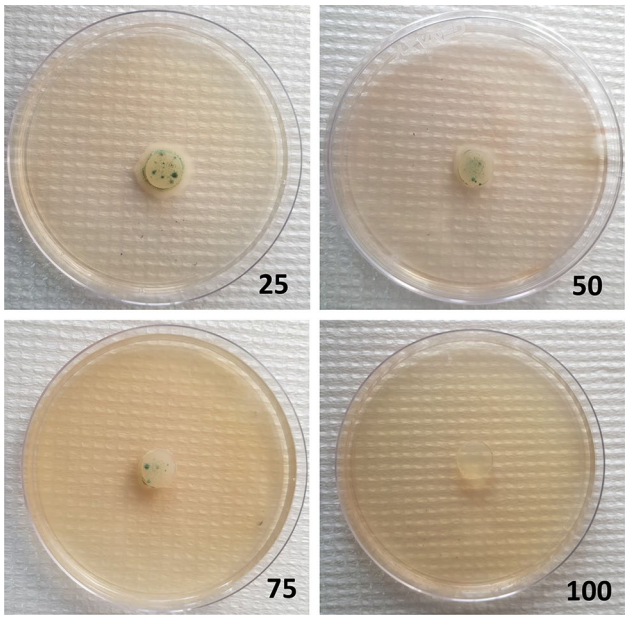

Fig. 10 Antimicrobial activity of different concentrations (25–100 ppm) of silver nanoparticles synthesized at room temperature against *Colletotrichum gloeosporioides*

4 Discussion

Silver and its compounds are effective and broad-spectrum antimicrobial agents [29]. Silver nanoparticles have improved antimicrobial activity due to their unique

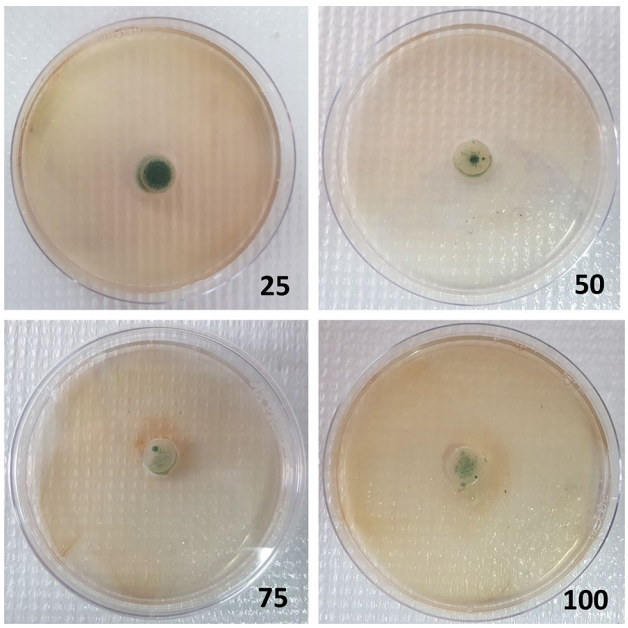

Fig. 11 Antimicrobial activity of different concentrations (25–100 ppm) of silver nanoparticles synthesized by heating at 60 °C against *Colletotrichum gloeosporioides*

Table 3 Antibacterial activity of silver nanoparticles against *Enterobacter* and *Bacillus*

Treatment	Zone of inhibition (mm)	
	Enterobacter	*Bacillus*
AgNP-RT25 ppm	5.5d	4.5 c
AgNP-RT50 ppm	9.5b,c	5.5b,c
AgNP-RT75 ppm	12.0a, b	8.0a
AgNP-RT100 ppm	14.5a	9.5a
AgNP-H25 ppm	5.0d	3.5c
AgNP-H50 ppm	7.0c,d	5.5b,c
AgNP-H75 ppm	8.5c,d	7.0b
AgNP-H100 ppm	9.0b,c	7.5a,b
Control	0.0e	0.0d
SE ($N=4$)	1.11	0.79
5% LSD	3.25	2.32

Mean ($N=4$) values in a column marked with different letters are statistically significant according to least significant difference (LSD) test at $P \leq 0.05$

AgNP-RT, silver nanoparticles synthesized at room temperature; AgNP-H, silver nanoparticles synthesized at 60 °C

property of large surface area to volume ratio [30]. Reactivity of silver nanoparticles is high due to the generation of Ag^+ ions as compared to unreactive metallic silver [31]. Silver has multiple modes of action against broad range of microbes and thus provides benefit of avoiding development of resistance as compared to issues with chemical management of pathogens. One of the potential applications in which silver nanoparticles can be utilized is in management of plant diseases. The present study reports the successful biosynthesis of silver nanoparticles by herbal plant of western Himalayan region, *O. kilimandscharicum*. Several studies have been reported on plant-assisted reduction of metal nanoparticles. The synthesis of silver nanoparticles using a wide range of plant extracts like *Aloe vera* [32], *Myrmecodia pendans* [33], *Desmodium gangeticum* [34], *Indigofera barberi* [35], *Euphorbia helioscopia* [36], *Artimisia vulgaris* [37], *Taraxacum officinale* [38], *Convolvulus arvensis* [39] is reported. The main phytochemicals responsible for biosynthesis of silver nanoparticles are alkaloids, terpenoids, phenolic compounds, enzymes, coenzymes, protein and sugars [17]. Phytochemical analysis of *O. kilimandscharicum* leaf extract was carried out to verify the role of various functional groups in the bioreduction of silver. The plant *O. kilimandscharicum was* found to be a good source of phenolic compounds, flavonoids and tannins which are responsible for the transformation of silver ions to silver nanoparticles. Biocompounds in the plant extract of *O. kilimandscharicum* reduce silver metal salt from positive oxidation state to zero oxidation state. Biomolecules also form a monolayer on the surface of nanoparticles to prevent the agglomeration.

The silver nanoparticles synthesized using *O. kilimandscharicum* showed bright reddish brown colour reflecting the reduction of Ag^+ into Ag^0. The characteristic reddish brown colour of colloidal silver solution is attributed to collective oscillation of free conduction electrons induced by an interacting electromagnetic field [40]. The colour density also depends on the size of the synthesized silver nanoparticles. The UV–Vis spectrum exhibited an absorption band at around 450 nm, which is characteristic for silver nanoparticles.

The effect of the variation of plant extract concentration and temperature on the biosynthesized silver nanoparticles was investigated using UV–Vis spectroscopy. The absorption increased with the increase in the concentration of plant extract from 20 to 30%. The nanoparticles synthesis and size reduction was optimum at these concentrations due to the availability of reducing functional groups in the leaf extract. Higher concentration of plant extract resulted in decrease in intensity of the plasmon band and broad peak due to larger size and aggregation of nanoparticles. An optimal concentration of plant extract is required as there could be aggregation, which leads to reduction in total number of nanoparticles in the presence of higher concentrations of plant extract. At higher concentrations of 40% and 50% plant extract, the absorption band becomes broader and shifts towards the red region. This may be attributed to the presence of large amounts of phytomolecules at higher concentration that causes the rapid reduction of silver ions. The fast reduction of silver ions results in further growth of nanoparticles that leads to an increase in the size of nanoparticles by a phenomenon called Ostwald ripening [41].

The formation of the silver nanoparticles was observed at room temperature (25 °C) with a characteristic plasmon band at 450 nm. An increase in the intensity of the nanoparticles and narrowing of the peaks was observed at temperatures from 40 to 60 °C which suggests reduction in particle size. A decrease in intensity and red shift was observed at higher temperature of 80 °C. Red shift or blue shift in the UV absorption peak characteristic of silver nanoparticles depends on the particle size, shape, aggregation and the surrounding dielectric medium [42]. A shift in the absorption maxima to longer wavelength and bandwidth increase, results with the increase in size of the nanoparticles [43]. The reduction in the concentration of nanoparticles and size increase may be due to desorption of the biomolecules at higher temperature, thereby reducing the concentration of the silver nanoparticles formed.

Fusarium oxysporum and *C. gloeosporioides* are the prevalent disease causing fungi affecting vegetable crops. Therefore, in this study we attempted to evaluate the

efficacy of biosynthesized silver nanoparticles for control of phytopathogens *F. oxysporum* and *C. gloeosporioides*. The results of the present study demonstrate that silver nanoparticles synthesized using *O. kilimandscharicum* have the capacity to inhibit the growth *F. oxysporum* and *C. gloeosporioides.* Growth of the fungi was inhibited at different concentrations of 25 to 100 ppm silver nanoparticles. The inhibition was increased with the increase in concentration of silver nanoparticles. The inhibitory effect was observed in a concentration-dependent manner in both fungi.

The highest antifungal properties were observed on treatment with 100 ppm silver nanoparticles in vitro. The results are in corroboration with other studies available on the effects of silver nanoparticles on phytopathogens. The antifungal effects of different concentrations of silver nanoparticles (20 to 100 ppm) were studied against aggressive isolates of *Botrytis fabae* and *Alternaria alternate* causing chocolate spot disease and Alternaria leaf spot disease of faba bean [44]. The highest reduction of chocolate spot and Alternaria leaf spot severity was obtained with treatment by 100 ppm of silver nanoparticles. Abdelmalek and Salaheldin [45] studied silver nanoparticles as a potent fungicide for citrus phytopathogenic fungi. In vitro assay was carried out on potato dextrose agar (PDA) media treated with 50, 100 and 150 ppm of silver nanoparticles. Results revealed that 150 ppm silver nanoparticle showed potent antifungal activity against the isolated fungi *Alternaria alternate, Alternaria citri* and *Penicillium digitatum*. Kim et al. [14] studied antifungal effects of silver nanoparticles against eighteen different plant pathogenic fungi on potato dextrose agar, malt extract agar and corn meal agar plates. The most significant inhibition of plant pathogenic fungi was observed on PDA with 100 ppm of silver nanoparticles. Application of silver nanoparticles for the control of *Colletotrichum* species in vitro and pepper anthracnose disease in field is reported by Lamsal et al. [16]. Silver nanoparticles were applied at various concentrations to determine antifungal activities in vitro and in the field. The application of 100 ppm concentration of silver nanoparticles produced maximum inhibition of the growth of fungal hyphae as well as conidial germination in comparison with the control in vitro.

The biogenically synthesized nanoparticles by leaves of *O. kilimandscharicum* were tested against two pathogenic bacteria, namely *E. cloacae* and *Bacillus* sp. Strong antibacterial activity was observed against the test bacteria. Antimicrobial mechanism of silver is attributed to disruption of a broad range of biological processes in micro-organisms. A number of studies have demonstrated the release of silver ions from silver nanoparticles that are responsible for the antimicrobial activity [46]. Silver ions inhibit the cell membrane structure and functions [47, 48] and also the expression of proteins associated with adenine triphosphate production [49]. Several studies have shown that silver nanoparticles may kill fungal spores by destructing the membrane integrity [50]. It has also been indicated that interaction of silver nanoparticles with phosphorus and sulphur-containing compounds may cause damage to DNA and proteins, resulting in cell death [51]. Reactive oxygen species are also produced by silver ions via their reaction with oxygen which causes damage to proteins, lipids and nucleic acids [52].

5 Conclusion

In this study, biosynthesis of silver nanoparticles using aqueous leaf extract of *O. kilimandscharicum* is reported. UV–Vis absorption spectra displayed surface plasmon resonance characteristic of silver nanoparticles. Biosynthesized silver nanoparticles exhibited potent antifungal and antibacterial activity against all the tested pathogens. Silver nanoparticles synthesized using 30% leaf extract demonstrated the highest antimicrobial activity. It is evident from the present study that silver nanoparticles synthesized using *O. kilimandscharicum* have great potential in agriculture as an effective antimicrobial agent against the harmful plant pathogens.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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