Khalid et al. Bulletin of the National Research Centre (2019) 43:18
https://doi.org/10.1186/s42269-019-0057-2

Bulletin of the National Research Centre

RESEARCH Open Access

The prophylactic effect of *Ranunculus laetus* (Wall)-mediated silver nanoparticles against some Gram-positive and Gram-negative bacteria

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Abstract

Background: The possession of immense activities of the silver nanoparticles, due to which they have played an anomalous role in the various fields of bioscience, is of big concern of study in the recent era. So, the main aim of our study was to synthesize silver nanoparticles (AgNPs) using aqueous leaf extract of *Ranunculus laetus* using 0.1 M silver nitrate solution, and the synthesized silver nanoparticles were tested for their antibacterial activity.

Results: The dark brown color of reaction mixture preliminary confirmed the synthesis of AgNPs. Further, characterization of synthesized silver nanoparticles showed UV-vis absorption peak at 442 nm, infrared spectroscopy revealed nitro compounds and aromatic amines as reducing and capping agents while X-ray diffraction revealed face-centered cubic crystallites of AgNPs with an average size of 24.125 nm. Scanning electron microscopy images confirmed spherical shape of silver nanoparticles with diameter 7.21–17.62 nm. The synthesized silver nanoparticles revealed significant antibacterial activity $P < 0.05$, against *Escherichia coli* (MTCC No. 739), *Pseudomonas aeruginosa* (MTCC No. 1688), *Staphylococcus aureus* (MTCC No. 96), and *Bacillus subtilis* (MTCC No. 441). The order of antibacterial potential was *P. aeruginosa* > *S. aureus* > *E. coli* > *B. subtilis*.

Conclusion: The ionic silver was reduced to silver nanoparticles by the action of reducing agents present in the aqueous leaf extract of *Ranunculus laetus*, and these nanoparticles bare credible antibacterial activity against diverse bacterial strains.

Keywords: Silver nanoparticles, *Ranunculus laetus*, UV-vis, X-ray diffraction, Scanning electron microscopy, Antibacterial activity

Introduction

Nanotechnology has evolved as a very fast-growing field with diverse applications in science and technology. Metal nanoparticles have engrossed the attention of scientist for their extraordinary physical and chemical properties (Garg 2012). In current years, nanomaterials have gained exceptional consideration due to their remarkable applications (Azizinezhad et al. 2014). An eco-friendly green mediated synthesis of the inorganic nanoparticle is a fast-growing research in the limb of nanotechnology (Sathya and Ambikapathy 2012). Due to simplicity and environment friendliness, nanoparticle synthesis by the plant-mediated method has gained importance in recent years (Elumalai et al. 2010).

Among diverse nanomaterials, the popularity of silver nanoparticles has gained the attention of biologists, researchers, and scientists, due to their incredible physicochemical and natural characteristics (Li et al. 2014). Many study reports have shown that silver nanoparticle synthesis can be achieved by using root, leaf, seed, bark, and fruit as reducing agents (Kalidasan and Yogamoorthi 2014). Synthesis of silver nanoparticles by using plant extract has been explored by innumerable researchers, and the reports have revealed that these methods are quite simpler and nontoxic in contrast to physicochemical methods

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(Raja et al. 2012). Furthermore, silver nanoparticles have been used in the energy sector, sensing, electronics, and health care (Rycenga et al. 2011; Konop et al. 2016). From early times, the silver particles have been used as antimicrobial agents (Silver and Phung 1996). The silver nanoparticles have been verified as an efficient biocide against Gram-negative and Gram-positive bacteria (Jones and Hoek 2010; Anuj and Ishnava 2013).

In keeping view the importance of silver nanoparticles, the present study was carried out to elucidate the synthesis of silver nanoparticles using *Ranunculus laetus* leaf extract. The plant extract acts as reducing and capping agent, converting ionic silver to metal silver nanoparticles. Moreover, the antibacterial activity of synthesized silver nanoparticles using *Ranunculus laetus* leaf extract was evaluated against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Bacillus subtilis* bacterial strains.

Materials and methodology

Plant collection and authentication

Ranunculus laetus shoots were collected from the neighboring vicinity of Tehsil Lar district Ganderbal Jammu and Kashmir India during the month of August (2014). The plant was authenticated by Dr. Akhtar H. Malik (Curator, Centre for Biodiversity and Taxonomy, Department of Botany University of Kashmir). A voucher herbarium specimen was submitted to the Department of Taxonomy University of Kashmir bearing specimen number 2078- KASH for future references.

Ranunculus laetus is a perennial herb belonging to family Ranunculceae (Butter cup family) attaining a height of 30–70 cm. The creeping rootstock possesses numerous fibrous roots and flowering stems. The leaves at the base have long petioles (12–15 cm) as the height of the plant increases, the size of the petioles decreases. So, the top leaves of the stem are sessile. The flowers are yellow in color with 17–25 mm diameter. The leaf paste of *R. laetus* is applied to affected parts of warts and wounds (Hanief et al. 2013) and for treating skin infection (Abbasi et al. 2010). Also, the plant decoction is used to cure indigestion (Rachna et al. 2012)

Plant extract preparation

Leaves of the plant were detached and washed with double distilled water until the foreign matter was removed. Then, the leaves were shade dried and grinded to powder with the help of electric grinder (Philips). Twenty grams of leaf powder was weighed by electric balance (Denver, Germany) and was placed inside of soxhlation apparatus (J-Sil, 50/42, Borosil glass). Ninety percent of ethanol was used as a solvent for extraction purpose. After 24 h of soxhlation, the crude extract was subjected to Vacuum Rotary Evaporator (Scientech) to obtain a semi-solid extract.

Table 1 Phytochemical investigation of ethanolic leaf extract of *Ranunculus laetus*

Phytochemicals		Tests	*R. laetus*
Primary metabolites	Carbohydrates	Molish's test	++
		Fehling's test	++
		Barfoed's test	++
		Benedict's test	++
	Amino acids and proteins	Ninhydrin test	++
		Biuret's test	–
		Millon's test	++
Secondary metabolites	Alkaloids	Mayer's test	–
		Wagner's test	++
		Hager's test	–
		Dragendroff's test	++
	Terpenoids	Salkowski test	++
		Libermann-Burchards test	++
	Flavonoids	Lead acetate test	++
		Alkaline reagent test	++
		Shinoda test	++
	Tannins and phenolic compounds	$FeCl_3$ test	++
		Nitric acid test	++
		Gelatin test	–
		Dilute iodine solution test	–
	Saponins	Froth test	++
	Glycosides	Legal's test	++
		Keller-Killani test	–
		Borntrager's test	++

++ present, – absent

Phytochemical investigation of crude extract

The crude extract was tested for the presence or absence of phytoconstituents, carbohydrates, amino acids and proteins, alkaloids, terpenoids, glycosides, steroids, triterpenoids, flavonoids, saponins, and tannins according to the standard procedures (Trease and Evans 1989; Kokate et al. 2006).

Synthesis of silver nanoparticles (Huang et al. 2011)

Twenty milligrams of semi-solid extract was dissolved in 100 ml of double distilled water in a beaker and was boiled for complete dissolution. The aqueous extract obtained was filtered through Whatman filter paper no. 1. For the synthesis of silver nanoparticles, take 10 ml of aqueous plant extract and add to 90 ml of silver nitrate solution (0.1 M) in a beaker. The beaker was kept on a magnetic stirrer (Remi). After 10 min, the color of the solution changed. For the stabilization of silver nanoparticles (AgNPs), the solution was kept as such for 24 h.

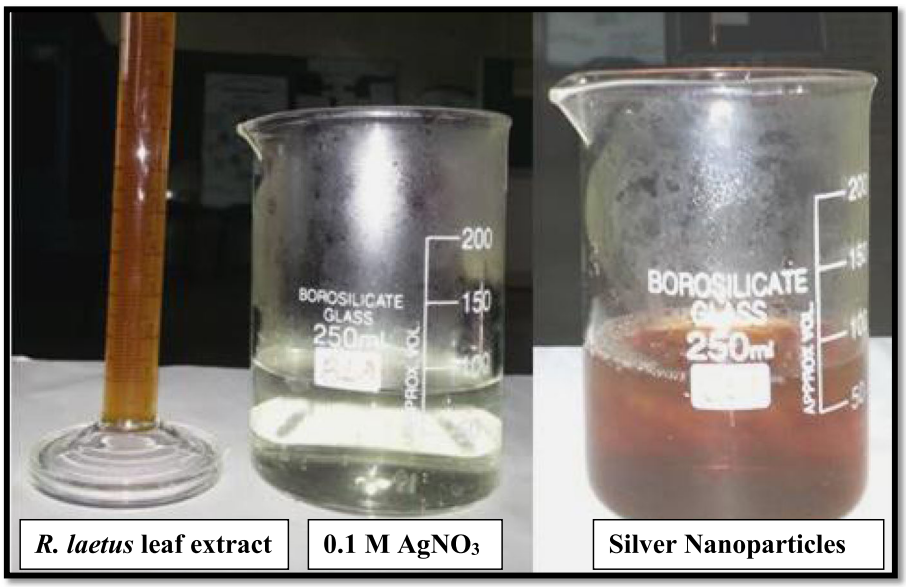

Fig. 1 Dark brown color of AgNPs

Characterization of silver nanoparticles

UV-vis spectroscopy

The optical property of AgNPs was determined by double-beam UV-vis spectrophotometer (UV-vis Analyst- 0001, Electronic India). After the addition of $AgNO_3$ to the plant extract, the spectrum was taken after 24 h between 300 to 700 nm using double distilled water as a blank.

Fourier transformation infrared spectroscopy

FTIR spectrometer (Shimadzu) was used for the analysis of synthesized AgNPs. The sample was prepared by dispersing the silver nanoparticles of leaf extract of *Ranunculus laetus* uniformly in a matrix of dry KBr, compressed to form an almost transparent disc. KBr was used as standard to analyze the sample. The percentage transmittance of synthesized AgNPs was recorded in the range of 4000-400 cm^{-1}.

X-ray diffraction

The shape and size of silver nanoparticle crystals were carried out by X-ray diffractometer (RIGAKU Japan Miniflex) equipped with CuKα radiation (0.154 nm) operated at a voltage of 30 kV and 10 mA current. The sample was scanned in the region of 20° to 80° for 2θ at 0.04°/min scanning speed. The diffraction patterns of synthesized silver nanoparticles were obtained with the help of integrated X-ray powder diffraction software (PDXL). The average crystal size of synthesized silver nanoparticles was calculated by Debye-Scherrer equation:

$$D = \frac{0.94\,\lambda}{\beta\,\mathrm{Cos}\theta}$$

Where D is the crystallite size of AgNPs, λ is the wavelength of the X-ray source (0.1541 nm), β is the full width at half maximum of the diffraction peak, 0.94 is the Scherrer constant, and θ is the Bragg angle.

Scanning electron microscopy

The shape of synthesized silver nanoparticles was revealed by the scanning electron microscope (JEOL, Japan JSM 6390A). A thin film of the sample was prepared on a carbon-coated copper grid by dropping a very small amount of the sample on it, and the extra solution was removed by blotting paper. Then, the film on the SEM grid was allowed to dry by putting them under a mercury lamp for 5 min. The sample was characterized at an accelerating voltage of 20 kV, and a high-resolution image of the sample was obtained.

Antibacterial activity

Disc diffusion method and minimal inhibitory concentration

The antibacterial activity of *Ranunculus laetus*--mediated silver nanoparticles was evaluated by Kirby-Bauer Disc diffusion technique (Bauer et al. 1966). Also, the minimal inhibitory concentration (MIC) of the synthesized silver nanoparticles was determined by spectrophotometric macrodilution method (Devienne and Raddi 2002). The antibacterial activity was carried out against Gram-negative bacteria *Escherichia coli* (MTCC No. 739) and *Pseudomonas aeruginosa*

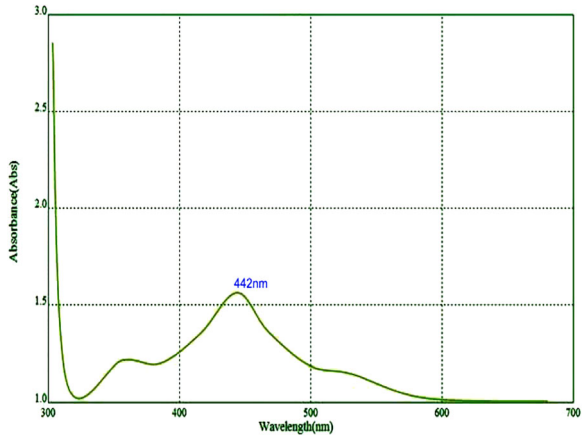

Fig. 2 UV-vis analysis of *R. laetus*-mediated AgNPs

(MTCC No. 1688), Gram-positive bacteria *Staphylococcus aureus* (MTCC No. 96), and *Bacillus subtilis* (MTCC No. 441).

Results

The results revealed that the *Ranunculus laetus* crude extract contained carbohydrates, amino acids and proteins as primary metabolites, and alkaloids, terpenoids, flavonoids, tannins and phenolic compounds, saponins, and glycosides as secondary metabolites as shown in Table 1.

The preliminary synthesis of silver nanoparticles was confirmed by the dark brown color of the mixture when the plant extract was added to 0.1 M $AgNO_3$ solution (Fig. 1). Further characterization, done by UV-vis spectroscopy revealed surface plasmon peak at 442 nm of synthesized AgNPs which confirmed the synthesis of AgNPs (Fig. 2). The infrared analysis revealed two important peaks of 1339.61 cm^{-1} and 1228.92 cm^{-1} which corresponded to nitro compound (N–O symmetric stretch) and aromatic amine (C–N stretch) respectively (Fig. 3). The X-ray diffraction pattern of our sample

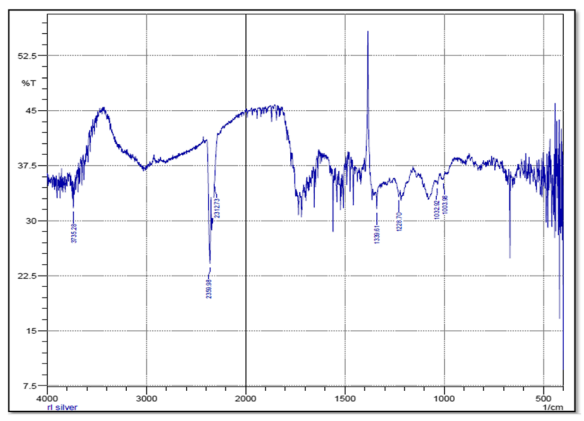

Fig. 3 Infrared analysis of *R. laetus*-mediated AgNPs

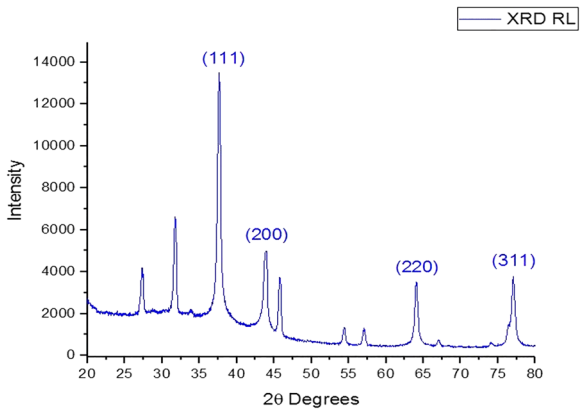

Fig. 4 X-ray diffraction of *R. laetus*-mediated AgNPs

was indexed with standard XRD spectrum of Joint Committee on Powder Diffraction Standards (JCPDS Card No. 53-61386). The XRD spectrum of *Ranunculus laetus* leaf extract-mediated AgNPs revealed four main 2 angle peaks at 38°, 44.006°, 64.057°, and 77.025° (Fig. 4), which were indexed as 111, 200, 220, and 311 planes respectively, and correspond to the face-centered cubic lattice of the silver nanocrystals. Moreover, *Ranunculus laetus* leaf extract-mediated silver nanoparticle crystals revealed sizes between 17.65 and 39.79 nm with an average size of 24.125 nm. The scanning electron microscopic image showed agglomeration, spherical shape, and the polydispersed nature of AgNPs, and the nanoparticles were 7.21–17.62 nm in diameter (Fig. 5).

Three different concentrations of *Ranunculus laetus* leaf extract-mediated silver nanoparticles, i.e., 100 mg/ml, 50 mg/ml, and 25 mg/ml, were tested against *E. coli*, *S. aureus*, *P. aeruginosa*, and *B. subtilis*. Streptomycin (100 mg/ml) was used as a standard drug (Table 2). At 100 mg/ml concentration of *R. laetus* AgNPs, zone of inhibition (ZOI) against *E. coli* was measured 19.00 ±

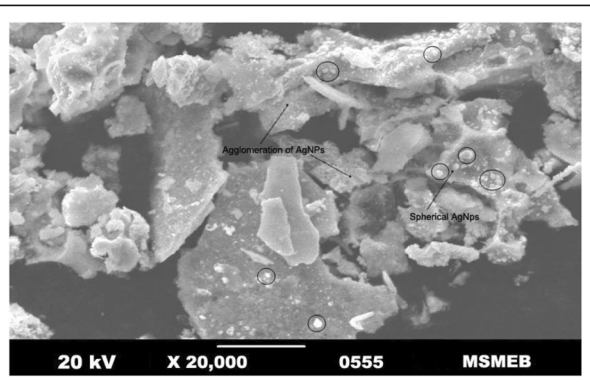

Fig. 5 SEM analysis of *R. laetus*-mediated AgNPs

Table 2 Antibacterial activity of *R. laetus*-mediated AgNPs and leaf extract

Concentration	*E. coli*	*S. aureus*	*P. aeruginosa*	*B. subtilis*
25 mg/ml AgNPs	15.33 ± 4.61 mm	15.66 ± 2.51 mm	17.00 ± 1.73 mm	12.66 ± 0.57 mm
50 mg/ml AgNPs	16.33 ± 3.60 mm	17.66 ± 3.05 mm	18.66 ± 1.52 mm	15.66 ± 2.08 mm
100 mg/ml AgNPs	19.00 ± 1.00 mm	20.66 ± 2.51 mm	22.00 ± 1.73 mm	18.33 ± 0.57 mm
R. laetus leaf extract (100 mg/ml)	12.33 ± 0.34 mm	15.69 ± 0.47 mm	17.12 ± 1.30 mm	10.99 ± 0.61 mm
Streptomycin (100 mg/ml)	30.66 ± 0.57 mm	30.66 ± 2.30 mm	25.00 ± 3.60 mm	28.00 ± 1.00 mm

1.00 mm while 50 mg/ml and 25 mg/ml ZOI were measured 16.33 ± 3.60 mm and 15.33 ± 4.61 mm respectively as shown in Figs. 6 and 7. Standard streptomycin showed 30.66 ± 0.57 mm ZOI against *E. coli*. Moving to *S. aureus*, the ZOI was measured 20.66 ± 2.51 mm, 17.66 ± 3.05 mm, and 15.66 ± 2.51 mm against 100, 50, and 25 mg/ml concentrations respectively. while as, for the same strain streptomycin showed 30.66 ± 2.30 mm ZOI. Furthermore, against *P. aeruginosa*, the zone of inhibitions was found larger than others. At 100, 50, and 25 mg/ml concentrations, the ZOI were calculated 22.00 ± 1.73 mm, 18.66 ± 1.52 mm, and 17.00 ± 1.73 mm respectively but streptomycin revealed 25.00 ± 3.60 mm ZOI (Fig. 7). The *R. laetus* AgNPs at 100, 50, and 25 mg/ml concentrations showed 18.33 ± 0.57 mm, 15.66 ± 2.08 mm, and 12.66 ± 0.57 mm ZOI against *B. subtilis* while as for streptomycin, it was measured 28.00 ± 1.00 mm.

The 100 mg/ml of aqueous leaf extract of *R. laetus* showed ZOI of about 12.33 ± 0.34 mm, 15.69 ± 0.47 mm, 17.12 ± 1.30 mm, and 10.99 ± 0.61 mm against *E.coli*, *S.aureus*, *P. aeruginosa*, and *B. subtilis* respectively.

The MIC, determined by spectrophotometer macrodilution method was calculated by a sharp decline in the absorption value. The *Ranunculus laetus* AgNPs showed a MIC value of 1×10^{-1} mg/ml against *E.* col; 1×10^{0} mg/ml were recorded against *S. aureus.* Against *P. aeruginosa*, it was 1×10^{-1} and 1×10^{0} mg/ml was recorded against *B. subtilis* as shown in Table 3.

Discussion

Various methods have been employed for the synthesis of nanoparticles which include physical, chemical, and biological methods. The physical and chemical methods involve the use of high temperatures, pressure, high current, a toxic substance like arsenic, sodium borohydride, hydrazine, and other chemical substance which possess very harmful and toxic implications (Ahmed and Ikram 2015). On the other hand, the biological method provides a very safe and easier method for nanoparticle synthesis with no side effects (Ahmad et al. 2011). Plants have been proven very efficient and alternative for the synthesis of nanoparticles in the past few decades (Bhati-Kushwaha and Malik 2013). Metal nanoparticles are synthesized by phytochemicals present in plants, which work as reducing and capping agents (Swarnalatha et al. 2013). For the synthesis of gold, silver, and gold-silver-copper alloy nanoparticles, plants have been exploited to a greater extent (Joerger et al. 2000; Sathishkumar et al. 2009). Our results revealed *R. laetus* leaf extract reduced the ionic silver into silver nanoparticles which were confirmed by UV-vis peak at 442 nm. FTIR demonstrated the necessary compounds responsible for the reduction and stabilization of nanoparticles were nitro compounds and aromatic amines. Furthermore, XRD confirmed their crystalline nature with face-centered cubic lattice and SEM revealed their spherical shape. A similar type of results was found in the case of *Eruca sativa* and *Spinacia oleracea*-mediated AgNPs (Alaraidh et al. 2014),

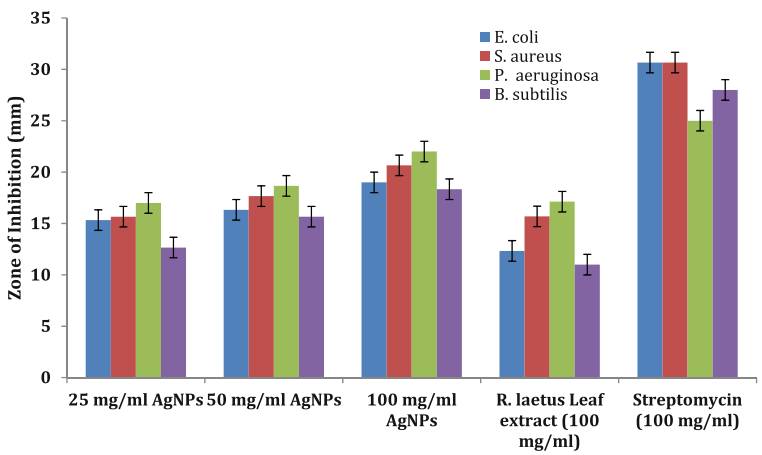

Fig. 6 Antibacterial activity of *R. laetus*-mediated AgNPs and *R. laetus* leaf extract

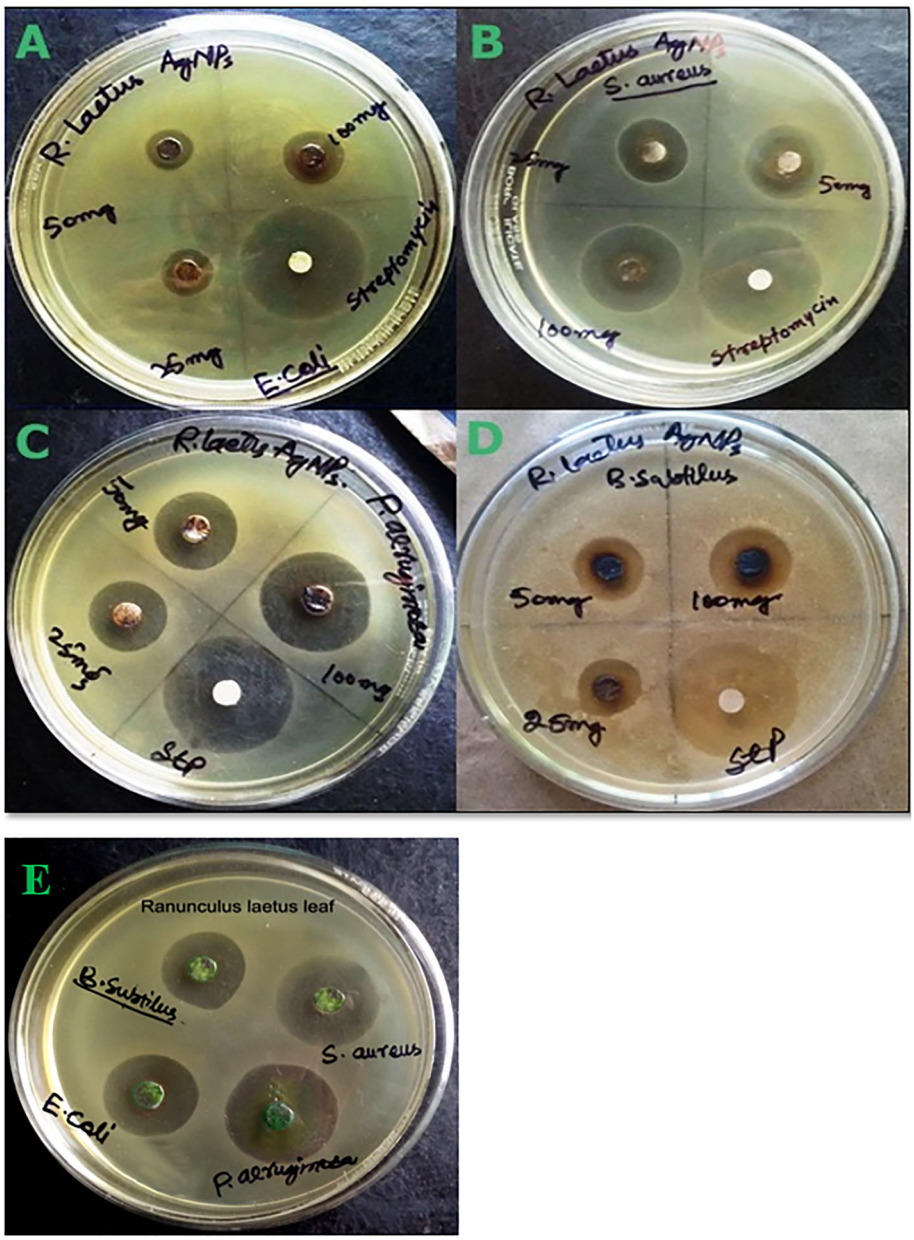

Fig. 7 *R. laetus*-mediated AgNPs. Zone of inhibition of against **a** *E. coli*, **b** *S. aureus*, **c** *P. aeruginosa*, **d** *B. subtilis*, and **e** Antibacterial activity of *R. laetus* aqueous leaf extract

Table 3 MIC of *R. laetus*-mediated silver nanoparticles

Concentration (mg/ml)	Absorbance (nm) using RL AgNPs *E.coli*	Absorbance (nm) using RL AgNPs *S. aureus*	Absorbance (nm) using RL AgNPs *P. aeruginosa*	Absorbance (nm) using RL AgNPs *B. subtilis*	Absorbance (nm) using streptomycin
1×10^{-5}	0.761 ± 0.027	0.798 ± 0.061	0.739 ± 0.043	0.381 ± 0.032	1.028 ± 0.048
1×10^{-4}	0.589 ± 0.045	0.641 ± 0.037	0.512 ± 0.053	0.307 ± 0.029	0.975 ± 0.052
1×10^{-3}	0.543 ± 0.034	0.487 ± 0.027	0.483 ± 0.029	0.262 ± 0.032	0.527 ± 0.025
1×10^{-2}	0.306 ± 0.023	0.426 ± 0.049	0.367 ± 0.052	0.215 ± 0.058	0.516 ± 0.035
1×10^{-1}	0.208 ± 0.047	0.355 ± 0.051	0.308 ± 0.056	0.179 ± 0.023	0.283 ± 0.040
1×10^{0}	0.197 ± 0.020	0.232 ± 0.023	0.252 ± 0.038	0.164 ± 0.035	0.247 ± 0.028
1×10^{1}	0.183 ± 0.016	0.204 ± 0.061	0.229 ± 0.031	0.128 ± 0.021	0.193 ± 0.041

mulberry AgNPs (Awwad and Salem 2012) and *Murraya koenigii* AgNPs (Vinothkumar et al. 2014).

The smaller size of silver nanoparticles leads to augmented membrane permeability and cell damage (Ankanna and Savithramma 2011). Silver nanoparticles show electrostatic attraction with the bacterial cell membrane, which leads to the formation of pits on the cell surface and ultimately causes cell expiration (Sondi and Salopek-Sondi 2004). AgNPs cause the destruction or death of the bacterial cell by directly interfering with metabolic and growth signaling pathways by modulating tyrosine phosphorylation of putative peptide substrates critical for cell viability and division (Shrivastava et al. 2007). However, other mechanism revealed that silver nanoparticles cause the formation of free radicals which leads to cell death (Danilczuk et al. 2006). Our results showed that *R. laetus*-mediated silver nanoparticles possessed significant antibacterial activity $P < 0.05$, against both Gram-positive and Gram-negative bacteria and the order of antibacterial activity was found *P. aeruginosa* > *S. aureus* > *E. coli* > *B. subtilis* and the antibacterial activity is concentration dependent. Furthermore, the antibacterial activity of the synthesized silver nanoparticles is very much enhanced as compared with the leaf extract antibacterial activity. However, the MIC revealed the lowest concentration of 1×10^{-1} mg/ml against *E. coli* and *P. aeruginosa* while against *S. aureus* and *B. subtilis*, it was 1×10^{0} mg/ml. Our results were in compliance with antibacterial activity of AgNPs of *Ocimum sanctum* (Rout et al. 2011), *Olea europaea* (Awwad et al. 2012), and *Withania somnifera* (Anbalagan et al. 2016).

Conclusion

The present study was aimed to synthesize silver nanoparticles using *Ranunculus laetus* leaf extract and their antibacterial activity was also elucidated. Our results revealed that *R. laetus*-mediated AgNPs were synthesized. Also, the synthesized silver nanoparticles possessed potential with enhanced antibacterial activity against pathogenic bacteria as compared to the *R. laetus* aqueousleaf extract.

Acknowledgements
This research work was supported by Pest Control and Ayurvedic Drug Research Lab, S.S.L. Jain P.G. College Vidisha, M.P. India.

Funding
Not applicable

Availability of data and materials
The manuscript contained all the necessary data obtained during the study. However, on the recommendation or request addition information will be provided by the corresponding author.

Authors' contributions
The work was conceived and premeditated by SK, SAM, and MAA, and the experiment was conducted by SK. The data obtained was analyzed by SK, SAM, and MAA. The compilation of the first draft, as well as editing, was done by SK. The final manuscript was read and approved by all authors.

Ethics approval and consent to participate
Ethical approval had been granted by the Pest Control and Ayurvedic Drug Research Lab, S.S.L. Jain P.G. College Vidisha, M. P, bearing animal ethical committee registration number 804/03/ca/CPCSEA.

Consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interests.

Publisher's Note

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Received: 9 November 2018 Accepted: 18 January 2019
Published online: 01 February 2019

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