



Biosynthesis of Silver Nanoparticles Using *Rhus parviflora* Leaf Extract and its Antimicrobial Activity

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Abstract: In the present paper, synthesis of silver nanoparticles (AgNPs) using *Rhus parviflora* leaf extract is reported. The aqueous leaf extract is resourcefully used for the reduction and stabilization of AgNPs. UV–Visible spectroscopy demonstrated surface plasmon resonance peaks in the range 422–425 nm which confirmed the formation of AgNPs. XRD pattern showed the existence of face centred cubic phase AgNPs with average particle size (D) less than 03nm as calculated by Debye Scherrer formula. TEM analysis validated that the synthesized AgNPs were spherical in nature and ranged from 04 to 10nm in size. FTIR study suggested that the AgNPs must be capped and stabilized by the polyphenolic bio-components present in the *R. parviflora* leaf extract. Antibacterial and antifungal tests of AgNPs were also performed against the selected bacterial and fungal strains, respectively and the tests showed good results. Therefore, these biosynthesized AgNPs could be utilized further for their potential applications in biomedicines and especially for the encounter against the multidrug resistant microbes.

Keywords: AgNPs • *Rhus parviflora* • surface plasmon resonance • polyphenolic bio-components

Introduction

Nano-biotechnology is considered to be one of the greatest promising research field and biosynthesis of metallic nanoparticles (MNPs) is presently under exploitation as the plant materials for the fabrication of MNPs is pretty new and exciting scientific field. Furthermore, in last few years, the creation of nanoparticles has been grown due to distinctive properties of nanoparticles, they have earned reputation compared to bulk structures. A lot of biophysico-chemical and cross methods were employed efficiently in the fabrication of various MNPs, yet they are expensive and involve the use of hazardous chemical agents (Mittal *et al* 2013; Kumar and Yadav 2009; Gondwal and Pant 2018).

Today, there is a requirement to add the methods which should be environment benign, sustainable involving very less amount of toxic agents, severely lessen environmental contamination and decrease the danger to

living beings. The synthesis of bio-functional metallic nanoparticles is very important, and in recent times, it has attracted various researchers/ clinicians. The bio-functional part has different compositions which may be utilized instead of insecure chemical compounds as safe reducing and capping agents in generation methods. The bio-components can be extracted from thousands of flora and fauna like plants, microbes etc. available in the nature. Generally during chemical synthesis, agglomeration occurs due to the involvement of attractive forces among the nanoparticles. Therefore, the bio-components might be used as capping agents to avoid agglomeration (Kotakadi *et al* 2013; Krishnaraj *et al* 2010; Kumar *et al* 2012; Kumar R *et al* 2012) Previous research works use microbes such as bacteria (Lengke *et al* 2007), fungi (Kowshik *et al* 2002; Rautaray *et al* 2003) etc for the biosynthesis of nanoparticles and in addition to this, various



plant extracts involved the utilization of new resources for their ability to create harmless and non-toxic nanoparticles, including iron, cobalt, gold, silver, zinc oxide, etc. Various studies have proved that the reductive capabilities of the metabolites which are present in these bio-systems can transform metallic ions into novel metal nanoparticles (Park *et al* 2011; Kim *et al* 2010; Malik *et al* 2014; Mude *et al* 2009).

Over the decades, silver has been used for various antimicrobial applications (Nabikhan *et al* 2010; Niraimathi *et al* 2013) The use of silver was not limited to the microbial applications against bacteria, fungi, parasites, etc. These were also exploited for environmental remediation due to their photocatalytic activity (Arunachalam *et al* 2012). But nano sized silver opened up a lot of opportunities for its use in various fields. In addition to this, silver's dimensions and shapes can be controlled for a specific application (Akhtar *et al* 2013). These structural smoothness and varieties amplify the efficiency of the biological applications of the nano silver and it can be achieved by using biological means such as plant extracts due to the presence of the various bio-functional groups that assist in the stabilization and reduction of silver ions.

In a study, *Pelargonium graveolens* leaf extract mediated AgNPs were synthesized and average particle size of 16–40 nm having spherical shape were reported (Shankar *et al* 2003). In another study, Ag nanoparticles from the *Phoenix dactylifera* leaf extract created and its potential effects against *E. coli* and *K. pneumoniae* were investigated. It was reported that *P. dactylifera* leaves serve as a fast-acting and effective antibacterial agent (Rashid *et al.*, 2016). At room temperature, *Cassia tora* leaf extract was employed to generate Ag nanoparticles having 43nm size. Moreover, their antibacterial results against *E. coli*, *P. aeruginosa*, *S. aureus* and *B. subtilis* showed good activity (Saravanakumar *et al* 2015). In

other studies, the extract of plants like *Phyllanthus amarus* (Khushboo *et al* 2014), *Terminalia Cuneata* (Edison *et al* 2016), *Coleus aromaticus* (Vanaja *et al* 2013), *Argyrea nervosa* (Thombre *et al* 2014), *Aloe vera* (Tippayawat *et al* 2016), *A. indicum* (AshokKumar *et al* 2015), *Allium cepa* (Saxena *et al* 2010), *Alternanthera dentata* (Kumar *et al* 2014), *Allium sativum* (Rastogi and Arunachalam 2011), *Ananas comosus* (Ahmed and Sharma 2012), *Artocarpus heterophyllus* Lam. (Jagtap and Bapat, 2013), *Argemone mexicana* (Singh *et al.*, 2010), *Arbutus unedo* (Kouvaris *et al.*, 2015), *Azadirachta indica* (Ahmed *et al* 2015), *Boerhaavia diffusa* (Kumar *et al* 2014), *Brassica rapa*L. (Narayanan and Park, 2014), *Catharanthus roseus* (Ponarulselvam *et al.*, 2012), *Capsicum annum* (Li *et al* 2007), *Chrysanthemum morifolium* (He *et al* 2013), *Cinnamon zeylanicum* (Sathishkumar *et al* 2009), *Citrus limon* (Prathna *et al* 2011), etc were reported to create AgNPs associated with various applications such as antimicrobial, anticancer, catalytic etc. in various fields of science and technology.

Rhus parviflora, plant selected for this research work belongs to Anacardiaceae family, commonly known as Tungla or Tintideek and is abundant as gregarious shrub or small trees upto 4m high and available on open slopes of sub montane zones, ascending to 180m and found in W. Himalaya, C. India, Nepal and Sri Lanka. It's leaves are 3-foliolate, leaflets obovate, terminal one larger than two laterals, basal part entire, upper coarsely or irregularly toothed, hairy on nerves beneath but terminal leaflets often narrowed into marginate petiolule and lateral ones are sessile. Leaves mixed with tobacco; sometimes during famine fruit grinded and mixed with flour (Gaur 1999).

Phytochemical constituents were investigated and for the first time eighteen compounds viz. vagenatin, β -sitosterol, daucosterol, 7α -methoxy- β -sitosterol, 7β -methoxy- β -sitosterol,



ergosterol peroxide, α -amyrin, lupeol, linolenic acid methyl ester, glycerol, 1,2-dioleoyl-3-linolein, iso-liquirtigenin, quercetin, (-)-syringa-resin-ol, (-)-pinoresinol-4'-O- β -D-glucopyranoside, (+)-5,5'-dimethoxyl-ariciresin-ol, (+)-iso-lariciresin-ol-9'-O- α -L-rhamno-pyranoside, and methyl- β -D-fructo-furanose were reported this plant (Shrestha *et al.*, 2016). It is also recorded in Ayurvedic pharmacopoeia as having therapeutic uses for the complications related to neurological disorders including anxiety, insomnia, epilepsy, and rheumatoid arthritis (Shrestha *et al.*, 2012), flavonoids of its fruits attenuate glutamate-induced neurotoxicity in HT22 Cells (Shrestha *et al.*, 2013), infusions of leaves were used against cholera (Kumar *et al.*, 2011) and leaf extract showed in vitro anti-HIV activity (Modi *et al.*, 2013). That's why, *R. parviflora* was selected for the synthesis of AgNPs as it is very useful in various aspects and in combination with silver, it may yield better results in different areas of science and technology.

Materials and Methods

Silver nitrate was purchased from Sigma Aldrich company and deionized distilled water was used in all experiments. The integral parts of *Rhus parviflora* were collected from Nagdev Hills, Pauri (Garhwal), India and its authentication number i.e. GUH20754 was provided by Garhwal University Herbarium, Srinagar (Garhwal), Uttarakhand.

In this research work, the bacterial {*B. subtilis* (NCFT.583.08), *S. aureus* (NCFT.576.08), *L. plantarum* (NCFT.623.34) and *P. aeruginosa* (NCFT.645.11)} and fungal {*A. niger* (NCFT.623.11) and *C. albicans* (NCFT.1006.11)} strains were selected for analysing the microbicidal effects of the biosynthesized AgNPs.

Biosynthesis of silver nanoparticles: Various researchers have utilized different parts (stem, root, leaves etc.) of the plant to synthesize silver nanoparticles and it resulted a great boon to the mankind ((Khushboo *et al* 2014;

Edison *et al* 2016; Vanaja *et al* 2013; Thombre *et al* 2014; Tippayawat *et al* 2016; AshokKumar *et al* 2015; Saxena *et al* 2010; Kumar *et al* 2014; Rastogi and Arunachalam 2011). Therefore, in this research work, after extensive literature survey, the leaves of *R. parviflora* were selected to synthesize silver nanoparticles and also by following the principles of green chemistry. 05g fresh leaves taken, washed several times with deionized distilled water and then dried in the hot air oven at 30°C. Hot air oven dried leaves were crushed and transformed into powder form. This powder form of leaves were added in conical flask containing 100ml of deionized distilled water (water is a good solvent). It was heated for 30mins at on hot plate magnetic stirrer at 65°C followed by cooling at room temperature. The obtained solution was finally filtered through Whatman filter paper twice. Then, Ag nanoparticles were biologically synthesized by mixing *R. parviflora* leaf extract with 1mM AgNO₃ solution in 1:9 proportion in a conical flask. After mixing and shaking, the solution was kept for 24 hours at room temperature in the dark place of the lab. At regular interval of times, the change in colour of solution was observed and this change in colour to brownish red and finally to dark red assisted in predicting the formation of Ag nanoparticle. Then the solution was centrifuged at 5000rpm so as to collect the nanomaterial and during this process, the supernatant was discarded. Again, the nanomaterials was centrifuged with deionized distilled water to get rid of any useless material. Finally, nanomaterial/ nanopowder was oven dried and transferred into air tight sample tubes for further characterization and antimicrobial tests.

Characterization of biosynthesized Ag nanoparticles: Characterization of a sample is a very important step in the research work. It assists in collecting data to study various factors that are responsible for the unusual properties of the synthesized material.



In the starting, at regular intervals of time, amalgamation of nano-silver was predicted by observing the colour changes. But to assure the presence of nano-silver particles in the solution and their uniformity in shape, the spectroscopic technique was employed for this by using UV-visible spectrophotometer. The formation of AgNPs was examined from 300-700nm wavelength range against aq. leaf extract (as blank) and then plotted.

X-ray diffraction analysis of this Ag nano-powder was analyzed using X'PERT-PRO Diffractometer, PANalytical. In this technique, X-rays passed through the powder that generate a diffraction pattern (if, $n\lambda = 2d\sin\theta$). The morphological parameters of Ag nanoparticles were measured by transmission electron microscope using JEOL JEM 1011, 100kva. Biosynthesized Ag nanoparticles were sonicated in the solution form for measuring individual particle size and shape. And after confirming the size, shape, nature and presence of nano-silver in the sample, powder of biosynthesized Ag nanoparticles was further characterized for bio-functional groups that were responsible for the reduction of silver ions and also their stabilization using FT-IR Spectrophotometer-Perkin Elmer Model RZX. The frequency range is measured as wave numbers typically over the range 4000–400 cm^{-1} and the background emission spectrum of the IR source is first recorded, followed by the emission spectrum of the IR source with the sample in place.

Determination of Antimicrobial activity of biosynthesized AgNPs: The bacterial (*B. subtilis*, *S. aureus*, *L. plantarum* and *P. aeruginosa*) and fungal (*A. niger* and *C. albicans*) strains were selected for analysing the microbicidal effects of the biosynthesized AgNPs. Casein digest (Soyabean) and dextrose broth (Sabouraud) of Hi Media Pvt. Bombay, India were used for antibacterial and antifungal trials, respectively.

Casein digest broth (Soyabean) was taken to inoculate the selected bacteria and incubation

temperature was maintained at 37°C for 18h and further it was checked to supply approx., 108CFU/mL. Similarly, fungal strains were investigated but inoculation was done in dextrose broth (Sabouraud) with incubation at 48 to 72h.

The antimicrobial activity was evaluated by measuring the diameter of zone of inhibition using Perez *et al* (1993) method with some modifications. Soyabean casein digest agar medium (SCDM) was used to inoculate the bacterial culture and then, separately suspended in broth. About 8mm diameter of wells were punched into agar and then, loaded with AgNPs solution (i.e. in DMSO with N-saline) and solvent blanks. Erythromycin, was simultaneously utilized as positive control and DMSO as negative control and then, the plates were incubated at 37°C for 18h.

Similarly, for assaying, antifungal potential of AgNPs solution, dextrose broth (Sabouraud) medium plates were used and same method was repeated with incubation at 48 to 72h. Fluconazole was utilized as standard. In triplicates, the steps for assaying antimicrobial potential were followed to validate the values of diameter of zone of inhibition for each case. In addition to this, AgNPs solution (100 μL) was prepared in DMSO (sterile) and then, successively diluted with N-saline (i.e. 0.85% NaCl) and similar amount of bacterial or fungal suspension was added to different test-tubes and kept for 48h incubation period for the determination of the minimum inhibitory and lethal concentrations (Usman and Ladan, 2007; Vollekova *et al* 2001).

Results and Discussion

UV-visible spectroscopic study: UV-Vis spectroscopic technique is an important instrument to monitor the formation of silver nanoparticles (Philip 2010; Sadeghi and Gholamhoseinpoor 2015; Zargar *et al* 2011) and could be utilized to study size of nanoparticles in aqueous medium (Wiley *et al* 2006). This analysis was carried out to study the progress of the reaction between silver ions



and the bio-components present in the leaf extracts. UV-visible spectra of Ag nanoparticles in aq. solution with different time intervals are shown in Fig. 1. Ag surface plasmon resonance absorption bands at 423nm were shown by *R. parviflora* leaf extract mediated synthesized Ag nanoparticles sample solutions. From the spectral peaks, it can be assumed that the reduction of silver ions and the formation of stable Ag nanoparticles occurred quickly and in addition to this,

uniform sized Ag nanoparticles present in the solution were also assessed from the sharpness of the peaks. The band gap of Ag nanoparticles was 2.93eV and it was calculated by the following equation i.e.

$$E_g = \frac{1240}{\lambda} \text{ (eV)}$$

Were,

Eg: Band gap energy, λ = observed absorption maximum wavelength (nm)

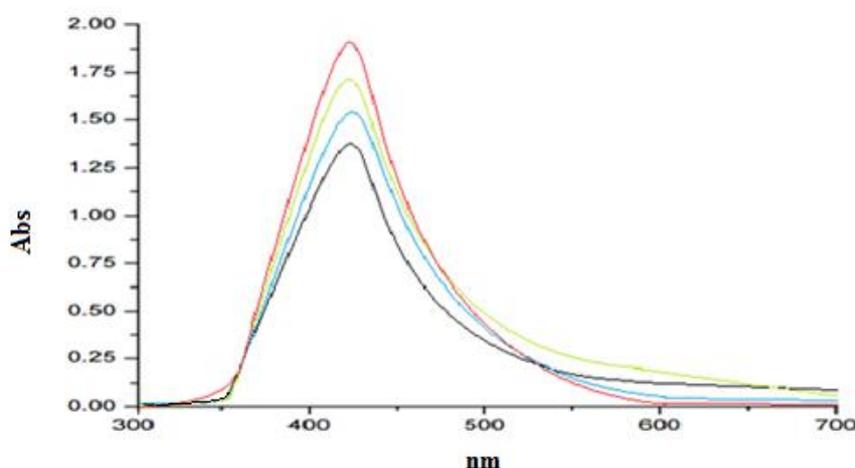


Fig. 1: UV-Visible spectra of AgNPs

X-ray diffraction analysis: This tool is applied for characterizing and identifying the phase, crystallinity and structure of silver nano-crystals. 2θ (degree) values with (hkl) planes at 37.08(111) and 42.96(200) were observed and with details are given the Table-1. The pattern as shown in the Fig. 2 validated the formation of face centred cubic phase Ag nanoparticles. The average particle size (D) is less than 03nm and it was calculated by Debye Scherrer equation:

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

where K is the Scherrer constant, λ is the X-ray wavelength, $\beta_{1/2}$ is the width of the peak at half maxima, θ is the Bragg diffraction angle

Transmission electron microscopic measurements: TEM is an important characterization technique in the area of nanotechnology; due to its high resolution and its capacity to determine the morphological parameters i.e. size and shape of metal based nanoparticles. TEM micro-image (Fig. 3) confirmed that Ag nanoparticles synthesized by using *R. parviflora* leaf extract are spherical shaped with maximum particles in the size range of 04-10 nm. In the TEM micro image, it is clearly visible that AgNPs are developed during this biological process are of nearly similar particle size and have average particle size 07nm. Even from the sharp peaks of nanoparticles as shown above in the UV-Visible spectrum, we can guess the uniformity in the nano-structures with very less size difference

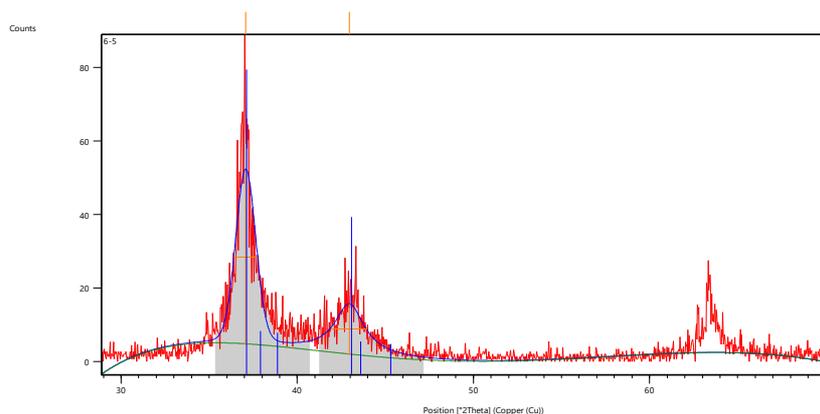


Fig.2: XRD pattern of Ag nanoparticles

Table-1: Important XRD peak list of Ag nanoparticles

Pos. [$^{\circ}$ 2Th.]	Height [cts]	FWHM Left [$^{\circ}$ 2Th.]	d-spacing [Å]	Rel. Int. [%]
37.08(2)	47(2)	1.34(6)	2.42282	100.00
42.96(6)	14(1)	2.0(3)	2.10368	28.87

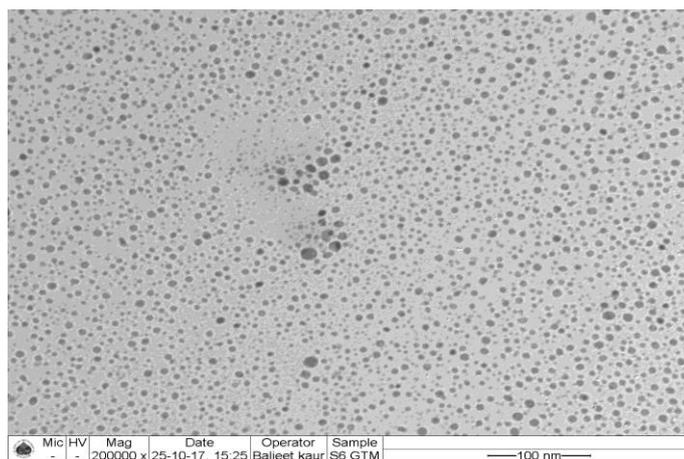


Fig. 3: TEM micro-image of Ag nanoparticles

FTIR spectral study

It is an important tool in predicting the presence of biomolecules along with the silver nanomaterial. It is the fact that the most molecules absorb light in the infra-red region and the absorption corresponds to the types of bonds present in the molecule. The resultant absorption spectrum from the bond natural vibration frequencies indicates the presence of various chemical bonds and functional groups present in the sample. Fig. 4 is showing the FTIR spectrum of AgNPs synthesized from *R. parviflora* leaf extract. Characteristic peaks at

3255.96 cm^{-1} correspond to -OH stretching vibrations but peaks between 1694.32, 1584.04 and 1345.80 cm^{-1} are due to C=O, C=C and Ar-OH functional groups, respectively. Thus IR spectroscopic study confirmed that mainly the hydroxyl and carbonyl groups could possibly bio-capped silver nanoparticles (i.e., capping of silver nanoparticles by bio-components) to prevent agglomeration and thereby stabilize the product. This further suggests that the bio-molecules could possibly perform dual



functions of amalgamation and stabilization of AgNPs in the aqueous medium

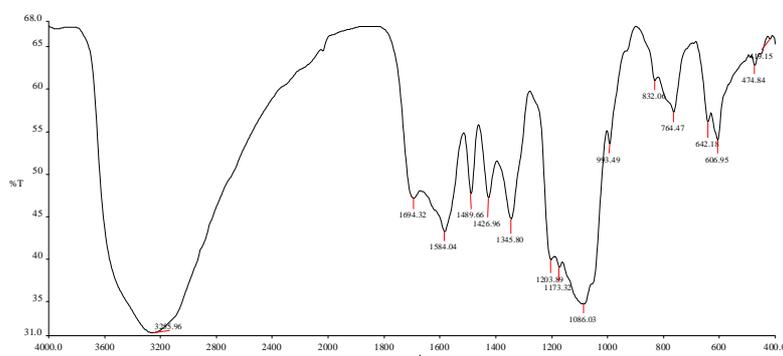


Fig. 4: FTIR spectrum of Ag nanoparticles

Antimicrobial activity: Human beings are trying to get rid of various deadly or harmful microbes which are surrounding them from all the sides. Human beings have tried so many drugs to kill them or to stop their further growth but its becoming tough day by day as the microbes are showing drug resistant behaviour. So to deal with these multidrug resistant microbes, the researchers have extended the use of AgNPs, as antimicrobial agents, but in combination with bioorganic capping and stabilizing material from plant, the enhancement in their antimicrobial activity has been reported (MubarakAli et al 2011; Prabhu and Poulouse 2012). From last few years, silver nanoparticles synthesized using various plant extracts have been exploited for their activity against thee multidrug resistant microbes and still, the researchers are exploiting them for a wide range of bio-applications to sustain life on this planet earth. And in this research work, the antimicrobial activity of AgNPs synthesized by using *R. parviflora* was carried out against microbial

(both bacterial and fungal) strains. Antibacterial and antifungal activities were determined separately by following standard and sensitive protocol as mentioned above in the methodology section. Our research work focused on the production of nano silver agents using green protocol with huge antimicrobial potential. Antibacterial activity has been determined by measuring the zone of inhibition, minimum inhibitory and lethal concentration. The synthesized silver nanoparticles from *R. parviflora* (*AgNPs-Rp*) displayed good response against *B. subtilis*, *S. aureus*, *L. plantarum* and *P. aeruginosa* as given in the table. 2. It has been observed that the nano agents were highly active against *L. plantarum* (43mm) and *P. aeruginosa*(41mm), good against *B. subtilis* (28mm) and to some extent against *S. aureus* (18mm). MIC and MLC results further supported that these can be utilized for action against the bacterial infections and also exploited for other multi drug resistant bacteria.

Table 2: Antibacterial activity of AgNPs

Sample	Diameter of zone of inhibition (mm)			
	<i>B. subtilis</i> (NCFT.583.08)	<i>S. aureus</i> (NCFT.576.08)	<i>L. plantarum</i> (NCFT. 623.34)	<i>P. aeruginosa</i> (NCFT.645.11)
<i>AgNPs-Rp</i> (100 µl)	28	18	43	41
Erythromycin (1 mg/ml)	45	34	35	38
	MIC and MLC (µl)			



	MIC	MLC	MIC	MLC	MIC	MLC	MIC	MLC
<i>AgNPs-Rp</i>	30	45	50	80	15	28	28	45

The antifungal activity of AgNPs synthesized by using *R. parviflora* leaf extract against *A. niger* and *C. albicans* strains were recorded using sensitive determination methods and is given in the table 3. The nano agents showed potential response against both selected pathogens {*C. albicans* (22mm) and *A. niger*

(18mm)}. MIC and MLC (μ l) values of the nanoparticles were also measured. From the antifungal results, we can assume that the biosynthesized nano agents are potentially active against the pathogens which are very harmful for living beings.

Table 3: Antifungal activity of AgNPs

Sample	Diameter of zone of inhibition (mm)			
	<i>A. niger</i> (NCFT.623.11)		<i>C. albicans</i> (NCFT.1006.11)	
<i>AgNPs-Rp</i> (100 μ l)	18		22	
Flucanazole (1mg/ml)	34		28	
	MIC and MLC (μ l)			
	MIC	MLC	MIC	MLC
<i>AgNPs-Rp</i>	75	90	50	70

Exactly, the mechanism of antimicrobial activity is not known/ clearly understood but some of the researchers state that AgNPs may affix to the surface of the cell membrane and perturb its permeability and lead to structural changes on cell wall/ membrane causing cell death (Sondi and Sondi 2004). In another study, it was suggested that the possibility of AgNPs may also pierce inside the bacteria and fungi causing destruction by interacting with phosphorous and sulphur having compounds such as DNA and proteins resulting in cell death (Morones et al., 2005; Baker et al 2005). Another study has reported that Ag nano agents penetrate microbial cell walls, resulting in structural harm to the cell walls and cell death due to the production of free radicals i.e. reactive oxygen species (ROS). The release of metallic ions once inside the cells (with lower pH), which generates free radicals (reactive oxygen species) and induce oxidative pressure; thus, further enhancing their antimicrobial activity (Aderibigbe 2017). Furthermore, phytochemicals associated with the metallic ions can also enhance their activity; not only by controlling the size of metallic nanoparticles but also by interacting with the microbial cell. Therefore, from the recorded antimicrobial activity of biosynthesized

nanoparticles as shown in the above tables 2 and 3, there is no hesitation in saying that the developed nano agents will definitely find great space in the field of science and technology and it may also attract other clinicians/ researchers to exploit them for various biological applications.

Conclusion

We can say that our attempt which is based on green method as it has many advantages such as, ease with which the process can be scaled up, cost effective, use of green solvents, low energy consumption, safe handling, etc. has created the uniformly distributed silver nanoparticles, very closely mono-dispersed, crystalline, spherical in shape with average size 07nm and stabilized by bioorganic material present in the *R. parviflora* leaf extract. The nanoparticles were found to be very active against the bacterial (*Bacillus subtilis*, *Staphylococcus aureus*, *Lactobacillus plantarum* and *Pseudomonas aeruginosa*) and fungal (*Aspergillus niger* and *Candida albicans*) strains. Overall, we can say that the synthesis of AgNPs from *R. parviflora* leaf extract and their toxicity against the selected pathogens opens a door for a new range of antimicrobial agents. This method could be



used as a competitive alternative to the already existing physical and/ or chemical methods and it may also develop the interest of researchers for the large-scale creation of other similar nano- structures using *R. parviflora* plant extract.

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