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ORIGINAL ARTICLE

# Biogenic silver nanoparticles: efficient and effective antifungal agents

**Vasudeva Reddy Netala[1] · Venkata Subbaiah Kotakadi[2] · Latha Domdi[1] · Susmila Aparna Gaddam[3] · Pushpalatha Bobbu[1] · Sucharitha K. Venkata[4] · Sukhendu Bikash Ghosh[5] · Vijaya Tartte[6]**

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**Abstract** Biogenic synthesis of silver nanoparticles (AgNPs) by exploiting various plant materials is an emerging field and considered green nanotechnology as it involves simple, cost effective and ecofriendly procedure. In the present study AgNPs were successfully synthesized using aqueous callus extract of *Gymnema sylvestre*. The aqueous callus extract treated with 1nM silver nitrate solution resulted in the formation of AgNPs and the surface plasmon resonance (SPR) of the formed AgNPs showed a peak at 437 nm in the UV Visible spectrum. The synthesized AgNPs were characterized using Fourier transform infrared spectroscopy (FTIR), Transmission electron microscopy (TEM), and X-ray diffraction spectroscopy (XRD). FTIR spectra showed the peaks at 3333, 2928, 2361, 1600, 1357 and 1028 $cm^{-1}$ which revealed the role of different functional groups possibly involved in the synthesis and stabilization of AgNPs. TEM micrograph clearly revealed the size of the AgNPs to be in the range of 3–30 nm with spherical shape and poly-dispersed nature; it is further confirmed by Particle size analysis that the stability of AgNPs is due its high negative Zeta potential (−36.1 mV). XRD pattern revealed the crystal nature of the AgNPs by showing the braggs peaks corresponding to (111), (200), (220) and (311) planes of face-centered cubic crystal phase of silver. Selected area electron diffraction pattern showed diffraction rings and confirmed the crystalline nature of synthesized AgNPs. The synthesized AgNPs exhibited effective antifungal activity against *Candida albicans*, *Candida nonalbicans* and *Candida tropicalis*.

**Keywords** Biogenic synthesis · Silver nanoparticles · FTIR · TEM · Antifungal activity

✉ Venkata Subbaiah Kotakadi
kotakadi72@gmail.com

✉ Vijaya Tartte
tvijayasvu@yahoo.com

[1] Department of Biotechnology, Sri Venkateswara University, Tirupati, AP, India

[2] Research Scientist, DST PURSE Centre, S.V. University, Tirupati, AP, India

[3] Department of Virology, Sri Venkateswara University, Tirupati, AP, India

[4] Department of Home Science, Sri Venkateswara University, Tirupati, AP, India

[5] Nuclear Agriculture and Biotechnology Division, BARC, Mumbai, MH, India

[6] Department of Botany, S.V. University, Tirupati, AP, India

## Introduction

Nanotechnology is a broad area and has shed a ray of light on biotechnology and medicine fields too. Especially, noble metal nanoparticles with fascinating colors due to the excitation of surface plasmon vibrations and optical properties offer a very convenient surface bioconjugation with molecular probes (Kreibig and Vollmer 1995; El-Sayed 2001). Most notable among them, use of silver in medical preparations is known since Neolithic age. However, the first recorded medical use of silver traces back to the eighth century (Moyer 1965). As the nanotechnology advances, silver races upfront in recent times due to its tunable photophysical attributes, its property being efficiently addressed via optical and spectroscopic techniques. Silver

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nanoparticles (AgNPs) now occupy the broader scientific interest from photonics to biomedicine.

Silver nanoparticles find innumerable applications due to their remarkable physicochemical properties which include optical, thermal (Karimzadeh and Mansour 2010), electronic, magnetic (Cobley et al. 2009; Feiner 2006) and catalytic properties (Edison and Sethuraman 2012). AgNPs have been found to possess different applications such as optical receptors (Vilchis et al. 2008), electric batteries (Wang et al. 2010), catalysts in many chemical reactions (Campelo et al. 2009), SERS based enzyme catalysis (Wei et al. 2007), biosensors (Dubas and pimpan 2008; Schultz et al. 2000), DNA delivery (Chen et al. 2012), antioxidants (Mittal et al. 2006), anticancer (Jacob et al. 2011), and antimicrobial agents (Jaidev and Narasimha 2010; Netala et al. 2014a, b). AgNPs as antimicrobial agents are of great interest today due to the increasing tendency of microbes to develop either resistance against antibiotics and transform themselves to resistant strains (Rai et al. 2009). For instance, *Candida albicans* and *Candida nonalbicans* are fungal pathogens of immuno-compromised host that produce a broad spectrum of diseases, ranging from chronic mucocutaneous candidiasis to invasive illnesses, such as systemic candidiasis, hepatosplenic candidiasis and peritonitis (Singhi and Deep 2009). Treating infections caused by *Candida species* has become a hectic problem due to its resistance to the less toxic azole drugs (Fluconoazole and Voriconazole) (White et al. 2002). Polyenes (Amphotericin B and Nystatin) cannot be employed because they are highly toxic and produce serious side effects like liver and renal dysfunction (Hoeharner et al. 2010). Hence researchers have been investigating and found metal nanoparticles, especially AgNPs, as alternate antimicrobial agents.

Silver nanoparticles can be synthesized by various physical and chemical methods including radiation-assisted (Huang et al. 2009; Abbasi et al. 2012), microwave-assisted (Nadagouda et al. 2011), electrochemical (Hosseini and Momeni 2010) photochemical (Kutsenko and Granchak 2009) polyol (Byeon and Kim 2012) and polyaniline processes (Bouazza et al. 2009). But most of these methods involved the application of toxic, hazardous and costly materials. Ecological impact and safety being considered the utmost objectives of any sort of research, nowadays, biological molecules and methods form the meat of it. Hence, the supramolecular complexes of bio-macromolecules and their electrostatic and topographic properties are being used widely in pharmaceutical and medical research areas. Peptides to proteins, Nucleic acids, sugars to polysaccharides, viral particles, plant derived compounds, etc., are being constantly explored for synthesis of novel nanoparticles and carriers. Hence the biological methods for the synthesis of AgNPs have gained significant interest in the field of nanobiotechnology. Biological methods involve the synthesis of clean, non-toxic, biocompatible and eco-friendly AgNPs. The biological methods, particularly plant-based approaches, do not involve hazardous chemicals, high-energy requirements and wasteful purifications (Vijayaraghavan et al. 2012; Venkatesham et al. 2014). A number of plant extracts including *Andrographis Paniculata* (Kotakadi et al. 2014a, b), *Azadirachta indica* (Tripathy et al. 2009), *Cassia alata* (Gaddam et al. 2014), *Cathranthus roseus* (Kotakadi et al. 2013), *Centella asiatica* (Netala et al. 2014a, b), *Coleus aromaticus* (Kotakadi et al. 2014a, b), *Melia dubia* (Netala et al. 2014a, b), *Ocimum tenuiflorum* (Patil et al. 2012), and *Pulicaria glutinosa* (Khan et al. 2014) have been reported.

*Gymnema sylvestre* is a woody climber belonging to the Asclepiadaceae family. It is mainly distributed in India, Srilanka, China, Japan and Australia. The leaves of this plant, popularly known as 'Gur-mar' in India, are renowned for distinctive property of suppressing the taste of sweetness. Hence the leaves of the *G. sylvestre* have been used for the treatment of diabetes in India since ancient times (Agarwal et al. 2000). Many scientists proved the antidiabetic property of *G. sylvestre* leaf extract both in mouse and human (Chattopadhyay 1999; Okabayashi et al. 1990). The mechanisms of antidiabetic property of leaf extract includes reduction of blood glucose levels by stimulation of insulin release (Sugihara et al. 2000), inhibition of glucose absorption (Hirata et al. 1992) and increased glucose tolerance (Kar et al. 1999). The leaf extract of this plant reduces elevated serum lipids, total cholesterol, and high-density lipoproteins and hence it is considered as anti-hyperlipidemic (Rachh et al. 2010). The leaves also possess hepatoprotective (Rana and Avadhoot 1992), anti-inflammatory, free radical scavenging and antioxidant properties (Nadkarni 1992; Agarwal et al. 2000). Besides, this the plant is also used in the treatment of hemorrhoids, jaundice, bronchitis (Masayuki et al. 1997), cardiopathy, asthma, dyspepsia, constipation and snake bite (Nadkarni 1992; Selvanayagam et al. 1995). Due to these wide medicinal values, the plant is called as "Miracle fruit plant". The pharmacological and biological activities of the *G. sylvestre* are mainly attributed to the active bioactive compounds present in the leaves including triterpene saponins belonging to oleanane and dammarene classes which are glycosides containing many hydroxyl groups, polyphenols including flavones, d-quercitol, lupeol, gymnemasides, gymnemagenols, and other constituents including oleanoleates, butyric acid, stigma sterols, tartaric acids, butyric acid, phytins, resins, various proteins, amino acids and their derivatives (Rao and sinsheimer 1971; Sahu et al. 1996). The total oleanane type saponin fraction of the leaves commonly known as 'gymnemic acids' have antidiabetic (Bakrudeen et al. 2010; Masayuki et al. 1997),

anti-hyperlipidemic (Rachh et al. 2010), anti-inflammtory, antioxidant (Diwan et al. 1995) and anti-atherosclerotic activities (Bishayee and Chatterjee 1994). Development of in vitro callus cultures from the leaves is identified as alternative and very effective approach for the optimization of bioactive compounds (Kanetkar et al. 2006). This callus cultures are explored for the biosynthesis of AgNPs as another important pharmaceutical or biomedical application of *G. sylvestre*. In the present study, we report for the first time the successful synthesis of AgNPs from the aqueous callus extract of *G. sylvestre*. The synthesized AgNPs are well characterized by spectral analysis using different spectroscopic techniques. The synthesized AgNPs were evaluated for biomedical applications by checking antifungal activity against different *Candida* species.

## Materials and methods

### Collection and sterilization of plant material

Healthy plants of *Gymnema sylvestre* were collected from Tirumala hill region of Eastern Ghats, Andhra Pradesh, India. The plants were authenticated by Taxonomist, Department of Botany, Sri Venkateswara University, Andhra Pradesh, India. In this study the fresh, young and healthy leaves were used as explants. Leaves were excised and thoroughly washed with sterilized double distilled water (SDDW) for 5 min to remove the dust. Then the leaves were surface sterilized using 70 % ethanol for 3 min. Finally, the leaves were rinsed with SDDW for 5 min and blotted on sterile paper to remove water drops.

### Induction of callus culture

The sterilized leaves were cut into small fragments of size 0.6 × 0.8 cm. The leaf fragments were inoculated on Murashig-Skoog media supplied with 2,4-dichlorophenoxyaceticacid (2.0 mg $L^{-1}$) and Kinetin (1.0 mg $L^{-1}$) as callus promoting plant growth hormones (Bakrudeen et al. 2010; Murashige and Skoog 1962) The cultures were maintained at 23–25 °C under the photoperiod of 16 h light/8 h dark.

### Preparation of aqueous callus extracts

Fresh callus was collected in the Petri-dishes and dried at 40 °C for about 24 h. The dried callus was crushed into very fine powder. 10 gm of callus powder was boiled in 100 mL of SDDW for 15–20 m at 70 °C and then cooled to room temperature. This aqueous solution was filtered by Whatman no.1 filter paper. The filtrate was named aqueous callus extract and used for the synthesis of AgNPs.

### Biogenic synthesis of AgNPs

Silver nitrate ($AgNO_3$) was purchased from Himedia. Aqueous solution of 1 mM $AgNO_3$ was prepared and used for the synthesis of AgNPs. 10 mL of aqueous callus extract was added to 90 mL of 1 mM $AgNO_3$ solution in a 250-mL flask and incubated at room temperature in dark condition for about 24 h or until the solution turned dark brown in color. The synthesis of AgNPs was primarily detected by observing the solution for color change from yellow to dark brown.

### Characterization of AgNPs

The synthesis of AgNPs was confirmed using UV–visible spectrophotometer 2060+ (Analytical Technologies Ltd,) with a resolution of 1 nm between 200 and 700 nm. After the biosynthesis, the AgNPs were separated by centrifugation of the solution at 15000 rpm for 15 min. AgNPs were redispersed in water and purified by repeated centrifugation for five times to remove the unused callus extract and the pellet obtained was dried in hot air oven. Further, the air-dried powder of AgNPs was used for characterization. FTIR analysis of the purified AgNPs was carried out to study the possible functional groups involved in synthesis and stabilization of AgNPs. The analysis was done using FTIR (Model: ALPHA interferometer, Bruken,) in the range of 500–4000 $cm^{-1}$ with the resolution of 2 $cm^{-1}$. The size and shape of the AgNPs were determined using transmission electron microscopy (TEM). TEM analysis was carried out using FEI Tecnai F12 (Philips Electron Optics Ltd) operated at 100 kV. Particle size and Zeta potential measurement experiments were carried out using a Nanopartica (HORIBA instrument). XRD analysis was carried out to reveal the crystalline nature of synthesized AgNPs using Ultima IV X-ray diffractometer (Rigaku Ltd, Tokyo).

### Antifungal activity of AgNPs

Disc diffusion method was employed to evaluate the antifungal activity of AgNPs against *Candida albicans*, *Candida nonalbicans* and *Candida tropicalis* (Kumar et al. 2013; Monali et al. 2009). 200 µL of fungal inoculum was spread evenly on the surface of Potato Dextrose Agar (PDA) plates. Each PDA plate consists of four discs. To compare the antifungal activity of synthesized AgNPs, one sterile disc impregnated with less toxic azole drug, voriconazole and another sterile disc impregnated with toxic polyene, amphotericin B were used as positive controls. Sterile disc impregnated with 25µL (1 mg/1 mL) of AgNPs was used as tested concentration. The plates were then incubated at 28 °C for 24 h. After the incubation, the

plates were examined for growth inhibition zones. The diameter of zone of inhibition (ZOI) was measured using ruler and recorded. The test was repeated three times to ensure reliability.

## Results and discussion

In the present experiment, biogenic synthesis of AgNPs using aqueous callus extract of *G. sylvestre* was carried out followed by their characterization and antifungal activity. Callus was initiated on 12th day of inoculation on Murashig-Skoog media supplied with 2,4-dichlorophenoxyaceticacid (2.0 mg $L^{-1}$) and Kinetin (1.0 mg $L^{-1}$) and good amount of callus biomass was observed between 20 and 35 days. After 35 days, the friable and light yellow colored callus (Fig. 1). was harvested and used for the preparation of the aqueous callus extract which is rich in plant secondary metabolites and proteins responsible for the bioreduction process. Hence this aqueous callus extract was used for biogenic synthesis of AgNPs. Initial confirmation of the bioreduction of $Ag^{+}$ (silver ions) to $Ag^{0}$ (AgNPs) was done by observing the color change of the solution from yellow to dark brown (Fig. 2). It is well known that AgNPs exhibit a dark brown or yellowish brown color in aqueous solution due to excitation of characteristic surface plasmon vibrations (Kreibig and Vollmer 1995; Prathna et al. 2011). The bioreduction was further confirmed by UV–vis spectroscopy.

**Fig. 1** Friable and *light yellow* color callus raised from the leaves of *Gymnema sylvestre* on MS media containing 2,4-dichlorophenoxyaceticacid (2.0 mg $L^{-1}$) and kinetin (1.0 mg $L^{-1}$)

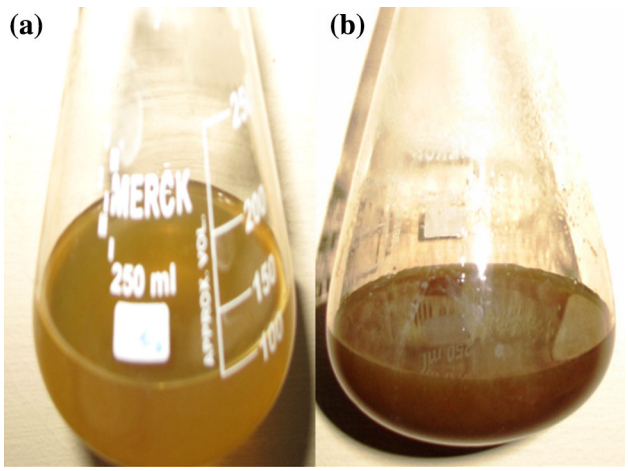



**Fig. 2** *Yellow* color of the aqueous callus extract of the *G. sylvestre* exposed to $AgNO_3$ (**a**) and *dark brown* color of the solution after 24 h of incubation indicates the of bioreduction (**b**)

### UV–vis analysis

The UV–visible spectra (Fig. 3) of the reaction solution showed an absorbance peak at 437 nm which is characteristic surface plasmon resonance (SPR) peak of AgNPs and hence confirmed their synthesis. The sharp and strong absorption peak is due to surface plasmon vibrations in the visible region ofexcited AgNPs. It is known that the size and shape of the nanoparticles reflect the absorbance peak. In fact the size has a linear correlation with the peak intensity, while the number of nanoparticles does not have such linear correlation (Jaidev and Narasimha 2010). SPR peak could correspond to the spherical shape of metal nanoparticles (Kotakadi et al. 2014a, b, 2015). Different metabolites and proteins present in the aqueous callus extract could be responsible for bioreduction and capping of AgNPs synthesized (Noginov et al. 2007; Stamplecoskie and Scaiano 2010).

### FTIR spectra analysis

The FTIR spectral analysis of AgNPs (Fig. 4). showed intensive peaks at 3333, 2928, 2361, 1600, 1357 and 1028 $cm^{-1}$, respectively. The sharp peak at 3333 $cm^{-1}$ can be assigned to N–H stretching of the primary amines and the peak at 2928 $cm^{-1}$ corresponding to C–H stretching of methylene groups of the protein (Bozanic et al. 2010). The peak at 2361 $cm^{-1}$ could be assigned to N–H stretching or C–O stretching vibrations (Ashok et al. 2010). Peak at 1600 $cm^{-1}$ corresponds to asymmetrical C–O stretching (Kora et al. 2012). The Peak observed at 1357 $cm^{-1}$ corresponds to C–N stretching vibrations of aromatic amines and aliphatic amines (Sanghi and Verma 2009). The peak at 1028 $cm^{-1}$ is responsible for O–H deformation/C–O stretch of phenolic and alcoholic groups (Valentina and

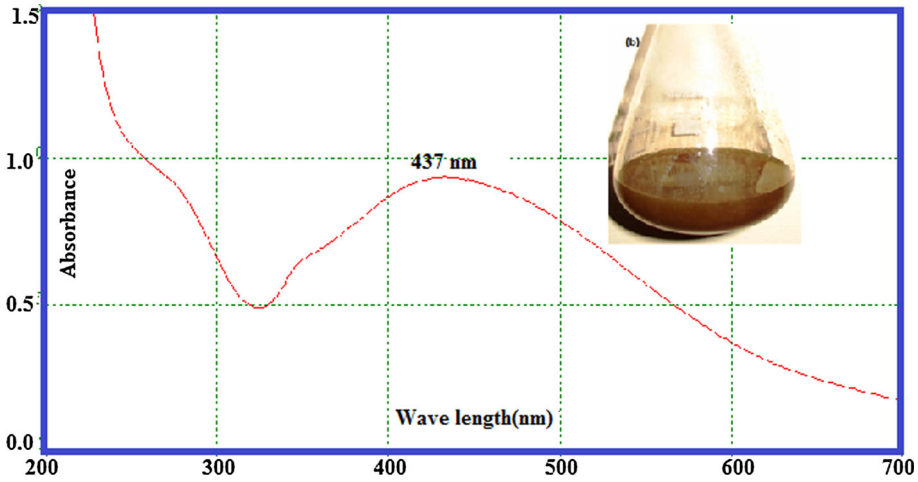



**Fig. 3** UV/vis spectra showing characteristic surface plasmon resonance peak of AgNPs synthesized using aqueous callus extract of *G. sylvestre*

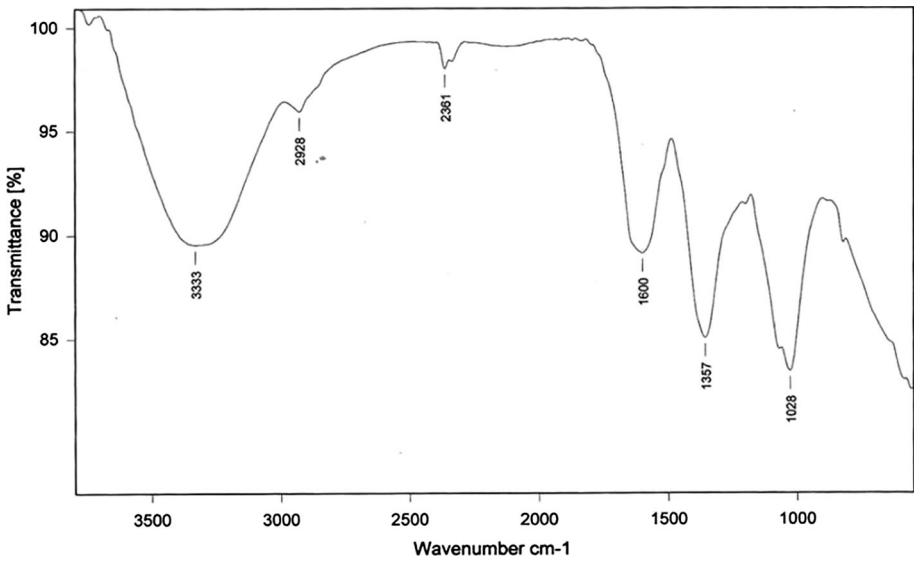



**Fig. 4** FTIR spectra of the biogenic synthesized AgNPs using aqueous callus extract of *G. sylvestre*

Boris 2013). FTIR analysis clearly revealed that phytochemicals like polyphenols present in the callus extract were mainly responsible for reduction of silver ions ($Ag^{+}$) into nanoscale silver particles ($Ag^{0}$). Proteins could play the main role in the stabilization of AgNPs by acting as capping agents.

## TEM analysis and SAED pattern

TEM studies determined the size and shape of the AgNPs synthesized. From the TEM micrograph it is clear that AgNPs are polydispersed, spherical in shape and ranged in size from 3 to 30 nm. TEM micrograph of AgNPs at different scale bars is represented in Fig. 5. The AgNPs in the solution were partially aggregated, uniform in size and stable. The TEM results were consistent with many reports for plant and plant-derived extract-mediated synthesis of AgNPs (Netala et al. 2014a, b; Kotakadi et al. 2014a, b). Figure 5b shows the SAED pattern of synthesized AgNPs, indicating polycrystalline diffraction rings which could be indexed to cubic-lattice of metallic silver and reveals the synthesized AgNPs are crystalline metallic silver.

## Particle size determination

The particle size of the biosynthesized AgNPs was detected by intensity and laser diffraction which are poly-dispersed in mixture solution. The average size (hydro dynamic radius) of synthesized AgNPs was found to be 25.3 nm with callus extract (Fig. 6i); after the analysis of AgNPs with callus extract, it has been revealed that the particles synthesized with aqeous callus extract are less than 3–30 nm in size. The results are similar to that of TEM results indicating that the average size of the AgNPs is 25.3 nm. The biosynthesized particles are poly-dispersed in nature. The stability was further confirmed by Zeta potential of the particles.

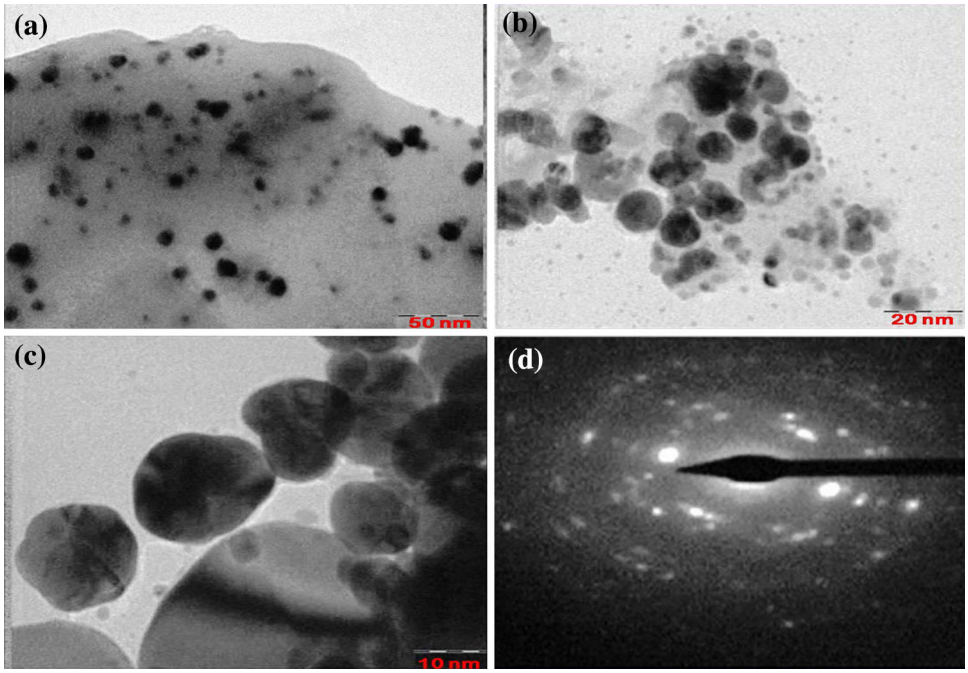
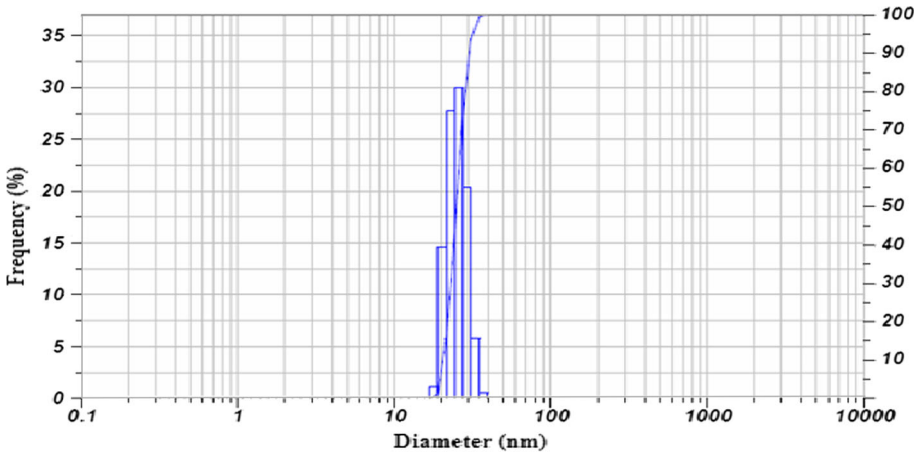



**Fig. 5** TEM micrograph of biogenic synthesized AgNPs; *scale bar* represents 50 nm (**a**); 20 nm (**b**); 10 nm (**c**). SAED pattern of the AgNPs (**d**)




Calculation Results

| Peak No. | S.P.Area Ratio | Mean | S. D. | Mode |
|---|---|---|---|---|
| 1 | 1.00 | 25.3 nm | 3.6 nm | 25.4 nm |
| 2 | --- | --- nm | --- nm | --- nm |
| 3 | --- | --- nm | --- nm | --- nm |
| Total | 1.00 | 25.3 nm | 3.6 nm | 25.4 nm |

**Fig. 6** Particle size analysis for AgNPs of callus extract

## Zeta potential of AgNPs

The electrostatic repulsive force between the nanoparticles depends on the charge which is present on the surface of the nanoparticle. When they are negatively charged, the process prevents the nanoparticles from agglomeration in the medium, leading to long-term stability. In the present study, the very high negative value of Zeta potential confirms the repulsion among the particles and thereby increases the stability of the formulation. The zeta potential of the AgNPs of fruit extract was found to be −36.1 mV. (Fig. 7) Based on the above results, it is concluded that the AgNPs synthesized with callus extract were very stable.

## XRD analysis

XRD is used to evaluate the crystalline nature of synthesized AgNPs. XRD pattern obtained (Fig. 8) showed that four characteristic diffraction peaks at 38.31°, 44.61°, 64.5° and 77.58° were, respectively, indexed to (111), (200), (220) and (311) planes of face-centered cubic crystal lattice of silver (JCPDS PDF card 87-0718). XRD pattern showed

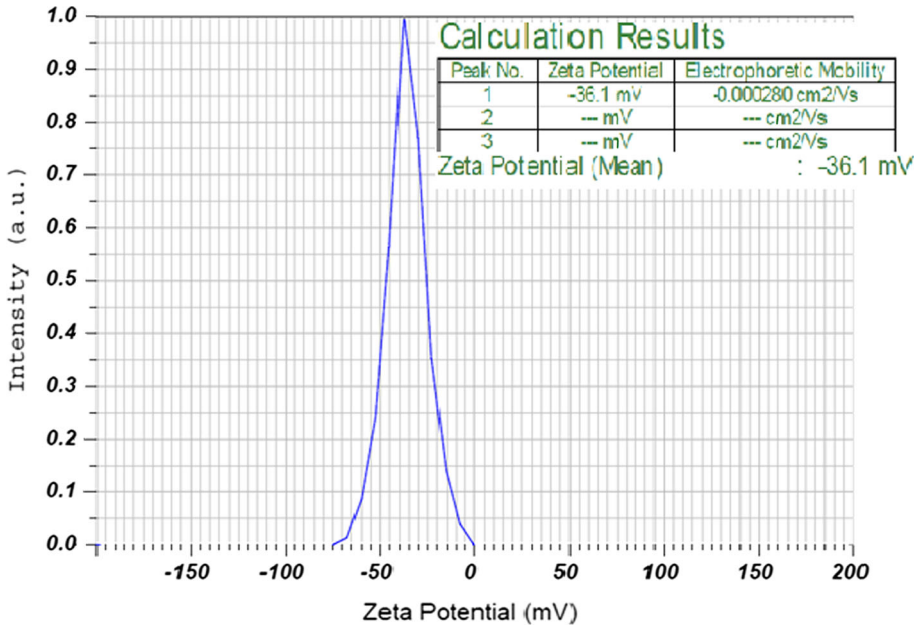



**Fig. 7** Zeta potential of synthesized AgNPs by callus extract

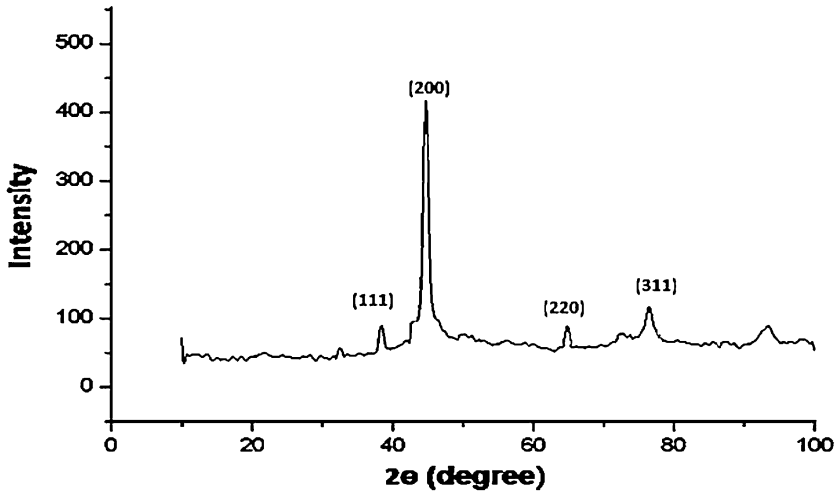



**Fig. 8** XRD pattern of biogenic synthesized AgNPs using aqueous callus extract of *G. sylvestre*

here are consistent with the earlier reports (Patil et al. 2012; Vijayaraghavan et al. 2012).

## Antifungal activity

To reveal the biomedical importance of the synthesized AgNPs antifungal activity was carried out against *C. albicans, C. nonalbicans* and *C. tropicalis*. The synthesized AgNPs exhibited excellent antifungal activity against *C. albicans, C. nonalbicans* and *C. tropicalis* and formed the ZOI of diameters 15.4, 14.2 and 15.7 mm, respectively (Fig. 9). Although, AgNPs showed strong antifungal activity against all the *candida* species, higher ZOI was noticed for *C. tropicalis* followed by *C. albicans*. Remarkably, AgNPs exhibited almost equal activity with that of voriconazole but showed less activity than the toxic drug Amphotericin B (Table 1). The results suggested that the antifungal activity of AgNPs can be further enhanced by increasing their concentration as these are nontoxic and biocompatible. The results of antifungal activity of the AgNPs are consistent with many earlier reports (Kumar et al. 2013; Monali et al. 2009) and proved that the biosynthesized AgNPs possess more potent antifungal activity when increasing the concentrations than the azole drugs which are toxic at higher concentrations and also provided the information on the mode of action of AgNPs on fungi (Kumar et al. 2013; Monali et al. 2009; Bankar et al. 2010; Kim et al. 2009; Vivek et al. 2011). AgNPs perturb the cell wall and result in the release of cell wall components like ions and intracellular proteins and as a result the growth of fungi is inhibited (Kim et al. 2009). In addition, they form pore-like structures or pits and dissipate the membrane electrical potential. As a result disruption of cell membrane occurs which in turn inhibits the normal budding process (Bankar et al. 2010; Kim et al. 2009; Vivek et al. 2011).

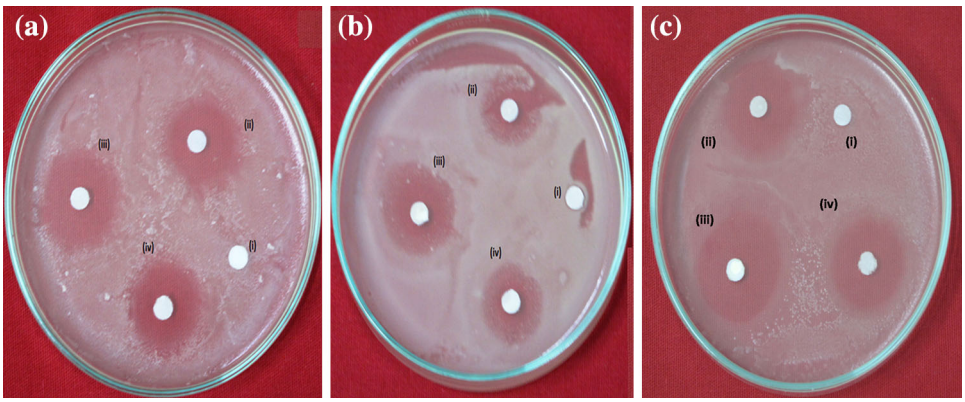



**Fig. 9** Antifungal activity of the AgNPs against *Candida albicans* (**a**), *Candida nonalbicans* (**b**) and *Candida tropicalis* (**c**). No inhibition zone is observed by aqueous callus extract (*i*), Inhibition zones formed by AgNPs (*ii*), by Amphotericin B (*iii*) and by voriconazole (*iv*) were represented

**Table 1** Antifungal activity of biogenic synthesized AgNPs compared with amphotericin B and Voriconazole

| S. no | Tested fungi | Amphotericin B | Voriconazole | AgNPs |
|---|---|---|---|---|
| 1. | *C. albicans* | 16.1 | 15.4 | 15.2 |
| 2. | *C. nonalbicans* | 14.8 | 13.4 | 12.9 |
| 3. | *C. tropicalis* | 16.8 | 16.2 | 15.8 |

Values are expressed as diameter of zone of inhibition (ZoI)

## Conclusion

In the present study, we have developed a novel, promising, ecofriendly and biotechnological approach for the synthesis of clean and biocompatible AgNPs. In this study, we have synthesized spherical shaped AgNPs of 3–30 nm in size using aqueous callus extract of *G. sylvestre* as a reducing agent for the first time. The synthesized AgNPs are very well dispersed, well defined in shape and polycrystalline in nature. The synthesized AgNPs possess very good antifungal activity against different *candida* species and hence clearly proved their pharmaceutical and medicinal importance as tropical antifungal agents and in the manufacturing of antifungal skin ointments.

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



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