

Research paper

# Enhanced antibacterial activity of streptomycin against some human pathogens using green synthesized silver nanoparticles

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## Abstract

The development of eco-friendly technologies in nanoparticle synthesis is of utmost importance in order to expand their biological horizons. In the present study, bioreduction of  $\text{AgNO}_3$  into AgNPs using various leaf extracts of *Ficus virens* is explained. The resulting AgNPs were characterized by UV–vis spectroscopy, dynamic light scattering (DLS), X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR) and transmission electron microscopy (TEM). Synthesis of AgNPs was confirmed by color change from transparent to brown with maximum absorption at 420 nm due to surface plasmon resonance of AgNPs. X-ray diffraction studies showed that the biosynthesized AgNPs were crystalline in nature, and TEM analysis showed spherical shape of the nanoparticles with size ranging from 4.98 to 29 nm. FTIR study indicates that mainly  $\text{C}=\text{O}$ ,  $\text{-OH}$  and  $\text{N-H}$  groups in leaf extracts are involved in the reduction of  $\text{Ag}^+$  ions to Ag atoms, and proteins are responsible for stabilizing the silver nanoparticles. The synthesized AgNPs showed significant antibacterial activity against Gram positive and gram negative human bacterial pathogens. The results showed that AgNPs also synergistically enhance (2.02–57.98%) the antibacterial activity of streptomycin, a common antibiotic. With this approach, AgNPs can be used as a new generation of antimicrobial agents for successful development of drug delivery.

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**Keywords:** Biosynthesis; Silver nanoparticles; *Ficus virens*; UV–vis; DLS; XRD; FTIR; TEM; Antibacterial; Synergism

## 1. Introduction

Recently, nanotechnology has emerged as a rapidly growing field with numerous applications in science and technology for synthesis of new materials [1]. In the current century, nanotechnology is likely to manipulate science, economy and day-to-day life [2], and potential effects of nanomaterials are being used in *in vivo* and *in vitro* biomedical applications and research [3]. Nanoparticles are usually referred as particles with a size up to 100 nm [4,5]. Metal nanoparticles have promising applications in nanosensors, electronics, nanodevices, absorbents *etc.* [6]. Nanoparticles can be synthesized by physical, chemical and biological methods. Physical and chemical methods sometimes require extreme conditions such as high pressure, energy, temperature and toxic chemicals [7]. Biological methods are cost-effective, eco-friendly and rely on use of plant extracts, enzymes

and microorganisms [8–10]. Among metal nanoparticles, silver has drawn the attention of scientists because of its extensive application in the development of new technologies in the areas of electronics, material sciences and medicine at the nanoscale [11]. Silver nanoparticles have proved to be most effective as it has good antimicrobial efficacy against most pathogenic bacteria. It has been reported that silver nanoparticles (SNPs) are non-toxic to humans and most effective against bacteria, viruses and other eukaryotic microorganisms at low concentrations and without any side effects [12]. Thus, silver nanoparticles play a major role in the field of nanotechnology and nanomedicine [13,14].

Many reports are available on the biogenesis of silver nanoparticles using plant extracts such as *Azadirachta indica* [15], *Gliricidia sepium* Jacq. [16], *Carrica papaya* [17], *Opuntia ficus indica* [18], *Lippia citriodora* [19], *Murrayako enigii* [20], *Citrus sinensis* [21], *Osmium sanctum* [22], *Saururus chinensis* [23], *Eucalyptus hybrida* [24], *Coriandrum sativum* [25], *Memecylon edule* [26], *Prunus armeniaca* [27], and *Magnolia kobus* [28]. *Ficus virens* is a medium-sized Fig tree belonging to family Moraceae. Various plant parts of *Ficus*

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species, such as leaves, stems, bark, roots, flowers and seeds, have long been used as drugs in traditional medicine systems [29]. It is used in diabetes, diarrhea, leucorrhoea, menorrhoea, nervous disorder, vaginal diseases, in the treatment of skin diseases, ulcer and soreness in the mouth *etc.* [30,31]. *F. virens* possess antioxidant properties [32–34] and do not show any toxicity even at higher and repeated doses [35]. So it is a suitable candidate for reduction of silver to silver ion.

The present study aims at biosynthesis and characterization of silver nanoparticles using methanolic, ethanolic and aqueous extracts of *F. virens* leaves. Further, an attempt was made to synergistically enhance the antibacterial activity of streptomycin using silver nanoparticles.

## 2. Materials and methods

### 2.1. Media and chemicals

Silver nitrate ( $\text{AgNO}_3$ ) was purchased from Qualigens. Nutrient agar, LB medium and marine agar medium were purchased from HiMedia laboratories Pvt. Ltd. Mumbai, India. All other reagents used were of analytical grade.

### 2.2. Microorganisms

Pathogenic microorganisms (*Bacillus subtilis* (MTCC-121), *Staphylococcus epidermidis* (MTCC-3382), *Klebsiella pneumoniae* (MTCC-3384), *Vibrio cholerae* (MTCC-3904), *Enterococcus faecalis* (MTCC-6845) and *Vibrio vulnificus* (MTCC-1145) used for studying antibacterial activity of silver nanoparticles were purchased from Microbial Type Culture Collection (MTCC), IMTECH, Chandigarh.

### 2.3. Collection of plant material

Fresh and healthy leaves of *F. virens* were collected from the campus of Chaudhary Devi Lal University, Sirsa, Haryana, during the month of August 2014. The leaves were rinsed thrice with distilled water followed by double distilled water to remove the dust and other contaminants. The leaves were separated, cut into small pieces and dried under shade.

### 2.4. Preparation of the leaf extracts

Dried leaves were grinded to fine powder using a kitchen blender. Five grams of leaf powder was added into each three Erlenmeyer flasks containing 100 ml solvents (methanol, ethanol and water). Mixture was boiled in water bath at 42 °C, and the extracts obtained were filtered through Whatman No. 1 filter paper. The filtrate was collected and stored at 4 °C for further studies.

### 2.5. Synthesis of silver nanoparticles

Two milliliters of respective leaf extract was added to 8 ml of silver nitrate (1 mM) solution in a test tube. The mixture was stirred continuously for 5–10 minutes and incubated in the dark for 4 hrs. The reduction of  $\text{AgNO}_3$  into AgNPs was confirmed visually by the change in color from colorless to various shades of brown (Fig. 1). Biosynthesized silver nanoparticles were separated by centrifugation at 10,000 rpm for 15 minutes at

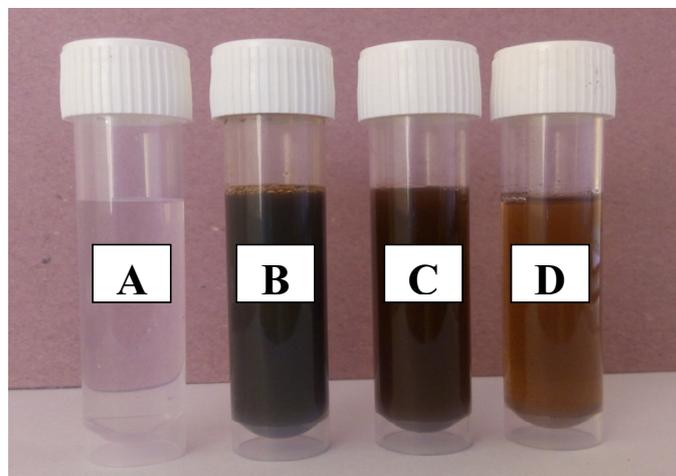


Fig. 1. Silver nitrate ( $\text{AgNO}_3$ ) solution (A) and color change pattern of biosynthesized AgNPs using aqueous (B), ethanol (C) and methanol (D) extracts of *F. virens* leaves.

5 °C. Supernatant was discarded, and the pellet was dispersed in distilled water two to three times in order to remove extra debris. The AgNPs were dried in an oven at 40 °C, and such dried powder was further used for characterization and for antibacterial studies.

### 2.6. Characterization of nanoparticles

#### 2.6.1. UV–vis spectroscopy

UV–Vis spectroscopic analysis was carried out on Elico SL 159 at 600 nm. Path length of cuvette used was 10 mm. A mixture of leaf extract and silver nitrate solution (2 ml) was taken in cuvettes, and wavelength was recorded as a function of reaction time at room temperature for confirmation of the synthesis of silver nanoparticles.

#### 2.6.2. Particle size analysis (DLS)

The particle size and size distribution of the AgNPs synthesized using different leaf extracts of *F. virens* were determined with the help of Malvern Zetasizer Nano ZS90 (Malvern Instrument, UK) based on dynamic light scattering of silver nanoparticles synthesized in different extracts (aqueous, ethanol, methanol).

#### 2.6.3. X-ray diffraction analysis (XRD)

XRD measurements of the silver nanoparticles were recorded on a X'Pert Pro (PANalytical BV, the Netherlands) at a voltage of 40 kV and a current of 30 mA, with  $\text{Cu K}\alpha$  radiation in a  $\theta$ – $2\theta$  configuration.

#### 2.6.4. Fourier transform infra-red (FTIR) spectroscopy

FTIR spectroscopy measurements were taken on FTIR (Shimadzu IR Affinity) spectrophotometer at a range of scanning ( $4000$ – $450$   $\text{cm}^{-1}$ ). Dried and powdered AgNPs were mixed with potassium bromide (KBr) (2:98 ratios by weight) and pressed at 11,000 psi to make the disc. The detector was purged carefully using clean dry nitrogen gas to increase the signal level and reduce moisture. The discs were then introduced in the

spectrophotometer, and spectrum was recorded. The FTIR spectra were analyzed using online spectroscopic analysis.

### 2.6.5. Transmission electron microscopy

For transmission electron microscopy, AgNPs were sonicated for 5 minutes, and a drop of appropriately diluted sample was placed onto carbon coated copper grid and allowed to dry. The TEM images of synthesized silver nanoparticles were obtained for determination of size and shape using JEM-1200EX electron microscope (Hitachi H-7500).

### 2.7. Antibacterial activity

AgNPs synthesized using different leaf extracts (aqueous, ethanol and methanol) of *F. virens* were evaluated for their antibacterial activity by agar well diffusion method against different pathogenic bacteria as previously described [36]. Each bacterial strain was swabbed uniformly onto the individual plates using sterile cotton swabs. Wells of 8 mm diameter were made on nutrient agar plates using gel puncture. The concentration of samples (streptomycin, AgNO<sub>3</sub>, AgNPs and streptomycin + AgNPs) used in wells was kept at 1 mg/ml; 60 µl of streptomycin (positive control), silver nitrate and AgNPs (prepared with different extracts) was inoculated in different wells in the agar plate. A combined formulation of streptomycin (30 µl) and AgNPs (30 µl) was also added in the wells to assess the enhancement of antibacterial activity of streptomycin. The plates were allowed to remain undisturbed for 1 h to ensure even diffusion of samples into agar. The plates were incubated at 37 °C for 18–24 hrs. The zone of inhibition formed around the wells was measured with the help of antibiotic measurement scale and expressed in millimeters. Negative growth zones were measured only after 24 hrs. to avoid misleading results. All experiments were performed in triplicates.

## 3. Results and discussion

### 3.1. Biosynthesis of silver nanoparticles

Synthesis of silver nanoparticles by bio-reduction of silver nitrate in the presence of various leaf extracts of *F. virens* was confirmed from UV–Vis spectroscopy analysis. The maximum absorption peak occurred at 420 nm which is a typical absorption peak of metallic nanoparticles. The formation of various shades of dark brown color of colorless silver nitrate solution clearly indicated the synthesis of AgNPs (Fig. 1). Also, increased peak size over the time indicated the synthesis of AgNPs. The maximum and stable silver nanoparticle synthesis was achieved after 4 hrs. of reaction of leaf extracts with silver nitrate solution. Ethanol extracts showed more rapid synthesis of AgNPs compared to other extracts. The phytochemicals present in the extracts help in reduction of Ag<sup>+</sup> ions into Ag atoms which then join to form AgNPs and also stabilize the nanoparticles by preventing agglomeration [37,38].

### 3.2. Particle size analysis

Average size of silver nanoparticles obtained by DLS were 69.05 nm (aqueous), 23.63 nm (ethanol) and 21.91 nm (methanol) as shown in Fig. 2 (a–c). Sinha et al. [38] reported the

synthesis of silver nanoparticles (34.03 nm) using green alga *Pithophora oedogonia*. Similarly, Premanand et al. [39] showed the formation of silver nanoparticles of size in the range of 30–40 nm using *Nelumbo nucifera* leaf extract. It can be observed that in the present study, methanolic extracts were effective in producing even smaller size AgNPs.

### 3.3. X-ray diffraction (XRD)

To confirm the nature of nanoparticles, X-ray diffraction pattern of the biosynthesized AgNPs was studied using aqueous leaf extract and is shown in Fig. 3. The diffraction pattern showed four diffraction features on 2θ° of X-axis, i.e., 38°, 46°, 64.5° and 77° corresponding to 111, 200, 220 and 311 Bragg reflections of Y-axis respectively, and all the four peaks can be indexed to face centered cubic structure of AgNPs [40]. It is observed that reflections are sharp and high in intensity, indicating that the prepared silver nanoparticles are highly crystalline in nature. Similar results were found by Yugandhar et al. [41] using *Syzygium alternifolium* stem extracts mediated synthesis of silver nanoparticles.

### 3.4. Fourier transform infrared spectroscopy (FTIR)

The functional groups of *F. virens* responsible for the bio-reduction of AgNO<sub>3</sub> to AgNPs can be explained from FTIR analysis. The FTIR spectral analysis of AgNPs synthesized using aqueous extract is shown in Fig. 4(a). Strong absorption peaks at 3363.17 and 3304.14 cm<sup>-1</sup> show N-H stretching vibration of hydrogen-bonded NH group. Whereas peaks at 3045.16 cm<sup>-1</sup> (C-H asymmetrical stretching vibration), 2965.15 cm<sup>-1</sup> and 2923.17 cm<sup>-1</sup> (CH<sub>3</sub> symmetrical stretching vibration), 2821.19 cm<sup>-1</sup> and 2737.18 cm<sup>-1</sup> (C-H stretching vibration) can be assigned to methyl, methylene and methoxy groups [42]. The peak that appeared around 1689.30 cm<sup>-1</sup> and 1596.11 cm<sup>-1</sup> is related to C=C stretching vibration. The appearance of peaks at 1520.10 cm<sup>-1</sup> (N-H deformation vibrations), 1457.20 cm<sup>-1</sup> (CH<sub>3</sub> deformation vibration), 1272.60 cm<sup>-1</sup>, 1352.12 cm<sup>-1</sup>, 1210.18 cm<sup>-1</sup>, 1148.19 cm<sup>-1</sup> and 1279.20 cm<sup>-1</sup> (C-H deformation vibration) was observed. Similarly, the peaks at 1074.30 cm<sup>-1</sup> and 1062.31 cm<sup>-1</sup> (N-S stretching vibration), 904.12 cm<sup>-1</sup> (C-C skeleton vibration), 872 cm<sup>-1</sup> and 858.22 cm<sup>-1</sup> (C-H deformation vibration), 826.21 cm<sup>-1</sup> (C-C skeletal vibration), 768.3 cm<sup>-1</sup> (C-C rocking skeleton vibration), 661.29 cm<sup>-1</sup> (C-H wagging vibration), 587.32 cm<sup>-1</sup> (C-S stretching vibration) were observed and are reported for native proteins [43].

In ethanol extracts (Fig. 4b), peak at 3366.12 cm<sup>-1</sup> shows N-H stretching vibration; 2921.13 cm<sup>-1</sup>, CH<sub>3</sub> symmetrical stretching vibration; 2851.14 cm<sup>-1</sup>, C-H stretching vibration; 2394.15 cm<sup>-1</sup>, O-H stretching vibration; 1607.13 cm<sup>-1</sup>, C=C stretching vibration; 1383.2 cm<sup>-1</sup>, C-H deformation vibration-symmetrical; 1066.12 cm<sup>-1</sup>, C-C stretching vibration; 824.14 cm<sup>-1</sup>, C-C skeletal vibration; and 610.18 cm<sup>-1</sup>, C-H wagging vibration. Similarly, the methanol extracts (Fig. 4c) showed absorption peaks at 3390.25 cm<sup>-1</sup> (N-H stretching vibration), 2919.25 cm<sup>-1</sup> (CH<sub>3</sub> symmetrical stretching vibration), 2850.26 cm<sup>-1</sup> (C-H stretching

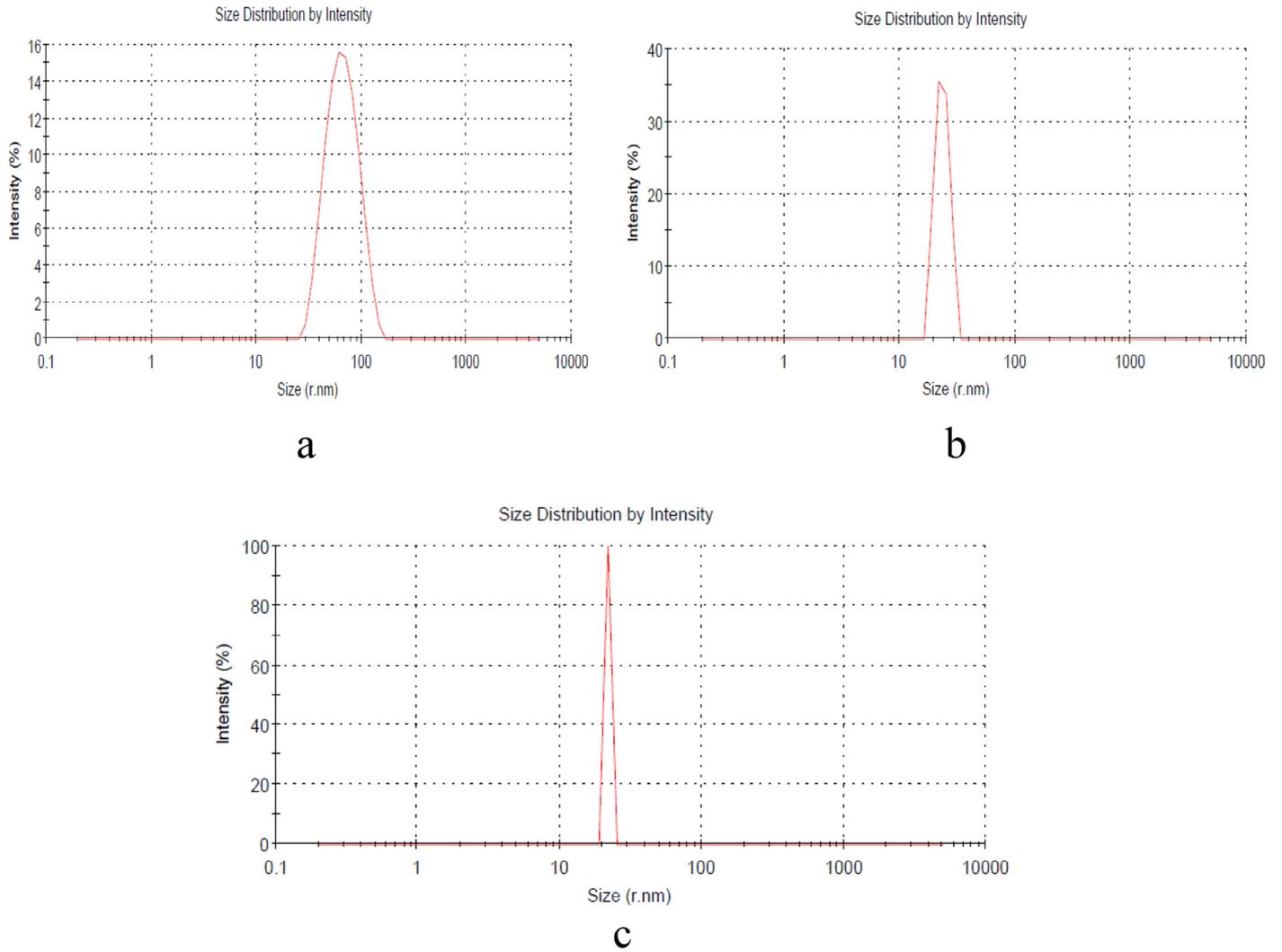


Fig. 2. Particle size analysis (DLS) of AgNPs synthesized using leaf extracts (a) aqueous, (b) ethanol, (c) methanol of *F. virens*.

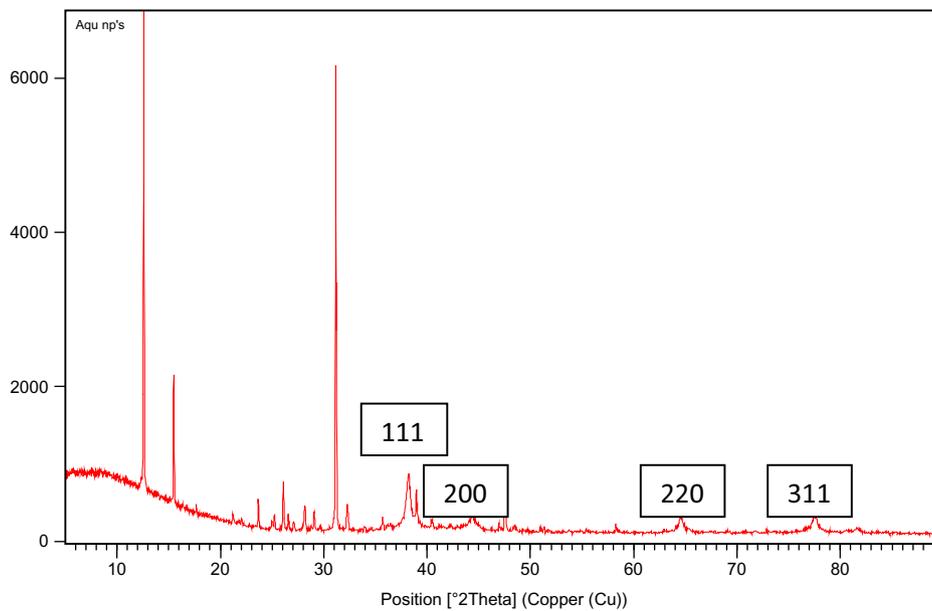


Fig. 3. XRD pattern of silver nanoparticles synthesized using leaf extracts (aqueous) of *F. virens*.

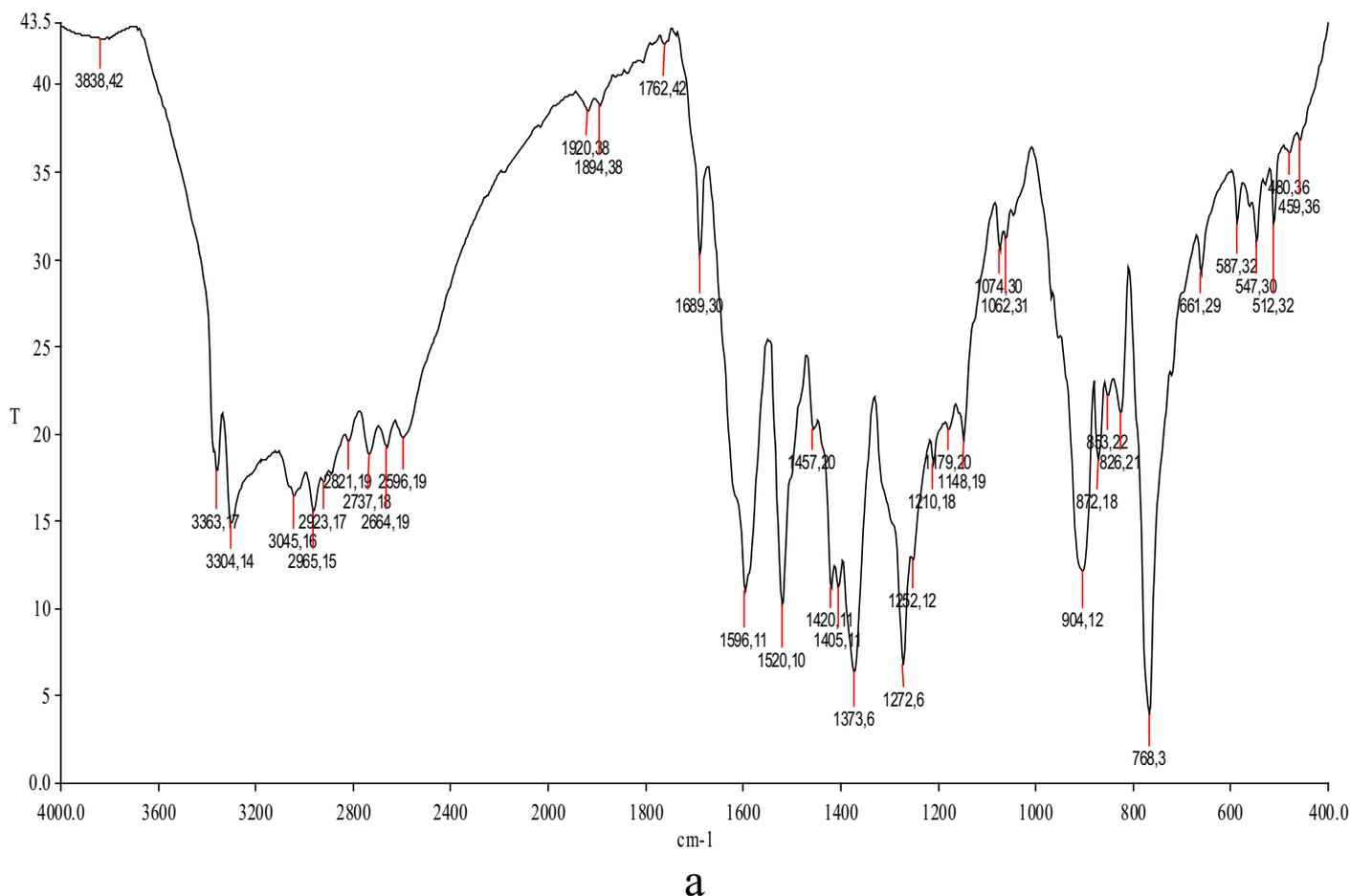


Fig. 4. FTIR spectrum of biosynthesized AgNPs using leaf extracts (a) aqueous, (b) ethanol, (c) methanol of *F. virens*.

vibration), 2426.31 cm<sup>-1</sup> (O-H stretching vibration), 1709.30 cm<sup>-1</sup> (O-H deformation vibration), 1615.27 cm<sup>-1</sup> (C=C stretching vibration), 1384.4 cm<sup>-1</sup> (C-H deformation vibration), 1069.26 cm<sup>-1</sup> (C-C stretching vibration), 824.31 cm<sup>-1</sup>, 718.34 cm<sup>-1</sup> (C-C skeletal vibration) and 606.34 cm<sup>-1</sup> corresponding to C-H wagging vibration. FTIR study indicates that mainly -C=O, -OH and N-H groups in leaf extracts are involved in the reduction of Ag<sup>+</sup> ions to Ag atoms, and proteins are responsible for stabilizing the silver nanoparticles [39].

### 3.5. Transmission electron microscopy (TEM)

The size of synthesized silver nanoparticles was analyzed using TEM. The silver nanoparticles synthesized from aqueous extract were roughly spherical in shape and morphology (Fig. 5a). The size of silver nanoparticles synthesized using aqueous extracts of *F. virens* varied from 16–27.7 nm. In ethanolic extract, TEM images of silver nanoparticles were also roughly spherical in shape with smooth edges, and their dimensions ranged from 13 to 29 nm as shown in Fig. 5(b). The size of silver nanoparticles synthesized using methanolic extract was 4.98–12.5 nm, and nanoparticles were spherical in morphology as shown in Fig. 5(c).

### 3.6. Antibacterial activity

Antibacterial activity of the synthesized AgNPs was investigated against three Gram-positive (*Bacillus subtilis*, *Staphylococcus epidermidis*, *Enterococcus faecalis*) and three gram negative (*Klebsiella pneumoniae*, *Vibrio cholerae* and *Vibrio vulnificus*) bacterial strains growing on nutrient agar medium using agar well diffusion method. Antibacterial activity of AgNO<sub>3</sub> (negative control), AgNPs, streptomycin (positive control) and streptomycin supplemented with AgNPs and their percent increase in antibacterial activity was evaluated. The diameter of each zone of inhibition was measured, and the results are presented in Table 1.

Silver is an important metal, and its salts have been used as an effective antimicrobial agent before the dawn of silver nanoparticles. However, the overuse of silver agents has decreased their efficiency as antimicrobial agent [41]. The results of present investigation revealed a considerable antibacterial activity of synthesized silver nanoparticles for all bacteria. It was observed that Gram negative bacteria were more susceptible to AgNPs as compared to Gram positive bacteria (Table 1). This may be due to the presence of thick layers of peptidoglycans present in the cell membrane of Gram positive

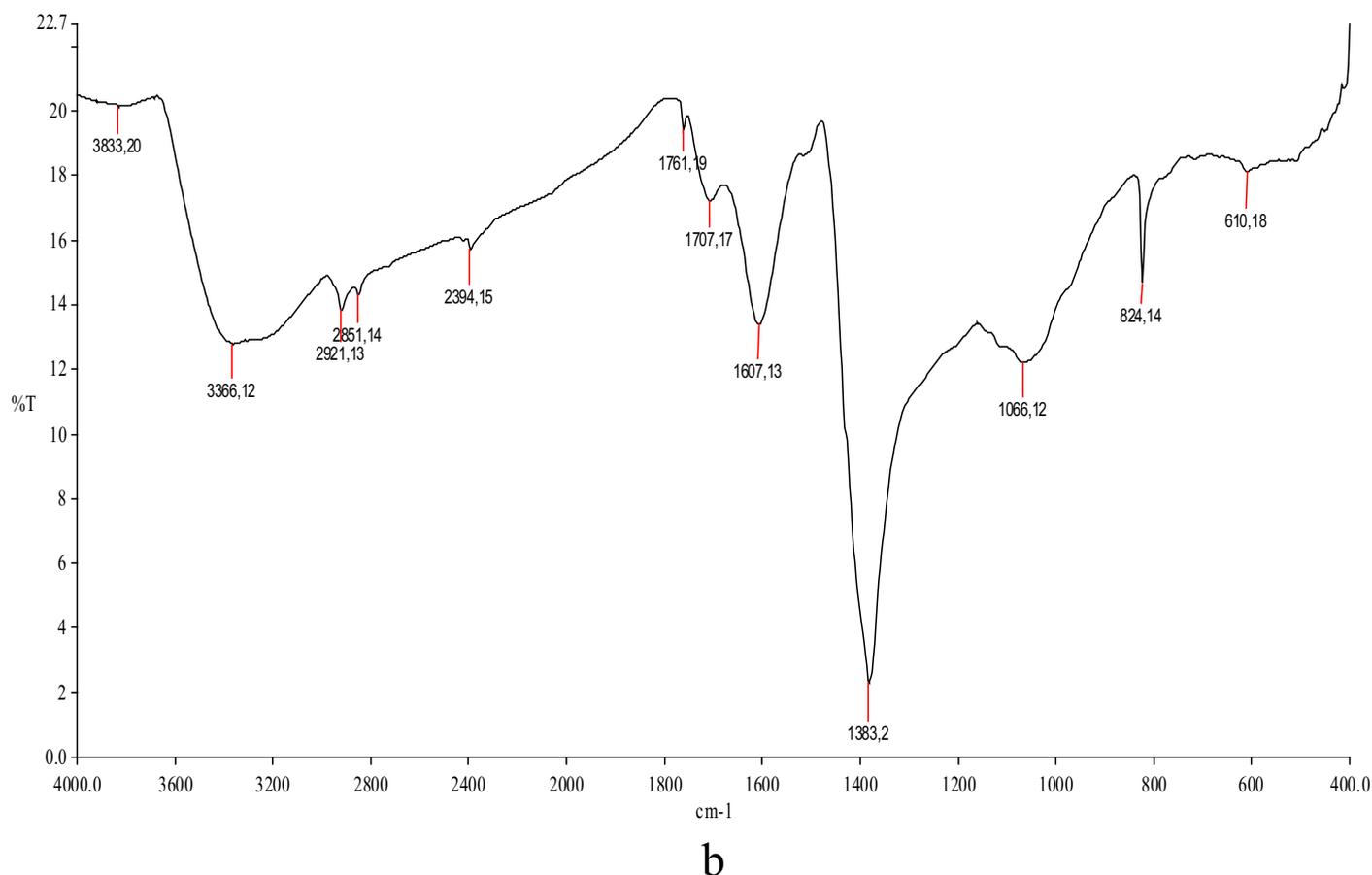


Fig. 4 (continued)

bacteria. When antibacterial activity of AgNO<sub>3</sub> (negative control) and AgNPs were compared against the studied bacteria, an increase in antibacterial activity of AgNPs up to 36.6% over the use of AgNO<sub>3</sub> was observed (Table 1). Similarly, when the

antibacterial activity of streptomycin (positive control) and streptomycin supplemented with AgNPs was compared, an increase in antibacterial activity ranging from 2.02 to 57.98% was observed (Table 1). Clearly, the AgNPs abet the antibacterial

Table 1

Zone of inhibition of synthesized silver nanoparticles using *F. virens* extracts against some human pathogenic bacterial strains.

Bacteria	Extracts	Zone of inhibition (mm)					
		AgNO <sub>3</sub>	AgNPs	% Increase	Streptomycin	Streptomycin + AgNPs	% Increase
<i>Bacillus subtilis</i> (MTCC-121)	Methanol	10.66 ± 0.58	11.66 ± 0.57	9.38	16.67 ± 1.54	18.33 ± 0.57	9.96
	Ethanol		12.33 ± 0.57	15.66		17.66 ± 0.57	5.95
	Aqueous		13.33 ± 0.57	25.05		20.3 ± 1.53	21.78
<i>Staphylococcus epidermidis</i> (MTCC-3382)	Methanol	11.0 ± 00	12.33 ± 0.57	12.09	16.33 ± 0.57	16.66 ± 0.57	2.02
	Ethanol		11.66 ± 0.57	6.00		17.66 ± 0.57	8.14
	Aqueous		13.0 ± 1.00	18.18		19.33 ± 1.54	18.37
<i>Enterococcus faecalis</i> (MTCC-6845)	Methanol	10.0 ± 00	12.66 ± 0.57	26.6	16.66 ± 0.57	18.0 ± 00	8.04
	Ethanol		13.66 ± 0.57	36.6		17.66 ± 0.57	6.00
	Aqueous		13.66 ± 0.57	36.6		18.33 ± 1.52	10.02
<i>Klebsiella pneumoniae</i> (MTCC-3384)	Methanol	10.33 ± 0.57	12.66 ± 0.57	22.56	16.0 ± 00	19.0 ± 00	18.75
	Ethanol		12.66 ± 0.57	22.56		19.66 ± 0.57	22.88
	Aqueous		15.66 ± 0.57	51.60		19.66 ± 0.57	22.88
<i>Vibrio cholerae</i> (MTCC-3904)	Methanol	12.66 ± 0.57	13.33 ± 0.57	5.29	16.33 ± 0.57	17.66 ± 0.57	8.14
	Ethanol		13.66 ± 0.57	7.89		21.33 ± 1.52	30.61
	Aqueous		14.0 ± 00	10.58		21.66 ± 0.57	32.64
<i>Vibrio vulnificus</i> (MTCC-1145)	Methanol	16.33 ± 0.57	18.33 ± 1.54	12.25	12.66 ± 0.57	18.66 ± 0.57	47.39
	Ethanol		16.33 ± 0.57	0.00		19.33 ± 2.51	52.69
	Aqueous		19.0 ± 1.73	16.35		20.0 ± 00	57.98

± Standard deviation (n = 3).

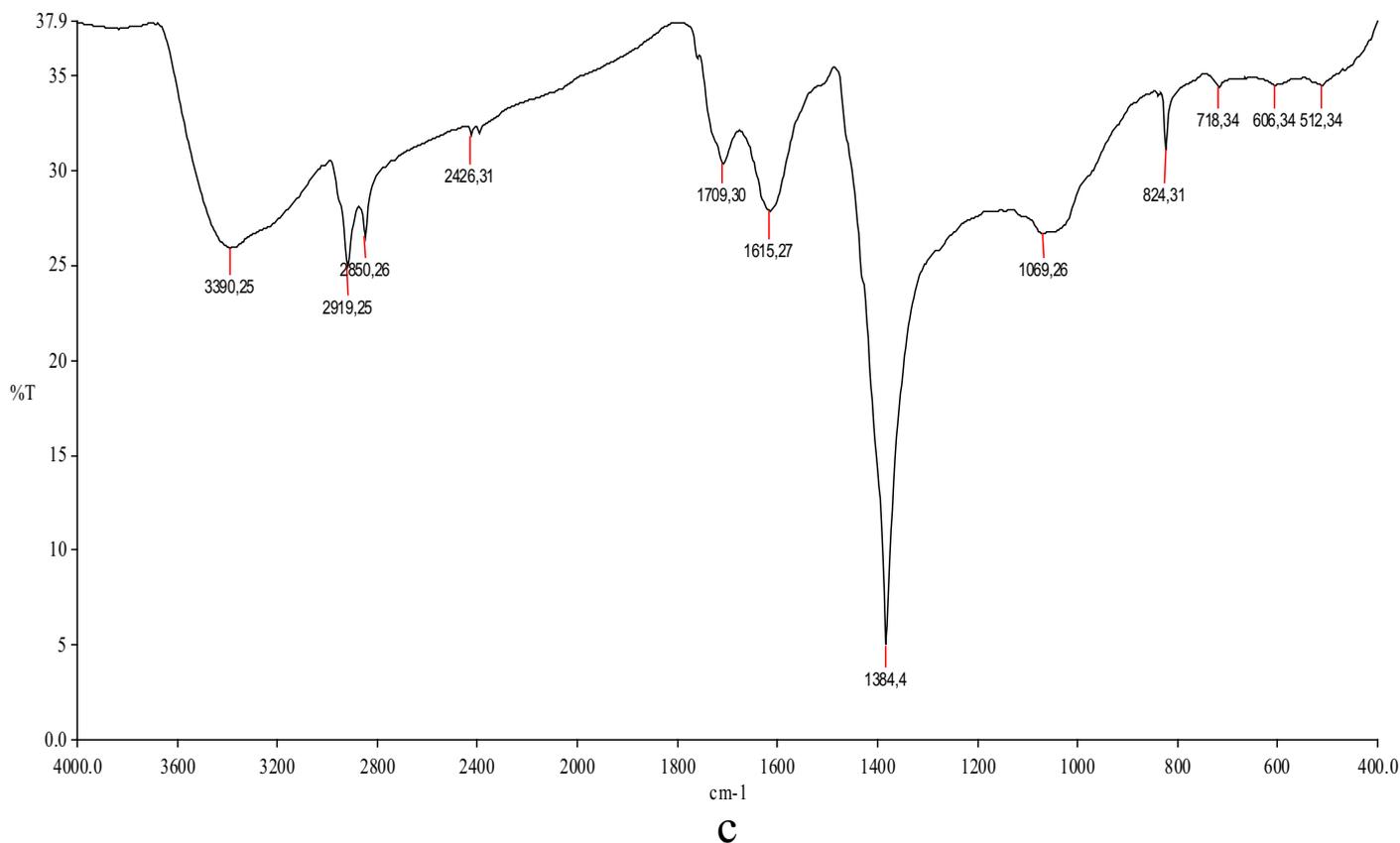


Fig. 4 (continued)

activity of streptomycin. Further, spherical shaped AgNPs (size, 16–27.7 nm) synthesized using aqueous extracts of *F. virens* showed higher antibacterial activity as compared to AgNPs synthesized using methanol or ethanol extracts of *F. virens*. Spherical-shaped SNPs synthesized from stem bark of *Syzygium cumini* [44] and *Cochlospermum religiosum* stem bark [45] have also been reported to demonstrate significant antibacterial activity. Small size, spherical shape and high surface area to volume ratio to interact with bacterial cell walls give them better antimicrobial activity [46]. In the present study, an interesting observation was made with *Vibrio vulnificus*, an extremely virulent bacterium that causes acute gastroenteritis, necrotizing wound infections and invasive septicemia. This bacterium is comparatively less susceptible to streptomycin; however, when streptomycin was supplemented with AgNPs, its antibacterial activity was increased up to 57.98%. This may be caused due to the binding of streptomycin with AgNPs. Firstly, AgNPs attach to the surface of the bacterial cell membrane and then penetrate into the bacteria. After penetration, they cause damage by interacting with phosphorous and sulphur containing compounds such as DNA and proteins resulting in bacterial cell death [47].

#### 4. Conclusion

The present study was aimed to synthesize silver nanoparticles using cost effective and ecofriendly method using various extracts of *F. virens*. The synthesis of AgNPs was

confirmed by UV–Vis spectroscopy at 420 nm. X-ray diffraction pattern of the biosynthesized AgNPs confirmed the crystalline nature of the AgNPs. FTIR study indicates that mainly –C = O, –OH and N–H groups in leaf extracts are involved in the reduction of Ag<sup>+</sup> ions to Ag atoms, and proteins are responsible for stabilizing the silver nanoparticles. These biosynthesized AgNPs showed no agglomeration and have size ranging from 4.98 to 29 nm with roughly spherical shape. This investigation clearly reveals that AgNPs show a broad spectrum of antibacterial activity against human pathogens and further synergistically enhance the antibacterial activity of streptomycin. In conclusion, a novel approach of synergistically enhancing the antimicrobial activity of antibiotics supplemented with AgNPs can be suggested. In future, this approach can also be used for successful development of drug delivery.

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#### Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest.

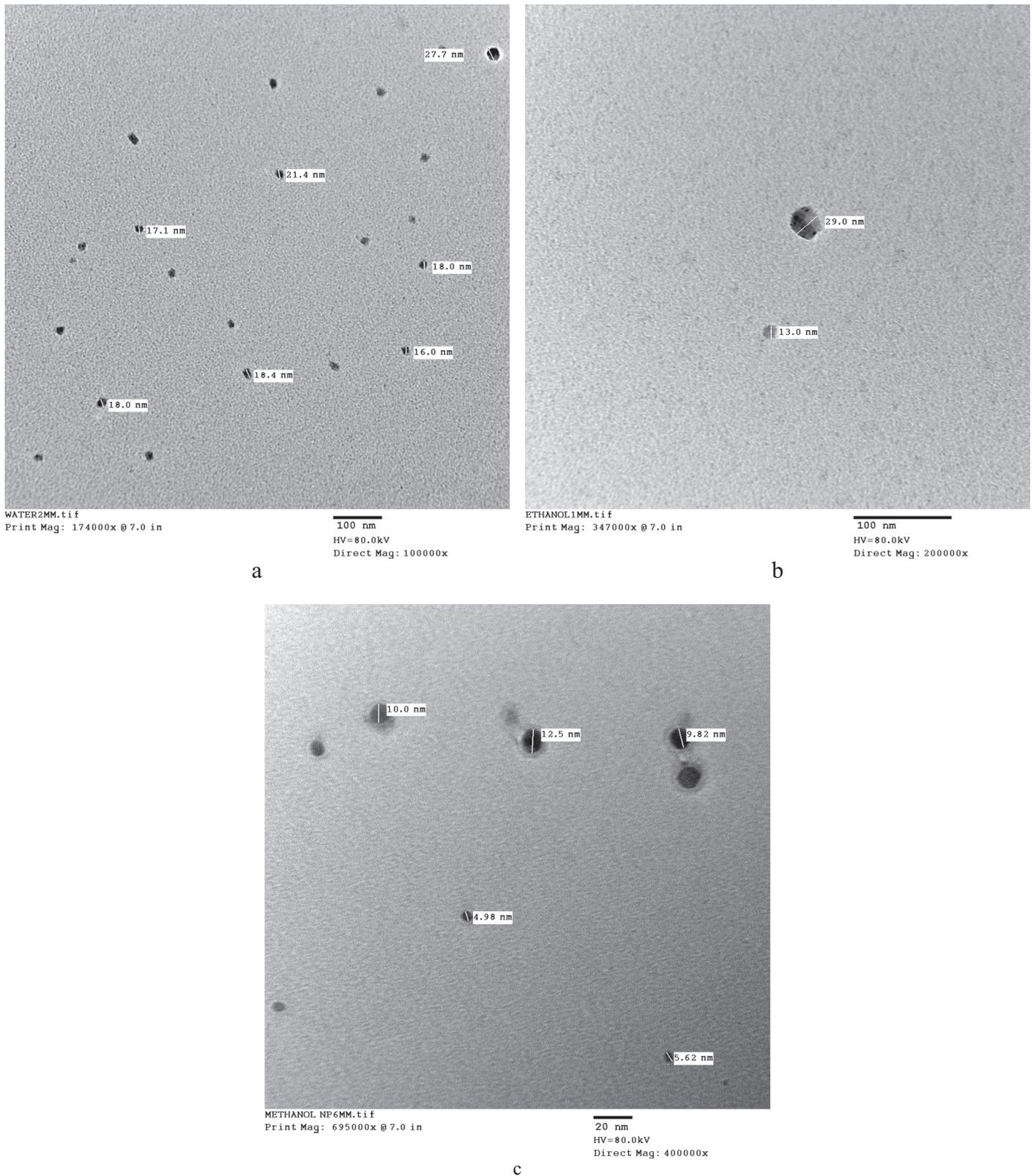


Fig. 5. TEM images of biosynthesized silver nanoparticles using leaf extracts (a) aqueous, (b) ethanol, (c) methanol of *F. virens*.

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