Synthesis of biogenic silver nanoparticles (bAgNPs) using leaf extract of Mirabilis jalapa and evaluation of anti-vibriocidal, anti-oxidant properties and cytotoxicity

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Abstract

Acute hepatopancreatic necrosis disease and luminescent vibriosis are two major bacterial diseases of penaeid shrimp which are caused by gram negative pathogenic bacteria *Vibrio parahaemolyticus* (*Vp*) and *Vibrio harveyi* (*Vh*) respectively. These diseases cause massive mortality and huge economic loss worldwide in shrimp aquaculture. Extensive and inappropriate usage of antibiotics against these pathogens resulted in antibiotic resistant strains. Drug repurposing appears to be an appropriate solution to eliminate the antibiotic resistance in pathogens. In the present study, biogenic silver nanoparticles (bAgNPs) are synthesized by reducing $AgNO_3$ using aqueous extract of *Mirabilis jalapa* (MJ) leaves. The anti-oxidant, cytotoxic and anti-vibriocidal activity of bAgNPs against *Vp* and *Vh* are evaluated. The formation of silver nanoparticles was confirmed by the appearance of dark brown colored solution and with a maximum absorption peak at 434nm. The characterization of bAgNPs using FESEM, TEM, XRD, FTIR, and DLS has confirmed that the nanoparticles are crystalline, spherical in shape with an approximate diameter of 50nm, and have capping agents. The diameter of microbial growth inhibition zones for *Vp* and *Vh* are 26mm and 23mm respectively. Further, the MIC values for *Vp* and Vh are 31.25µg/mL and 93.75µg/mL respectively. The DPPH and FRAP assays showed substantial anti-oxidant activity with IC_{50} values of 67.39µg/mL and 5.509µg/mL respectively. MTT assay to check cytotoxicity effect of bAgNPs on Vero cells resulted very less toxicity at maximum concentration tested with an IC_{50} value of 293.5µg/mL. Therefore, the biogenic silver nanoparticles synthesized from leaves of MJ showed effective anti-vibriocidal and anti-oxidant properties with negligible cytotoxic effect.

Introduction

Shrimp farming is accounted for multibillion-dollar industry worldwide that continues to show significant development (Srinivas and Venkatrayulu 2019). Penaeid shrimp culture is dominated by *Litopenaeus vannamei* (Pacific white leg shrimp), *Penaeus monodon* (tiger shrimp) and *Macrobrachium rosenbergii* (Giant fresh water prawn) etc., species because of their fast growth rate, adaptation to wide salinity ranges and high yield (Santos et al. 2020) (Jayasankar 2018) (Ngasotter et al. 2020). The high profitable value of shrimp has created excellent employment opportunities which plays a significant role in supporting their economic livelihoods of people working in aquaculture sector (Salunke et al 2020). Penaeid shrimps are more susceptible to diseases due to high stocking density, excess feed and inappropriate maintenance of physicochemical parameters in culture ponds (Istiqomah et al.2020) (Karunasagar and Ababouch 2012).

Vibriosis is a major bacterial disease affects penaeid shrimp caused by an opportunistic gram negative *vibrio* bacteria of *vibrionaceae* family (Novriadi, 2016). There are several vibrio species cause shrimp vibriosis among them *Vibrio parahaemolyticus* and *Vibrio harveyi* are the two major pathogens which are highly prevalent among others and causes Acute hepatopancreatic necrosis disease (AHPND) or early mortality syndrome (EMS) and luminescent vibriosis respectively, resulting in massive mortality and billion dollar economic loss in shrimp aquaculture sector (Istiqomah et al.2020) (Novriadi, 2016). The shrimp affected with Vibriosis are identified with symptoms such as stomach lethargy, discolouration of

hepatopancreas and hemocytic infiltration etc. (Letchumanan et al. 2015). *Vibrio parahaemolyticus* exhibit virulence to the shrimp by producing a binary toxins Photorhabdus insect-related A (Pir A) and Pir B which are present on an extra chromosomal plasmid pVA1, whereas the virulence of *Vibrio harveyi* caused by Extra cellular products (ECP's) which are lethal to the shrimp (Letchumanan et al. 2016).

There are several traditional treatment methods are available to control the shrimp *vibriosis*. Antibiotics such as Azithromycin, Oxytetracycline, oxolinic acid, and florfenicol etc. are used to inhibit both *Vibrio parahaemolyticus* and *Vibrio harveyi* but inappropriate and extensive use of antibiotics leads to the development of antibiotic resistance to almost all the antibiotics and also leaving adverse impact on environment (Holmström et al. 2017). There is an appropriate solution to eliminate the antibiotic resistance in pathogens is to replace the antibiotics with biogenic, eco-friendly methods to achieve the sustainable aquaculture practices (Boyd et al. 2020).

One of the eco-friendly technologies to reduce the adverse environmental impact is Nanotechnology. Nanotechnology is the novel science which deals with nanomaterial which has larger surface area to volume ratio and it is one of the powerful emerging science (Ahmad et al., 2019). Metal nanoparticles such as silver, gold, titanium and magnetic nanoparticles etc. have shown anti-microbial, anti-fungal, anti-viral, anti-tumour and anti-parasitic etc. activities. Since 1000 B.C silver has been used as antiseptic agent and also has the antibacterial and antitumor properties (Zhang et al., 2015). Silver nanoparticles (AgNPs) are the nanometer sized silver particles with few to 100nm diameter size are produced by physical, chemical and biological methods (Sivolella et al., 2012). Gamma irradiation, ultrasonic irradiation, evaporation-condensation are physical methods to synthesize AgNPs. In chemical methods sodium borohydride, polyol etc. compounds are used as reducing agents for synthesis of AgNPs. Secondary metabolites of plants, algae, fungi and bacteria are used as reducing agents to synthesize the biogenic or green AgNPs, that possess less toxicity than physically and chemically synthesized AgNPs (Van Hengel et al., 2020) (Samanta et al. 2019). In recent decades biogenic silver nanoparticles have gained great attention of the researchers and opened new insights in science and technology which are reported more efficient anti-bacterial activity than chemical or physically synthesized AgNPs (Ahmad et al. 2019) (Journal et al. 2017).

A thorough study of literature have been carried out to find appropriate methods using biogenic or green AgNPs against shrimp vibriosis, but unexpectedly very few studies were done on the effect of biogenic AgNPs on shrimp vibriosis. There is a need to synthesize biogenic or green AgNPs using different plant species or other biological sources in order to meet the demand of the aquaculture farmers who are depending exclusively for livelihood on the shrimp aquaculture (Geetha et al., 2020).

The present study proposed to synthesize biogenic AgNPs using *Mirabilis jalapa* aqueous leaf extract as reducing agent and assessment of anti-vibriocidal activity on *Vibrio parahaemolyticus* and *Vibrio harveyi*. The Antioxidant activities (DPPH, FRAP) and Vero cell cytotoxicity were also evaluated.

Materials And Methods

Preparation of leaf aqueous extract:

In the present study, plant *Mirabilis jalapa L.* of *Nyctaginaceae*, perennial herb with bushy in nature was selected for the synthesis of biogenic silver nanoparticles and leaves were collected from Rajahmundry, East Godavari, Andhra Pradesh, India. Aqueous leaf extract was prepared using decoction method according to the Singh et al. 2012. Leaves were subjected to shade drying under sunlight for 2–3 days and grounded into fine powder. 5% of leaf powder was dissolved in distilled water, boiled for 60 minutes at 50^{o}C and the resultant solution was filtered using Whatman number 1 filter paper and final aqueous leaf extract was stored in screwed bottle at 4^{o}C temperature until further analysis (Singh et al. 2012).

Biogenic synthesis of Silver nanoparticles (bAgNPs): Biogenic synthesis of silver nanoparticles was done according to the protocol of Chand et al. 2020. Aqueous leaf extract of Mirabilis jalapa was added to the 10mM of Silver Nitrate ($AgNO_3$) solution in 1:9 ratio was prepared with distilled water. The $AgNO_3$ solution and aqueous leaf extract were added and kept in constant magnetic stirrer up to 4 hours. The synthesis of silver nanoparticles was confirmed by changing of colour from yellowish green to dark brown. The solution was incubated for 24 hours in dark chamber and after incubation, the solution was centrifuged at 12000 rpm for 10 minutes and pellet was washed with distilled water for 2–3 times and dried into powder using hot air oven.

Characterization of biogenic silver nanoparticles (bAgNPs):

UV–Vis spectroscopy technique was used to confirm the formation of nanoparticles from the precursor 10mM $AgNO_3$ solution reduced by leaf aqueous extract. The absorbance spectrum of the sample was obtained in the range of 200–800 nm wavelength, using UV–Vis spectrometer Shimadzu-UV 1800 at different time intervals such as 0 hour, 2hours and 4hours.Fourier-transform infrared spectroscopy (FTIR) analysis was performed to analyse and identify the functional groups responsible for the reduction and stabilization of biogenic silver nanoparticles using ALPHA II, a compact FT-IR spectrometer (Bruker) in the wavelength range 4000–400 cm^{-1}. X-ray diffraction (XRD) method is an efficient technique to identify the crystalline phase of silver nanoparticles and it was conducted by XPERT-PRO using monochromatic Cu ka radiation (k = 1.5406 A$^{\circ}$) operated at 40 kV and 30 mA from 10^{o} to 90^{o} in 2θ angles. The obtained data was compared with the International centre for diffraction studies (ICDS) library to account for the crystalline structure. The morphology and shape of the silver nanoparticles were examined using Field Emission (FEI) Quanta 200 FEG MKII scanning electron microscope (SEM). The resolution of FESEM is 1.5 nm and with high output thermal field emission (> 100nA beam current). The FESEM has backscatter (BSE) detector with high sensitivity (18 mm) for atomic number contrast. TEM analysis was also used to determine the morphology, size and shape of the silver nanoparticles. TEM measurements were done by HITACHI H-800, operating at 200 kV. The TEM grid was prepared by placing a drop of the bio-reduced diluted solution on a carbon-coated copper grid and later drying it under a lamp. The hydrodynamic size and zeta potential of the bAgNPs was confirmed by using the Zeta sizer nano ZS90 with 90 degree scattering optics.

Anti-Vibriocidal efficiency of biogenic silver nanoparticles (bAgNPs):

The biogenic AgNP's synthesized from the aqueous leaf extract of *Mirabilis jalapa* was tested for anti-vibriocidal activity on *Vibrio parahaemolyticus* (MTCC No 451) and *Vibrio harveyi* (ATCC No 334). The anti-vibriocidal activities of the bAgNPs were determined by the well diffusion assay and micro broth dilution method using marine nutrient agar (MNA) medium. In this method, 10^5 CFU/mL of pathogenic organisms, *Vibrio parahaemolyticus* and *Vibrio harveyi* were taken from pure cultures and are swabbed on Marine nutrient agar (MNB) plates using sterile cotton swab. An approximately six wells with depth of 2.5mm were made on agar media using sterile gel puncture. The 6 wells were earmarked and WL1 was taken as negative control, and in WL2 20 µl of antibiotic (Azithromycin 20mg/mL) was added. WL2 to WL6 were added with 25 µl, 50 µl, 75 µl and 100 µl of bAgNPs (20mg/mL) respectively and incubated at 37^{o}C for 24 hours. The experiment was carried out in triplicates under aseptic conditions. The mean ± SD values of zone of inhibition were calculated (Ravichandran et al. 2019).

Micro dilution broth was performed to determine the minimum inhibitory concentration (MIC) of bAgNPs according to the Loo et al. 2018. This assay was carried out in 96-well micro titer plate using standard broth dilution method. The bacterial inoculums of two pathogens were adjusted to the concentration of 10^5 CFU / mL. Column-1 is taken as negative control and filled with 100 µl of Maine nutrient broth in micro titer plate. 100 µl of bAgNP's stock solution (20mg/mL) was added to the column 1 & 2, and column-11 and 12 served as negative control. Except negative control, 50µl (10^5 CFU / mL) of each pathogen were added to the all other columns and plates were incubated at 37^{o}C for 24 hours. After incubation the wells were observed for bacterial growth. Formation of turbidity indicates the growth of bacteria. The experiment was run in triplicates and The mean ± SD values of MIC was calculated (Loo et al. 2018).

Evaluation of Anti-oxidant activity:

2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

Free radical scavenging activity of biogenic silver nanoparticles was determined using DPPH assay. Biogenic AgNPs prepared at different concentrations – 20, 40, 60, 80 and 100 µg/mL were agitated thoroughly and added to 2mL of 3 x 10^{-5} M DPPH in methanol was added to all test tubes. The solutions were incubated at 37^{o}C in dark chamber for about 2 hours and the absorbance was recorded at 517nm wavelength using UV-Vis spectroscopy. The DPPH scavenging activity of each concentration was calculated by given below formula (Sumbal et al. 2017). Ascorbic acid was used as standard.

DPPH scavenging effect % Inhibition = A_0 -A_1 /A_0 × 100

Where A_0 = the absorbance of control.

A_1 = the absorbance of standard

Ferric reduction anti-oxidant power (FRAP) assay

FRAP assay was done according to the *Sumbal et al. 2019*. This method is depend upon the ability of bAgNPs to reduce Fe^{+3} to Fe^{+2} in the presence of 2, 4, 6-tripyridyl-s-triazine (TPTZ) and formation of intense blue complex (Fe^{+2} – TPTZ). Biogenic AgNPs prepared at different concentrations – 20, 40, 60, 80 and 100 µg/mL were agitated thoroughly and added to 2mL of FRAP reagent. The solutions were incubated at 37^{o}C in dark chamber for about 2 hours and the absorbance was recorded at 593nm wavelength using UV-Vis spectroscopy. The ferric reduction capacity of each concentration was calculated by given below formula. Ascorbic acid was used as standard.

% Inhibition of FRAP = A_0 -A_1 /A_0 × 100

Where A_0 = the absorbance of control.

A_1 = the absorbance of standard

Cytotoxicity assay:

The cytotoxicity of bAgNPs on Vero cell lines was carried out at Apex Biotechnology Training and Research Institute, Chennai, using 3- (4, 5-dimethyl thiazol-2yl)-2, 5- diphenyl tetrazolium bromide (MTT) assay. MTT is cleaved by mitochondrial Succinate dehydrogenase and reductase of viable cells, yielding a measurable purple product formazan, which is directly proportional to the viable cell number and inversely proportional to the degree of cytotoxicity. Dulbecco's Modified Eagle Medium (DMEM) medium was discarded from Vero cell sub culture and trypsinized separately. To this disaggregated cells in the flask 25mL of DMEM with 10% FCS (fetal calf serum) was added. The cells were homogenized in the suspended medium. One mL of homogenized suspension was added to each well in a 24 wells culture plate along with the addition of 0, 50, 100, 150, 200 µg/mL concentration bAgNPs in each column respectively and were incubated at 37°C in a humidified CO_2 incubator and allowed to reach 80% confluence.

After incubation, the treated cells were washed in phosphate buffered saline of pH 7.4 and cultured plate was loaded with 20 µl of MTT reagent and incubated at room temperature for about 3 hours. After incubation, the content was removed from the wells and 100µl Sodium dodecyl sulphate (SDS) in Dimethyl sulfoxide (DMSO) was added to the all wells to dissolve formazan crystals and absorbance was read at 540nm with Lark LIPR-96 micro titre ELISA reader. The percentage of cell viability and IC_{50} values were calculated using absorbance values (Sumbal et al. 1993).

Results And Discussion

Biogenic synthesis and characterization of bAgNPs:

The synthesis of bAgNPs was confirmed by observing the change in the color of solution from yellowish green to dark brown color. This color transition is due to the excitation of Surface Plasmon Vibrations of silver nanoparticles and was primarily confirmed by UV-Vis spectroscopy at different time intervals (0, 2 and 4 hours) and the absorbance peak was observed at 434 and 451 wavelengths, after 2 hours and 4 hours of synthesis respectively, which indicating the formation of biogenic silver nanoparticles (Fig. 1). Green AgNPs synthesized from *Prosopis farcta* fruit extract showed absorbance peak around 475nm wavelength (Salari et al., 2019). Another study showed that green AgNPs synthesized using *Brassica oleracea* leaf extract gave absorbance peak at 415nm wavelength (Ansar et al., 2020). The smaller the wavelength peak, the lesser the size of silver nanoparticles and more effective in its biological activity (Salari et al. 2019).

The XRD is a primary technique to determine the crystalline nature of silver nanoparticles (Rafique et al., 2017) by predicting spectrum of 2θ value ranges from 10^o to 90^o. The XRD pattern of bAgNPs in this study revealed intense peaks at 111, 200, 220 and 311 which strongly reflected the Bragg's reflection with face centred cubic (fcc) and confirming the crystalline nature of bAgNPs sample using ICDD (Crystallographic sheet 04-0783) shown in Fig. 2. The peaks which are unassigned in XRD pattern are due to the crystallization of bioorganic phase that occurs on the surface of silver nanoparticles. The intense peaks 111, 220 corresponds to the ultra-small nanoparticles and face centre cubic (fcc) (Mourdikoudis et al., 2018). XRD of Biogenic silver nanoparticles synthesized using sheep's blood (*Ovis aries*) showed intense peaks 111, 200, 220 and 311 which is similar to the present study (Kakakhel et al., 2021). Silver nanoparticles synthesized from *Prosopis chilensis* leaf extract XRD analysis showed four intense peaks 111, 200, 220 and 311 which strongly support Bragg's reflection and fcc (Kandasamy and Alikunhi 2013).

The phytoconstituents present in the plant extract have dual role which act as both reducing and capping agents. The presence of functional groups in the synthesized biogenic silver nanoparticles was confirmed through FTIR analysis in the spectral range of 400–4000 cm^{-1} and depicted in Fig. 3. The observed peak broadening and noise were probably macromolecules present in the plant extract which may be responsible for the reduction of silver nanoparticles. The FTIR band at 1008.50 cm^{-1} corresponds to Flouro halogen group (C-F stretching) which may be attached to the reducing or stabilizing agent, band at 1416.50 cm^{-1} corresponds to C-H bending (Alkane), band at 1636.95 cm^{-1} corresponds to C = O (ketones) and band at 3298.60 cm^{-1} corresponds to O-H stretching (alcohol). Gopalakrishnan et al., 2019 synthesized green silver nanoparticles using aqueous leaf extract of *Mirabilis jalapa* shown two peaks 3323 cm^{-1} and 1620 cm^{-1} corresponds to OH stretching and C = O ketones. *Sumbal et al., 2019* found OH stretching (3283.69 cm^{-1}), C-N group or alkyne (2122.60 cm^{-1}), C = C or NH group (1643.20 cm^{-1}) and C-O (1318.89 $cm^{-}1$) corresponds to the alcohols, amides and amine functional groups in the aqueous leaf extract of *Mirabilis jalapa.*

Selected area electron diffraction (SAED) of bAgNPs was determined by TEM analysis and it was found that diffraction rings are corresponding to the crystalline planes (Fig. 4a). The TEM images of the biogenic silver nanoparticles revealed variable shapes and most of are with spherical shapes with particle

size of 50nm and above (Fig. 4b) which was further confirmed by the Zeta size analysis. The TEM analysis of bAgNPs synthesized from *Prosopis farcta* fruit extract showed 60nm sized bAgNPs with corresponds to the UV-Vis absorbance wavelength around 475 nm, higher than the UV absorbance and size of bAgNPs of the present study (Salari et al., 2019). bAgNPs synthesized from leaf extract of *Erythrina suberosa* TEM analysis showed the size of nanoparticle between 15-34nm with corresponds to the UV-Vis absorbance peak at ~ 428nm (Mohanta et al., 2017). This clearly shows that, smaller the absorbance of UV-Vis spectroscopy then lesser the size of the nanoparticle. In the present study, TEM analysis of bAgNPs showed the particle size was around 50nm and a UV-Vis absorbance peak at 434nm (Fig. 4).

FESEM analysis was performed to determine the morphology of the bAgNPs. Figure 5 shows the SEM micrograph formed by the secondary electrons (SE) of silver nanoparticles. The FESEM analysis showed well dispersed bAgNPs at 0.5µm and most nanoparticles are with aggregated and spherical in shape. Green AgNPs synthesized from methanolic extracts of *Ipomoea carnea* shown spherical shaped and uniformly dispersed nanoparticles in FE SEM analysis (Singh, 2021) and gAgNPs synthesized from *Pseudoduganella eburnean* shown rod shaped and uniformly dispersed nanoparticles (Huq, 2020). In contrast FE SEM analysis of the present study reported the bAgNPs with Spherical and non-homogenous shapes and aggregated in form (Fig. 5).

The particle size and particle dispersion Index (PdI) was analysed using dynamic light scattering (DLS) which showed the size of biogenic synthesized silver nanoparticles to be > 50nm (Fig. 6a) and particle dispersion Index (PdI) was 0.202. The Z average (d.nm) is found to be 269. The zeta potential value of biogenic synthesized silver nanoparticles was found to be -24.9mV which indicating the higher degree of stability (Fig. 6b). The negative ZP value of bAgNPs may be due to the presence of capping agents. By monitoring the dynamic fluctuation from the light scattering intensity and velocity movement of the particles in suspension, the zeta size determines the average size of nanoparticles in aqueous suspension (Mat Yusuf et al., 2020). In this study, the computed PDI value of the bAgNPs was found to be in the range of 0–1, indicating that the bAgNPs were in a monodisperse phase with minimal particle aggregation (Mat Yusuf et al., 2020).

Anti-vibriocidal activity:

Over the years, antibiotic resistant strains of *Vibrio* species have emerged in the environment due to extensive use of chemotherapeutic agents in aquaculture. This resulted in the evolution of new pathogenic strains and more disease outbreaks which are difficult to treat with the chemotherapeutic agents (Letchumanan et al. 2015). *Vibrio parahaemolyticus* and *Vibrio harveyi* are more prevalent pathogens among the *Vibrio* species which cause *vibriosis* to shrimp (Campus 2018) (Mastan and Begum 2016). In recent decades, silver nanoparticles gained a lot of attention in different research and commercial sectors due to its targeted specific action and anti-microbial activity. Application of AgNPs in commercial aquaculture sectors have been increased to combat the various disease causing pathogens (Morones et al.2005) (Shankar et al.2004).

In this study well diffusion assay was performed to determine the anti-vibriocidal activity of biogenic silver nanoparticles (bAgNPs) and exhibited the maximum inhibition zone of 26.2 ± 0.54mm and 28.375 ± 0.47 mm wide respectively. The mean ± SD values of zone of inhibitions of *Vibrio parahaemolyticus* and *Vibrio harveyi* are depicted in Figs. 6 & 7 respectively. In contrast the control (antibiotic) used in this study showed inhibition zone of 23.1 ± 0.49 mm and 13.125 ± 0.29 mm wide of the both species respectively which are less than zone of inhibition of bAgNPs.

Micro dilution broth (MBS) was done to determine the Minimum Inhibitory Concentration (MIC) of green silver nanoparticles on *Vibrio parahaemolyticus* and *Vibrio harveyi.* The MIC values of bAgNPs against the growth of *Vibrio parahaemolyticus* and *Vibrio harveyi* are 31.25 ± 0 and 93.75 ± 44.19 µg/ mL respectively (Fig. 8)..

In contrast to the present study, colloidal silver nanoparticles synthesized by photo-assisted reduction method obtained two different sized nanoparticles 16.62nm and 22.22nm that showed 42.1mm and 43.12mm of zone of inhibition against *Vibrio harveyi* (Nafisi et al. 2017). Green silver nanoparticles synthesized by extracellular products of *Bacillus subtilis* produced size range of 10–25 nm showed zone of inhibition 21.25nm and 19.27nm on *Vibrio parahaemolyticus* and *Vibrio harveyi* respectively (Sivaramasamy and Zhiwei 2016). Green silver nanoparticles synthesized from leaf extract of *Prosopis chilensis* obtained size ranges from 5 to 25 nm with zone of inhibition 16nm and 19nm against *Vibrio harveyi* and *Vibrio parahaemolyticus* respectively (Kandasamy and Alikunhi 2013). 10mg of silver nanoparticles synthesized from tea leaf extract (*Camellia sinensis*) inhibited 70% of *Vibrio harveyi* growth in culture media (Vaseeharan et al. 2010). Biogenic silver nanoparticles synthesized from *Cheatomorpha antennia* shown anti-vibriocidal activity against *Vibrio harveyi* and exhibited 17.8mm zone of inhibition (Thanigaivel et al., 2021).

Anti-oxidant activity

Antioxidants are natural or synthetic compounds which delay remove or prevent the damage of a cell caused by free radicals or species which caused oxidative stress in cells. The antioxidant substance must be in low concentration and ability to scavenge free radicals or reactive oxygen/nitrogen species. In recent years, several spectrophotometric methods has been developed to determine the antioxidant capacity of both natural and synthetic substances (Bedlovicová et al., 2020). In this study DPPH and FRAP were used to evaluate the antioxidant activity of biogenic silver nanoparticles synthesized from aqueous leaf extract of *Mirabilis jalapa*.

DPPH assay:DPPH free radical scavenging activity was done for the assessment of antioxidant potential of green silver nanoparticles. The purple solution DPPH containing solution turned yellow after the addition of bAgNPs, which indicates the free radical scavenging activity. Figure-9 shows maximum inhibition at 500 µl/mL with 60.77% and the Inhibitory concentration 50 (IC_{50}) value was determined using the formula IC_{50} (0.5-b)/a, which was 67.3987 ± 202 µl/mL, indicating the concentration of bAgNPs required to inhibit the 50% of DPPH concentration.

FRAP assay: In FRAP assay, the maximum inhibitory percentage of bAgNPs required to reduce $Fe3^+$ to $Fe2^+$ ranges from 3.82 ± 0.65 to 22.92 ± 0.54%. The IC_{50} value of bAgNPs was 5.509 ± 152 µl/mL, indicates at this concentration 50% of $Fe3^+$ is reduced to $Fe2^+$ and is depicted in Fig. 10.

The significant anti-oxidant ability of bAgNPs was carried out by DPPH and FRAP assays showed IC_{50} value 67.39 µg/mL and 5.50 µg/mL respectively. Green silver nanoparticles synthesized from leaf extract of *Erythrina suberosa* shown significant free radical scavenging activity with IC_{50} value of 30.04 µg/mL (Mohanta et al., 2017). Green synthesized silver nanoparticles from *Prosopis farcta* shown significant DPPH free radical scavenging activity with IC_{50} value of 70 µg/mL which is higher than the present study (Salari et al., 2019). Green silver nanoparticles synthesized from *Cestrum nocturnum* shown 29.55% of anti-oxidant activity at 100 µg/mL, (Keshari et al. 2020), which is higher than our present study. Green silver nanoparticles synthesized from *Caesalpinia sappan* aqueous extract shown Ferric oxide anti-oxidant activity with 78.7 µg/mL (Suwan et al., 2018) and gAgNPs synthesized from *Nothapodytes nimmoniana* fruit extract studied for FRAP assay shown 214.89 µg/mL, (Mahendran and Ranjitha Kumari, 2016) and the concentration is higher than the present study.

Vero cell cytotoxicity:

The major consequence of nanoparticles is safety aspects. With the increasing applications of nanoparticles, there are possible adverse effects on environment and organisms. It is necessary to extensively evaluate the nanoparticles toxicity in both *in vitro* and *in vivo* (Saranya et al., 2017). In this study, the toxicity of biogenic silver nanoparticles was evaluated on Vero cell lines (African green monkey kidney cell lines) which are non-cancerous cell lines.

The *in-vitro* cytotoxicity activity results of the bAgNPs synthesized from leaf extract of *Mirabilis jalapa* against Vero cells showed minimal inhibition towards tested cell line. However, the increased concentration of bAgNPs, the cell viability decreased. The maximum cytotoxicity 69.49% of bAgNPs observed when 200 µg/ mL of bAgNPs were used against Vero cell lines. It was proven that the moderate cytotoxicity effect of the test sample showed no cell disintegration after 48 h of treatment against the selected tested cell lines even at higher concentrations (Figs. 11 & 12). The IC_{50} concentration of the tested sample against Vero cells was 293.5 µg/ mL.

MTT assay was done in the present study to evaluate the cytotoxic effect of bAgNPs on Vero cell lines, showed 73.87% of cell viability at maximum concentration of 200µg/mL and the IC_{50} value of bAgNPs on Vero cell lines is 293.5 µg/mL. Biogenic AgNPs synthesized from *Cassia fistula* shown Vero cell line cell death was 89.7% at 1000 µg/mL and IC_{50} value is 66.34 µg/mL, which is less than our current study (Remya et al., 2015). Colloidal silver nanoparticles synthesized using silver nitrate and citric acid showed mortality of 68.54% at 1000 µg/mL on Vero cell lines and IC_{50} value 10.68 µg/mL, which indicating the less concentration of colloidal silver nanoparticles cause 50% of cell death. Green AgNPs synthesized from leaf extract of copperrod plant (*Peltophoroum pterocarpum*) and studied cytotoxicity on Vero cell lines showed IC_{50} value of 90 µg/mL (Pannerselvam et al., 2021). Green silver nanoparticles synthesized

from *Alysicarpus monilifer* showed very limited toxicity of 25 µg/mL on Vero cell lines after 72 hours of incubation (Kasithevar et al., 2017).

Conclusion

The present work describes the synthesis of biogenic silver nanoparticles using aqueous leaf extract of *Mirabilis jalapa*. The leaf extract have bioactive compounds such as flavonoids, phenols, terpenoids, tannins, and alkaloids etc. which act as reducing, stabilizing and capping agents for the synthesis of biogenic silver nanoparticles. The formation bAgNPs was confirmed by UV-Vis spectroscopy, XRD, FTIR, TEM and FE SEM, Zeta size and Zeta potential showed spherical, cone shaped with around 50nm and above sized nanoparticles. The bAgNPs synthesized in this study have shown significant anti-vibriocidal activity on *Vibrio parahaemolyticus* and *Vibrio harveyi* which are more prevalent pathogens causes *Vibriosis* to the shrimp. The zone of inhibition for both pathogens 26nm and 28nm are respectively at 100µg/mL. Minimum Inhibitory Concentration (MIC) was determined as 31.25 and 187.5 µg/mL for both Vibrio species respectively and it was observed that, more concentration of bAgNPs are required to inhibit the *Vibrio harveyi* than *Vibrio parahaemolyticus*. Since, bAgNPs has free radical scavenging and ferric oxide reducing anti-oxidant activities. This is due to functional groups present on bAgNPs surface as capping agents. The cytotoxicity of bAgNPs on Vero cell lines has shown lesser toxicity at maximum concentration of 200µg/mL. These biogenic silver nanoparticles might be used as anti-microbial agents in future due to eco-friendly, less toxicity and highly effective against the various pathogenic organisms.

Abbreviations

AHPND – Acute Hepatopancreatic Necrosis Disease

EMS – Early Mortality Syndrome

Pir – Photorhabdus insect related

bAgNPs – Biogenic silver nanoparticles

FTIR – Fourier Transform Infrared Spectroscopy

XRD – X-ray diffraction

SEM – Scanning Electron Microscope

TEM – Transmission Electron Microscope

DLS – Dynamic Light Scattering

Declarations

Ethics approval and consent to participate

Not Applicable

Consent for Publication

Not Applicable

Competing interests

The authors declared no competing interest

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Authors Contributions

PVN were involved in supervision and conceptualization of project. AM and AMR were responsible for methodology, investigation, resources and writing the original draft manuscript. SRM were involved in data curation, editing and reviewing of the manuscript.

Additional information

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Figures

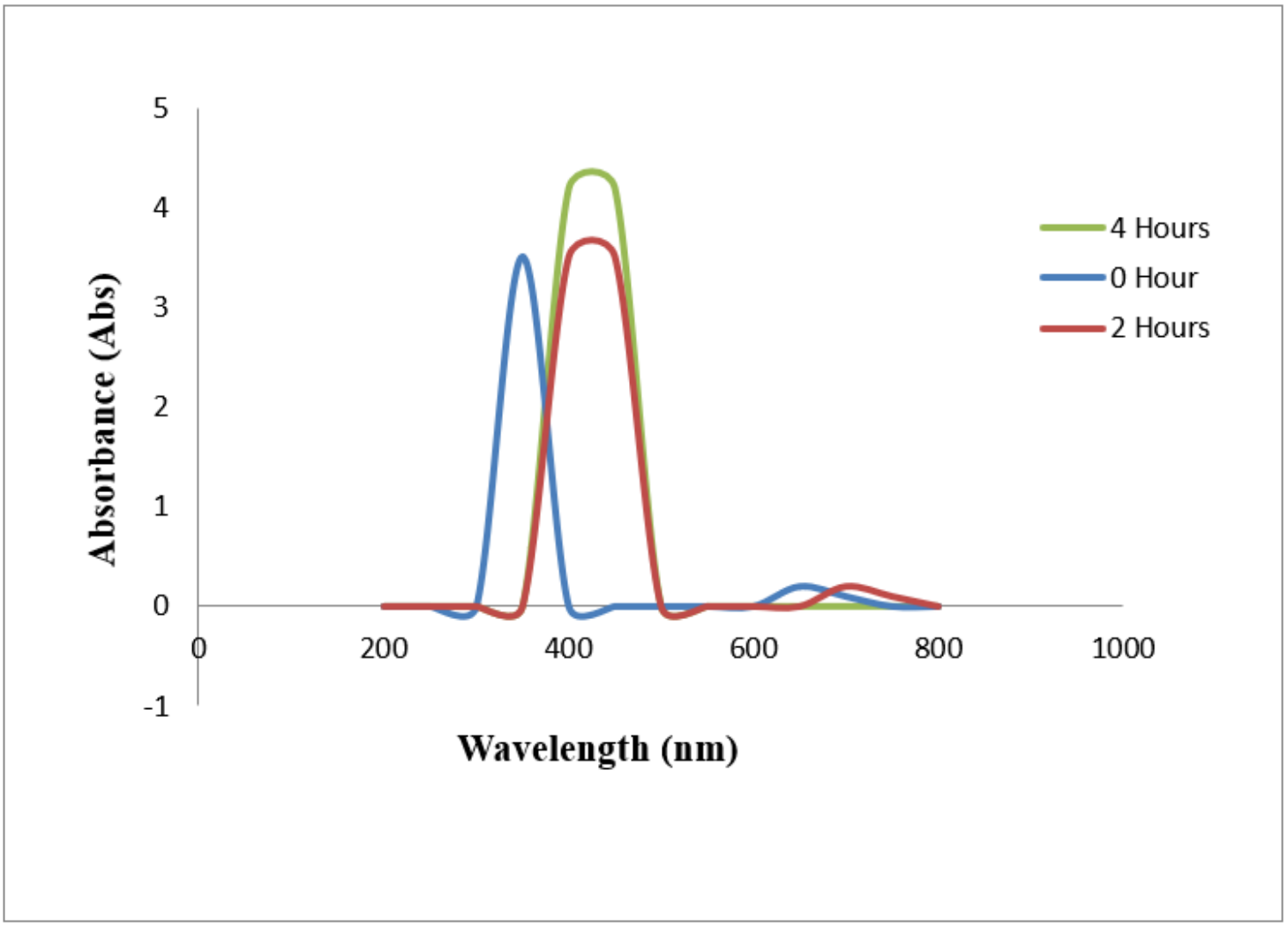


Figure 1

UV Vis spectra of biogenic synthesized silver nanoparticles from *Mirabilis jalapa* aqueous leaf extract at different time intervals.

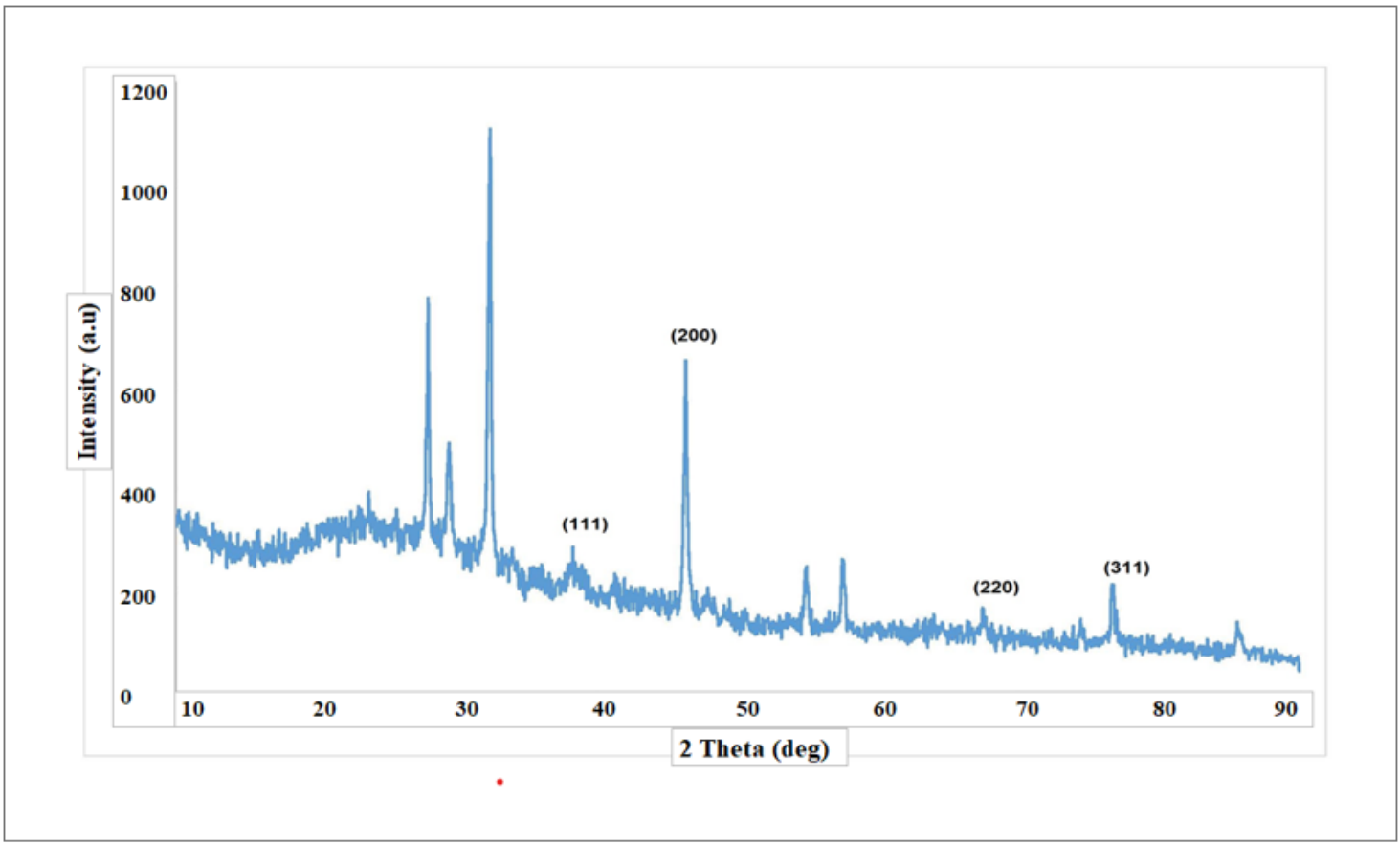


Figure 2

XRD pattern of biogenic silver nanoparticles synthesized from aqueous leaf extract of *Mirabilis jalapa* with intense peaks of 111, 200, 220 and 311 revealed the crystalline nature of bAgNPs

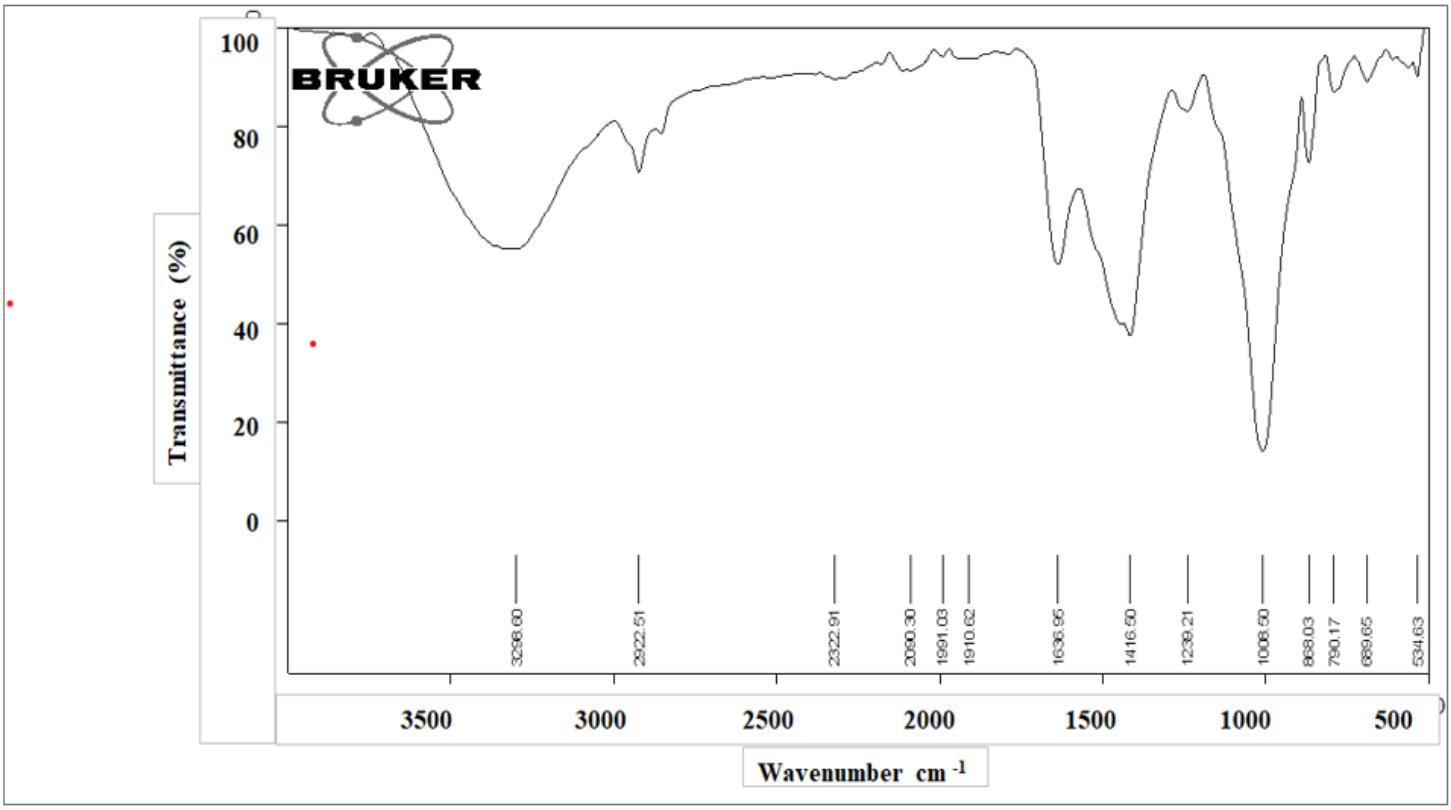


Figure 3

FTIR spectra of bAgNPs synthesized from leaf aqueous extract of *Mirabilis jalapa*.

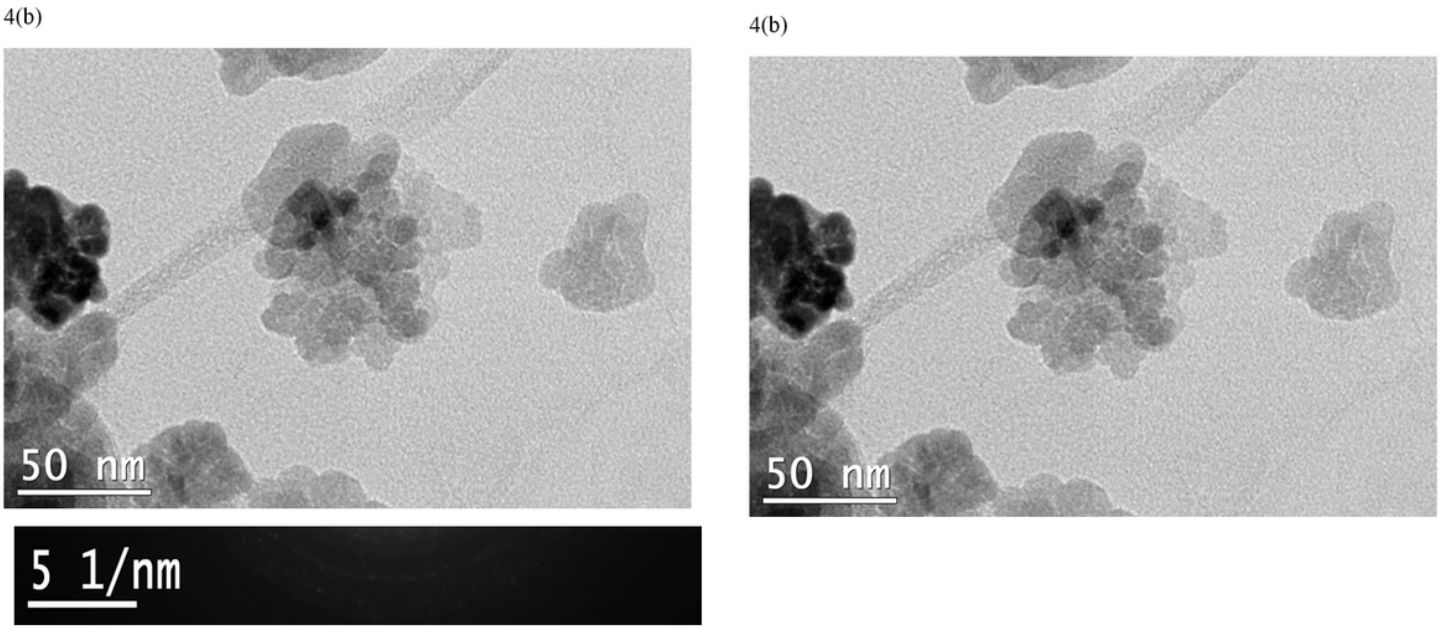


Figure 4

(a) SAED pattern of bAgNPs (b) Micrograph of biogenic silver nanoparticles showing the size and shape with TEM analysis

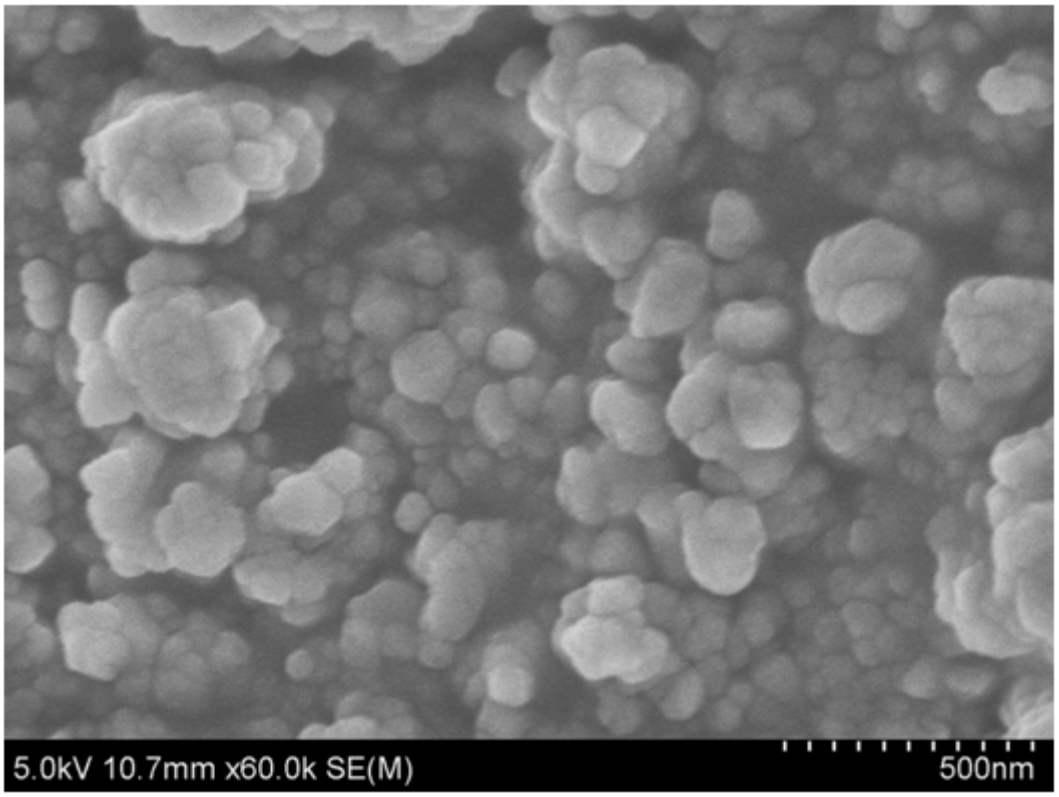
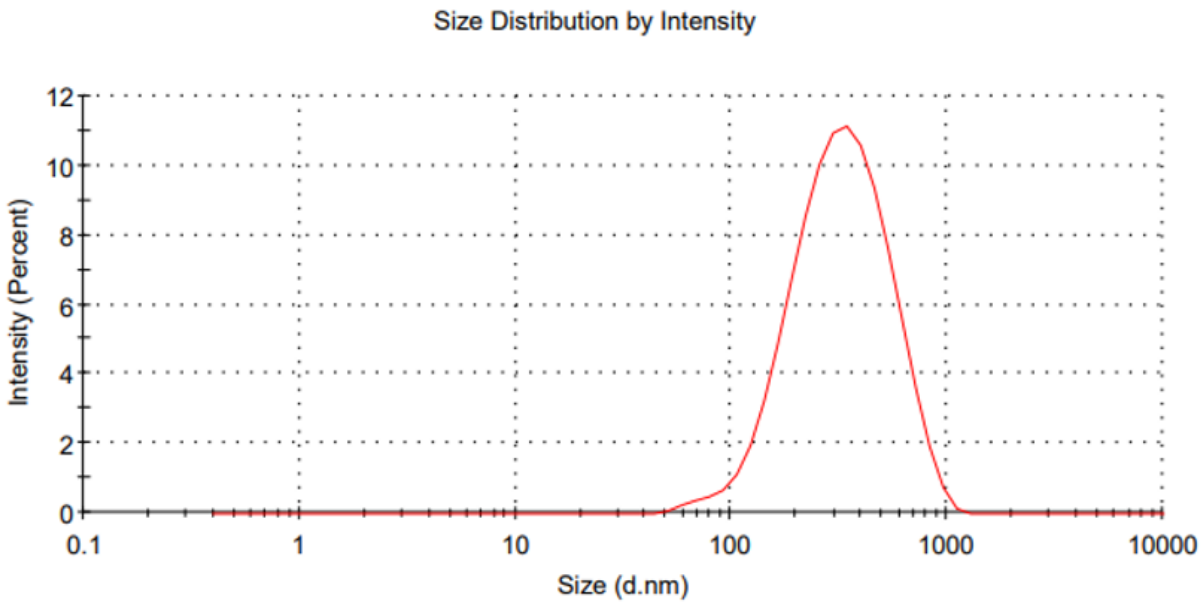


Figure 5

Scanning electron micrograph of biogenic silver nanoparticles

6(a)



(b)

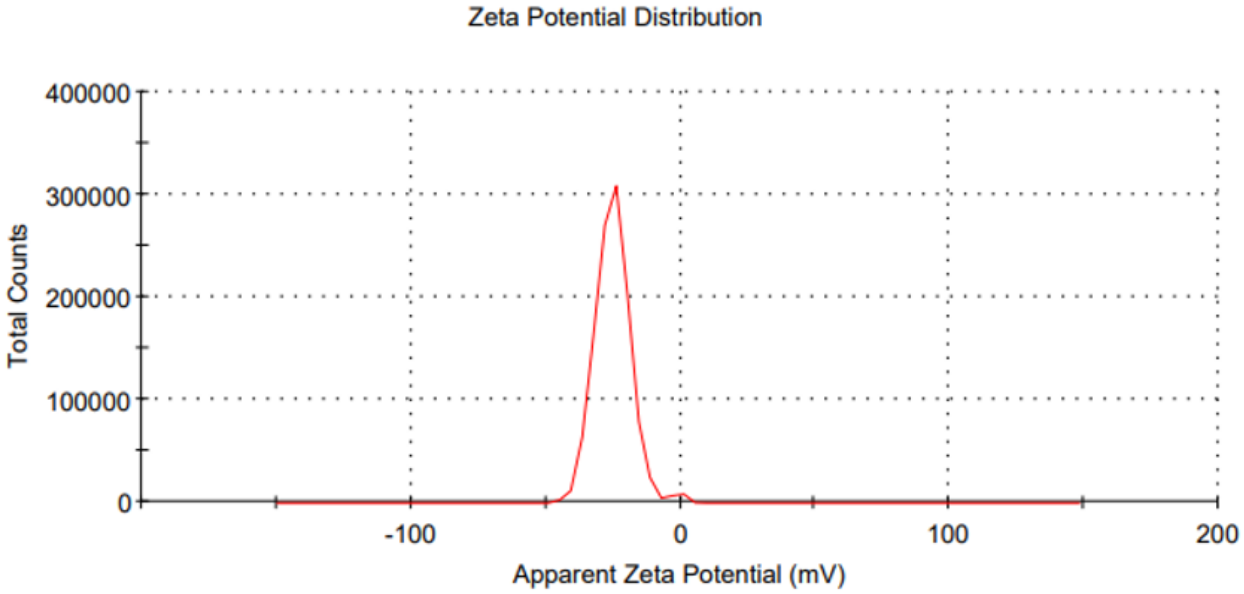


Figure 6

(a). The average size of biogenic AgNPs synthesized from *Mirabilis jalapa* by Zeta sizer (b) Zeta potential of bAgNPs.

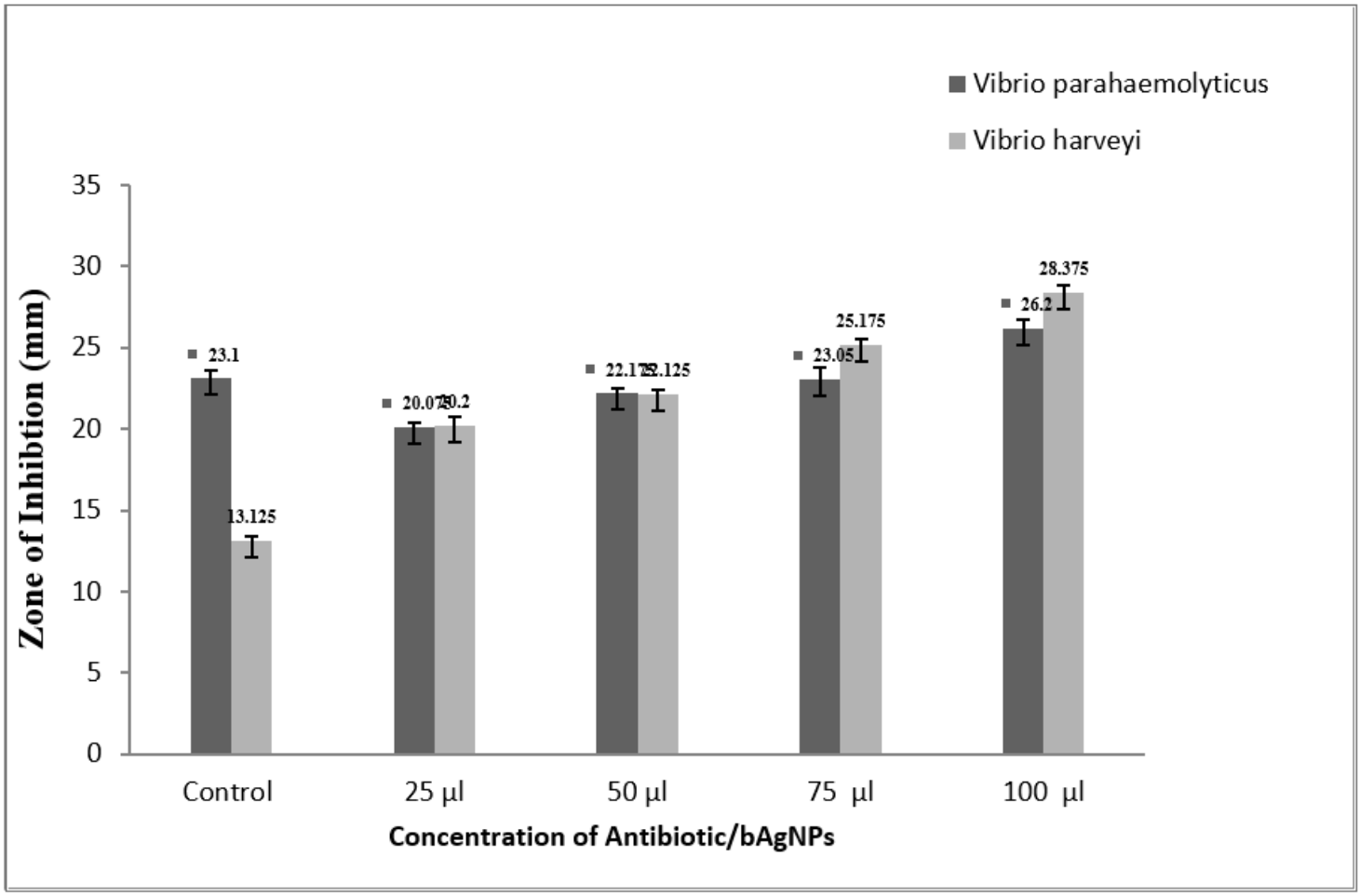


Figure 7

Antibacterial activity of bAgNPs against *Vibrio parahaemolyticus* and *Vibrio harveyi.*

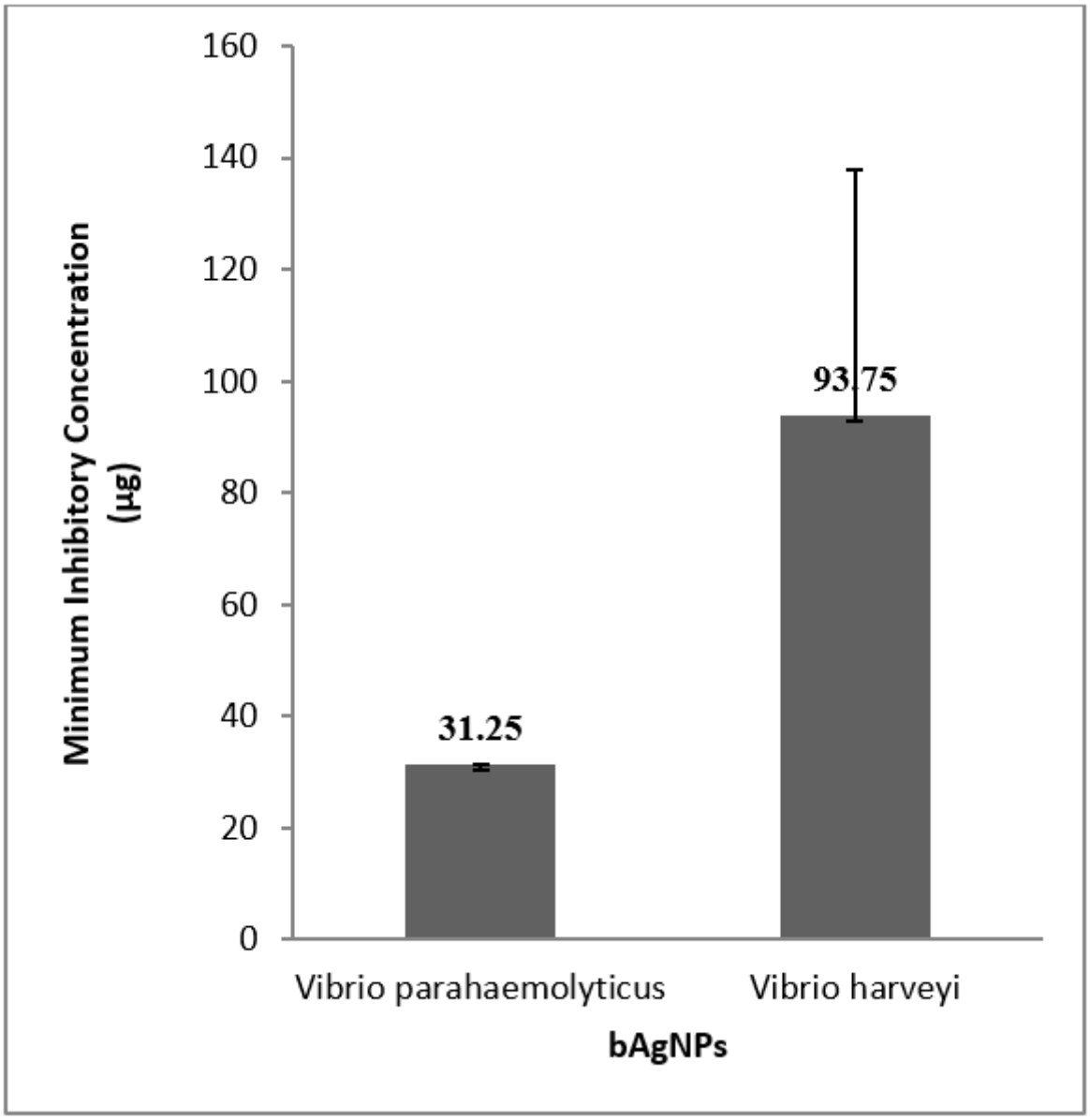


Figure 8

Minimum Inhibitory concentrations of bAgNPs on *Vibrio parahaemolyticus* and *Vibrio harveyi*

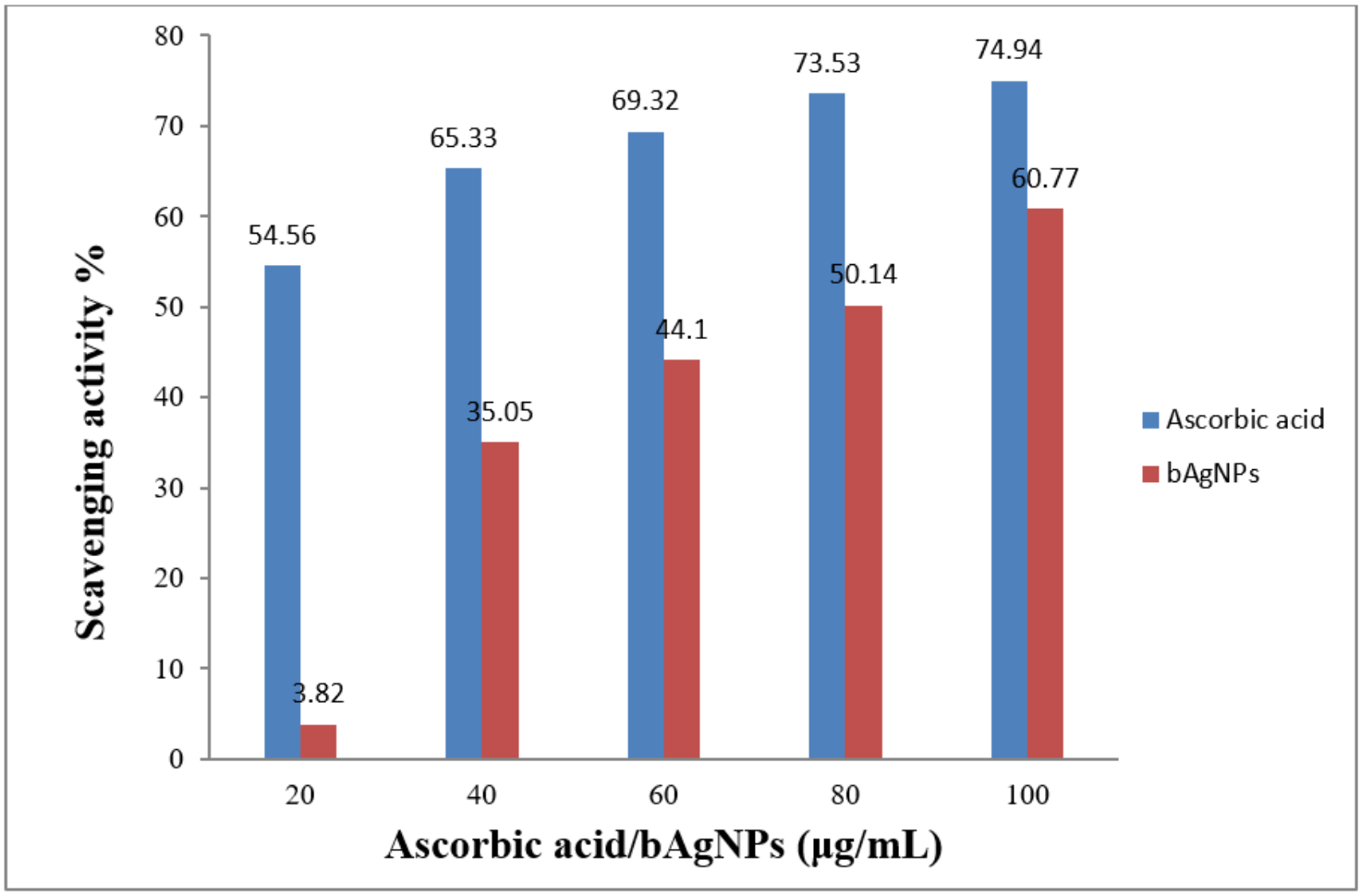


Figure 9

DPPH scavenging activity of Ascorbic acid and bAgNPs (µg/mL)

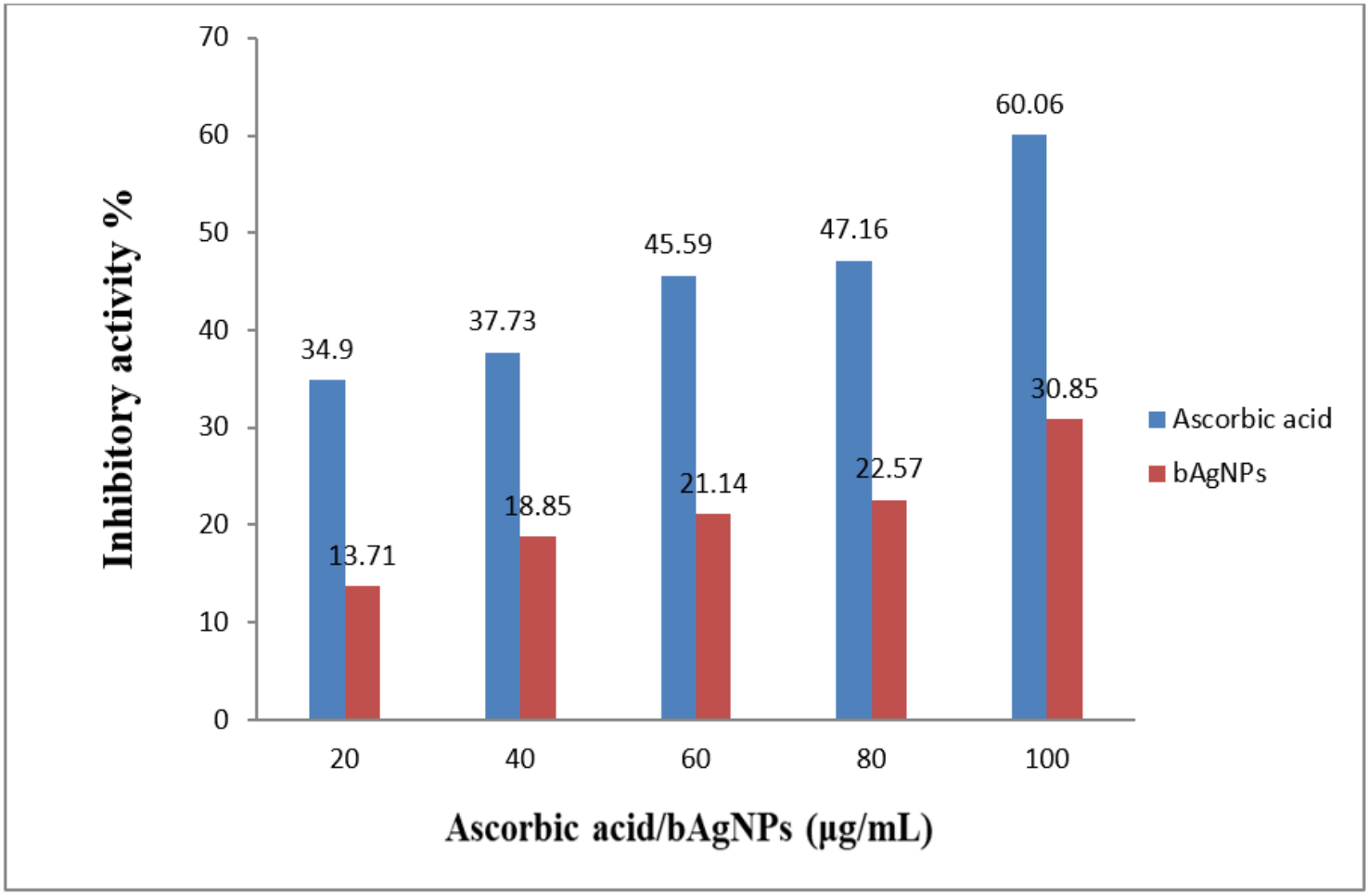


Figure 10

FRAP assay of Ascorbic acid and bAgNPs

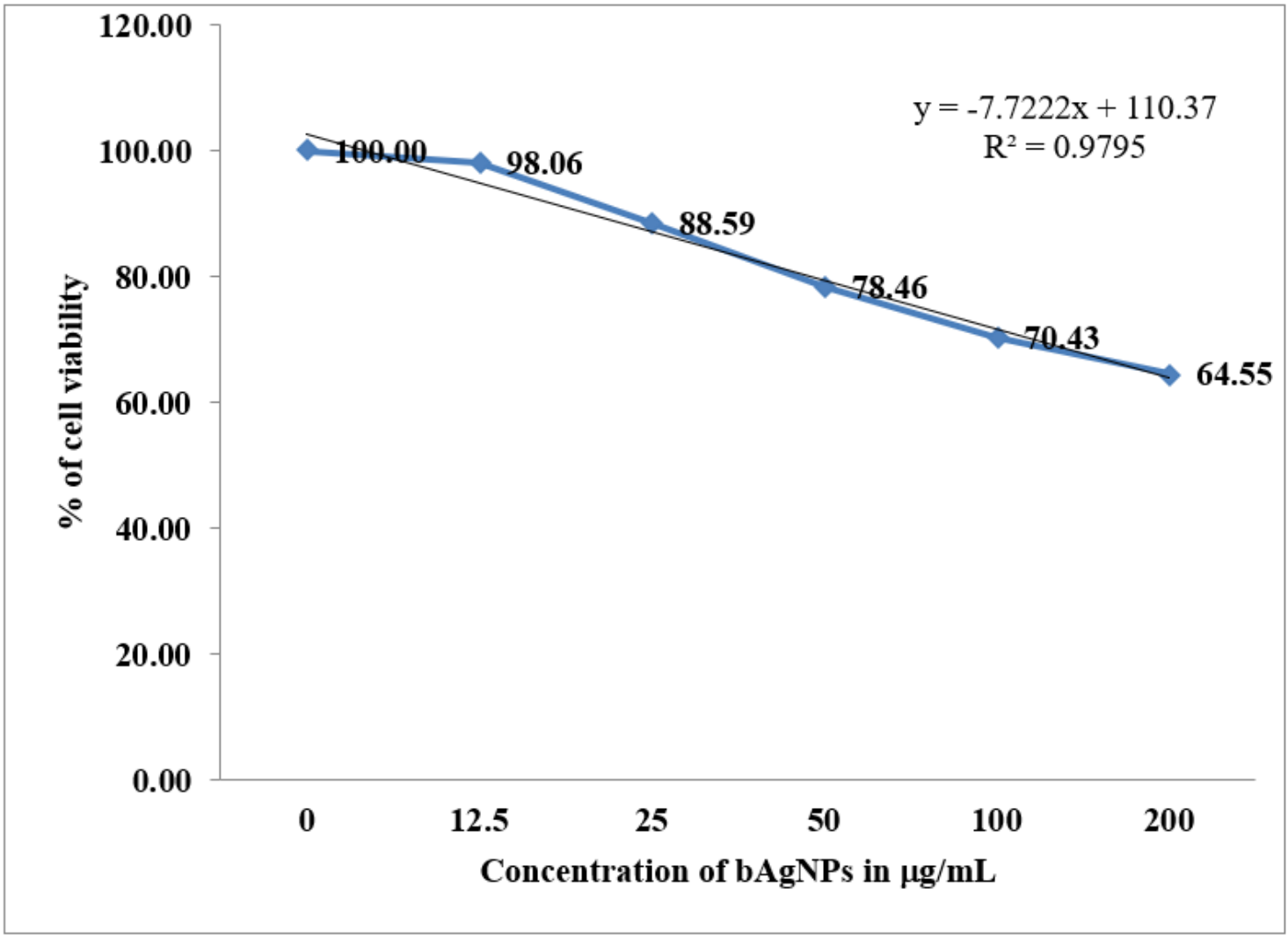


Figure 11

Cytotoxicity of bAgNPs on Vero cell line viability.

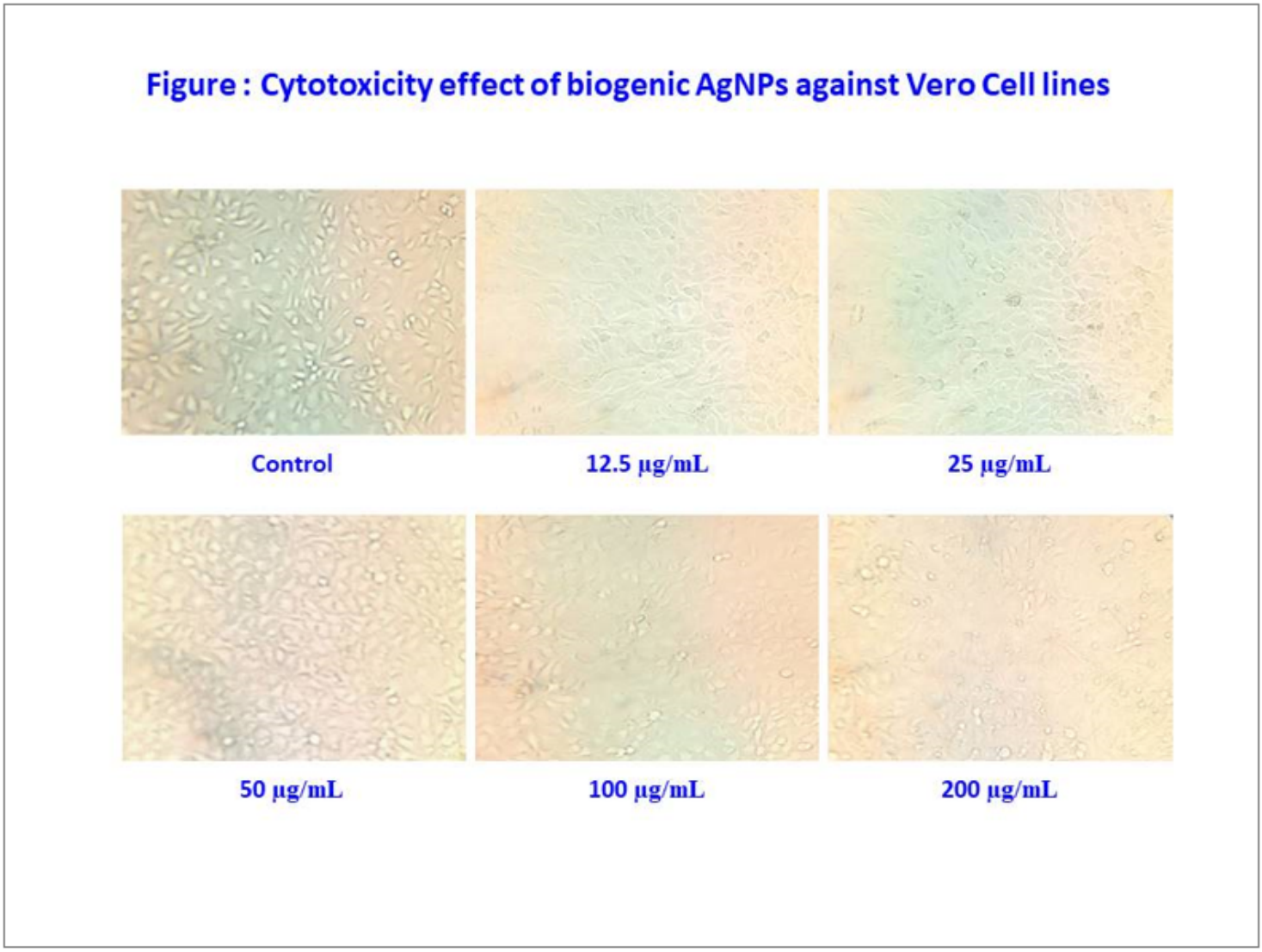


Figure 12

Cytotoxic effect of biogenic AgNPs against Vero cell lines.