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Growth inhibition activity of biogenic synthesized silver nanoparticles (Ag NPs) against mango anthracnose (*Colletotrichum gloeosporioides*) disease

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Abstract
The advantage of technology has provided better options to combat today's hurdles. In the era of modern agriculture, the unmanageable use of pesticides and fungicides are causing severe health problems. On the other side, phytopathogens are developing more résistance against traditional chemical measures. Thus, need of time to alternative cost-effective and eco-friendly technology for the solution to this modern problem. Furthermore, nanotechnology can be utilised to resolve these challenges. The green synthesis of nanoparticles has novel surface-dependent properties like catalytic and antimicrobial activity. Therefore, in present study the evaluation of silver NPs via the biogenic method of synthesis from mango leaf was carried out. These silver NPs were used to check the inhibitory effect against anthracnose disease in *in-vitro* conditions. The synthesized silver NPs exhibited 432 nm Surface Plasmon Resonance with calculated band gap energy of 2.87 eV. DLS analysis found an average particle size of 64.87 nm and the zeta potential peak at -14.0 mV. The TEM analysis revealed almost spherical shapes of synthesized silver NPs with an average size of 33.90 nm. In the present study aqueous biogenic silver NPs exhibited potent anti-fungal activity against *C. gloeosporioides* growth with inhibiting areas of 4.74±0.13 mm, 6.04±0.19 mm, 8.29±0.06 mm and 9.12±0.30 mm at 50, 100, 150 and 200 ppm concentrations, respectively. This was found very significant and advisable against positive control with higher concentrations of carbendazim (1000 ppm). Thus nanotechnology could be a better alternative approach to control anthracnose disease in the field and storage condition of mango.

Keywords: Biogenic, silver NPs, anti fungal, activity, *Colletotrichum gloeosporioides*

1. Introduction
Astonishingly, before 3000 BC, Romans, Greeks, Egyptians and Indians utilized silver in different forms to conserve food items (Block, 2001) [3]. As an example, during prehistoric usage of silver utensils for eating and drinking and preservation of eatable and drinkable items might be due to the familiarity with antimicrobial properties (William *et al*., 1940) [32]. A country rich in Ag resources, Argentina, was indeed named after this valuable noble metal Silver, with the symbol Ag, is derived from the Latin word Argentum, It is utilized in ancient civilization (Mohler *et al*., 2018) [17].

In eco-friendly nanotechnology, the process for bioreduction through biological molecules has been assessed. It comprises of complex biomolecules and functional groups i.e. ketone, aldehyde, carboxyl and hydroxyl moieties (Rana *et al*., 2020) [26]. Among various metallic nanoparticles, silver nanoparticles are widely utilized due to their excellent optoelectronic, anti- inflammatory and antimicrobial attributes (Mehta *et al*., 2021; Thirumagal and Jeyakumari, 2020; Mohler *et al*., 2018) [16, 30, 17]. The antimicrobial activities of silver NPs have been well-explored by studying their inhibitory effect on various microorganisms (Kotakadi *et al*., 2014, Rao *et al*. 2016) [12, 27]. Furthermore, the prevention of agricultural produces has been unmanageable use of pesticides and fungicides; which also spoil the ecosystem and cause hazardous effects in the food chain due to excess accumulation, globally (Hidalgo *et al*., 1998) [9]. Thus, we need to provide efficient, cost-effective, low toxic and environmentally friendly technology for the control of these fungal pathogens to save the ecosystem as well as human health (Li and Jennings, 2017) [14]. Usually, *In-vitro* applications of organic and inorganic antimicrobial nanoparticles such as Silver ion mediated NP is very popular to retain antimicrobial functionality (Barzegar *et al*., 2018) [2]. The green approach for synthesized nanoparticles from various plant extracts has been reported as a reducing agent to synthesize silver NPs (Vidhu and Philip, 2014; Rajakumar and Rahuman, 2012; Patil *et al*., 2012; Parthibavarman *et al*., 2019) [31, 25, 23, 19].

Therefore in the present study, the use of higher concentrations of silver ions for synthesized silver NPs from a phytochemicals pool of mango leaf. Further, we utilized the biogenic NPs to know the potent antifungal activity.

2. Materials and Methods

2.1 Plant material as a green precursor and chemicals

Mango (*Mangifera indica* L., 2n = 40, Anacardiaceae family, order Sapindales) fully matured green leaves of *cv*. Alphonso collected from the orchard (Fig.1) of Athwa Farm, Navsari Agricultural University, Ghod Dod Road, Surat, India situated at 8 m above sea level, North latitude 21.1722166 and East longitude 72.8029344, the average annual temperature is 27.2 ^{o}C, mean RH of 80-90%, average annual precipitation of 1143 mm in the south Gujarat province, West of India.

Chemicals were used in the experiment of analytical grade. Silver nitrate (MW-169.87 g/mol) was purchased from Himedia.

All glassware was cleaned with Acetone (Samir Tech-Chem Pvt. Ltd.) and rinsed several times with distilled water followed by deionized (DDW-Extrapure, ACS Chemicals, conductivity <0.5, neutral pH) water. pH adjustments of solutions were performed using 0.1 M HCL and 0.2 M NaOH. The solution was prepared in deionized water. For proper washing out of metal ions from used glassware, the aqua regia solution (corrosive acid) mixture is made by combining Nitric acid (HNO3-65%, MW-63.01 g/mol, Merk) and Hydrochloric acid (HCl-37%, MW-36.46 g/mol, Applichem) at ratio 1:3 in a fume hood with most of the precautions. This mixture is unstable, so it was usually prepared in small amounts and used immediately as fresh.

A typical total final volume for most applications is only 10 mL because it was usual to mix up a large volume of aqua regia. Also, Acetone (MW- 58.08 g/mol, Merk) was used for the removal of organic salt substances from glass wares followed by rinsing with DI water several times. In the end, all the glass wares were dried in a hot air oven (>60 °C) before being used in the biogenic synthesis of nanoparticles.

2.2 Apparatus and types of equipment

Samples were weighed on an Ohaus E- 225D weighing balance machine. For the nucleation process of silver NPs, a Digital ceramic magnetic stirring hot plate (Cole-Parmer) was used. The magnetic stirrer (needle size 8 x 35 mm, JJ Lab Wares) bar was used for biogenic synthesis.

The UV-Visible absorbance spectra were recorded on a UV-Visible spectrophotometer (UV-3000+ LABINDIA® Analytical) equipped with a 10 mm quartz cuvette and spectra were analyzed in UV-Win Spectrophotometer software in the range of 300 nm to 650 nm. The scanning was at medium speed and at 2.0 nm intervals. In the scan spectrum curve menu, the baseline was adjusted with a reference sample (DI water) prior to sample analysis. Data were saved by export files in the extension of ASCII and. doc format for further utilization.

Particle size distribution was obtained using the dynamic light scattering technique (DLS) and recorded using a Malvern Zetasizer Nano series followed by Zeta potential (mV). The measurement gave the average hydrodynamic diameter of the particles, the peak values in the hydrodynamic diameter distribution=, and the polydispersity index (PdI) that described the width of the particle size distribution (Elamawi *et al*., 2018) [6]. The measurement was carried out with a temperature equilibration time of 1 min at 25 °C with an angle of 90 °C. The data processing mode was calibrated at high multi-modal resolution.

The morphology and size of the biosynthesized silver NPs were measured with a Transmission Electron Microscopic examination (TEM; Technai-20, Phillips, Holland). For this purpose, a thin film of colloidal silver NPs was prepared and included in a carbon-coated copper grid, dried and kept under a vacuum before loading on a specimen holder, and TEM observations were made with (Zeiss-EM10C) operated at an accelerating voltage of 190 kV.

2.3 Extraction of a pool of Phytochemicals from Mango Leaf

The collected healthy leaves of mango were thoroughly washed several times using normal tap water. These leaves were dried for up to 48 hr at 50 °C temperature under the hot air oven followed by grinding to make powder. Powdered dried leaves (20.0 g) were added to 200 mL deionized water in a 500 mL flask and mixed well. The extract was prepared using a magnetic heating stirrer at 60 °C for 20 min. After cooling, at room temperature, the as-prepared solution was filtered through a Whatman filter paper no 4. The filtered extract was stored in the refrigerator within an amber color bottle for up to two weeks. This extract solution of the leaf was used as the stabilizing and reducing agent in the synthesis of silver nanoparticles.

2.4 Biogenic synthesis of silver NPs by leaf extracts of mango

Leaf extract of the *cv*. Alphonso was utilized for the phytochemical mediated nucleation process. In the experiment, $AgNO_3$ in deionized water was used as the source of silver (10.0 mM concentrations). Typical reaction mixtures contained 2.5 mL of the extract in 25 mL $AgNO_3$ solution under magnetic stirring at room temperature for the reduction of Ag^+ to Ag^0 until the colour of the mixture became reddish brown, indicating the formation of silver NPs. The Leaf extract was mixed in silver nitrate solution. The pH of the reaction mixture to 6.0 was adjusted.

For investigations through UV-Visible spectrophotometry, the equivalent amounts of the suspension were diluted in a constant volume of deionized water (1:10) and subsequently, its absorbance spectrum was measured at room temperature. The synthesized silver NPs were stored at chilling temperature in a clean amber colour bottle for further use. Silver NPs were sonicated in a sonicator (Bandelin sonorex) for 10 minutes before characterization.

2.5 Procurement of fungal sample and preparation of spore suspension

The fungal species *Colletotrichum gloeosporioides*, which is caused anthracnose disease in major fruit crops including *M. indica* L. Sometimes, it will lead to devastating and reduce yields by affected leaves, inflorescence, apical tips and fruits at different developing stages. Chemical control is caused problems in the food chain and also remains as a trace amount in fruit. Thus, bio-synthesized and natural extract-mediated nanoparticles emerge as a safe alternative with immense potential effects. Therefore, the pure culture of *Colletotrichum gloeosporioides* was procured from the Department of Plant Pathology, Navsari Agricultural University, Navsari, Gujarat, India and further used for

antifungal assay of synthesized nanoparticles. The fresh culture was prepared by diluted isolated spores for antifungal analysis (Jia, 2009) [11].

2.6 Antifungal Assay by Well Diffusion Method

Antifungal activity was assessed using well diffusion method. The prepared 5.0 ml Sterile distilled water (medium) containing fungal spores (*C. gloeosporioides*) by using a flamed loop was applied onto potato dextrose agar contains patry plates and spread evenly on it within aseptic conditions. The wells were made by cork borer (average diameter 6.0 mm) in PDA media that was sterile with alcohol and flame. Each well was impregnated with the silver NPs at different concentration (50.0 ppm, 100.0 ppm, 150.0 ppm, 200.0 ppm) using a micro pipette. Well were also made for the positive controls (0.1% Bavistin-50% W/P), Negative control (Sterile DIW). Also solvents like $AgNO_3$ (10.0 mM) used as a check materials. All were added with final volume 10 µL per well.

These plates are kept at 28 ˚C for one day to allow maximum diffusion of the test material to the surrounding media in a laminar air flow (Jagessar *et al*., 2008) [10]. The plates are then labeled, inverted and incubated at 30 °C for 48 h to obtain optimum growth of the fungus. The test materials having antifungal property inhibit fungal growth in the media surrounding the well and thereby yield a clear, distinct area defined as zone of inhibition. The antifungal activity of the test agent is then determined by measuring the mean diameter of three repetitions in completely randomized design.

3. Results and Discussion

3.1 Leaf Extracts of *M. indica* and biogenic synthesis of Nanoparticles and their Characterizations

Table 1 shows the characterization techniques utilized for present study and antifungal activity of the synthesized silver NPs.

In present study, we assessed green synthesis of silver NPs. Therefore this approach is totally environment-friendly, non-toxic and cost-effective. *M. indica* leaf extract contains phytochemicals including phenolics which can reduce cations and stabilize the as-formed silver NPs to prevent agglomeration (Samari *et al*., 2018) [28].

Table 1: Characterizations techniques used for synthesized Silver NPs

Sr. No.	Techniques	LE	Ag NPs
1	UV-Visible Spectroscopy	✓	✓
2	DLS and Zeta Potential	-	✓
3	TEM	-	✓
4	Antifungal Activity	✓	✓

Where,

LE= Leaf Extracts, ✓= Applied for analysis, - = Not applicable

3.3.1 *M. indica* Leaf extract as a reducing and capping agent for Ag nanoparticles synthesis

As shown in Fig. 2 the UV-Visible spectrum of aqueous extracts of the mango leaf with the band at 368 nm was depicted. This indicates that the leaf extracts have a rich content of flavonoids. Which is also reported for *Andrographis paniculata* plant extracts (Fardiyah *et al*., 2020) [7]. Moreover, this was supported by Costa *et al*., (2015) [4] that plant extracts usually are prominent in flavonoids efficient in absorbing ultraviolet rays, typically indicated by two peaks of ultraviolet absorption at ranges of 300–550 nm.

3.1.2 Biogenic leaf extract mediated silver NPs and their characterization

3.1.2.1 UV-Visible spectroscopy analysis

The silver NPs were synthesized using 10.0 mM of $AgNO_3$ concentrations in the presence of Mango leaf extract. The UV-Visible spectra of silver NPs synthesized at 10.0 mM $AgNO_3$ concentration using leaf extract were depicted in Fig. 2.

The formation of silver NPs was confirmed with visual colour changes from the green of leaf extracts to brownish red of silver NPs (Fig. 3). The direct effect of the proportion of phytochemicals including $C_{19}H_{18}O_{11}$ to the nucleation process of green synthesis of nanoparticles was observed. From UV-Visible spectroscopy, 10 mM concentration of silver salts can be able to turn into silver NPs which is depicted by the Surface Plasmon Resonance peak at 432 nm. In accordance with that Samari *et al*. (2018) [28] and Sarsar *et al*. (2013) [29] reported SPR at around 410 nm and 440 nm of synthesized Silver NPs from Mango leaf extracts, respectively.

The band gap energy was computed accordingly $Eg=1240/\lambda$; where λ was the maximum absorption wavelength (nm) as prescribed by Balcha *et al*., (2016) [1] and found 2.87 eV. Meanwhile, Parthibavarman *et al*., (2019) [19] found 2.83 eV Eg band gap energy of grape extract mediated silver NPs.

Finally, it can be confirmed that mango leaf extract has a potent aptness to reduce and stabilize silver NPs and reduction was far better than in another study that synthesized silver NPs using mango leaf extract (Sarsar *et al*., 2013) [29].

3.1.2.2 DLS and Zeta Potential analysis

Furthermore, Dynamic Light Scattering and Zeta Potential analysis were carried out to study particle size and stability of silver NPs in colloidal media. The silver NPs were found to have a size distribution ranging from 6.937 to 5283 nm with a poly-disparity index of 0.358 (Fig. 4). The estimated average particle size distribution of silver NPs was found 64.87 nm; the zeta potential of silver NPs was found as a peak at -14.0 mV (Fig. 5). That explicates their stability by electrostatic repulsion which will prevent the aggregation and agglomeration (Mani *et al*., 2021) [15]. Thirumagal and Jeyakumari (2020) [30] stated that the negative zeta potential of nanoparticles is a result of the adsorption of OH- ions from phytochemicals of the leaf.

3.1.2.3 TEM analysis

Further, silver NPs were characterized by TEM to estimate the particle size and particle distribution of NPs (Fig. 6). The TEM images demonstrated almost spherical shapes of silver NPs around the metrics. It was well distributed along with the size range of 14.42 nm to 56.36 nm with an average size of 33.90 nm as analysed by ImageJ software (Fig. 7). Similarly, Samari *et al*., (2018) [28] reported a range of 14 nm to 28 nm with the average size of 20.7 nm from mango leaf extracts.

3.2 Antifungal activity of biogenic silver NPs synthesized from *M. indica* leaf extract

The Zone of Inhibition (ZOI) is a scientific method used widely in applied microbiology for well diffusion methods in PDA media to evaluate the antifungal activities of any active substances (Radha, 2021) [24]. In the present study, 0.1% positive control as a systematic fungicide Bavistin (Carbendazim-50% W/P) depicted a Zone of inhibition around 23.01±0.58 mm (Fig. 8 and Fig. 9). It was found to be

most effective in inhibition of mycelia growth of *C. gloeosporioides* among different ranges of fungicides available in the market. Moreover, Kumari *et al*., (2017) [13] reported that the concentration of 0.1% of Carbendazim 50% WP found maximum inhibition of fungus growth of mango anthracnose during *in-vitro* conditions. In addition, regarding environment effects on flora and fauna, the toxicity of systematic fungicide carbendazim has raised serious concerns about safety (Hess *et al*., 1991; Muthuviveganandavel *et al*., 2008) [8, 18]. Therefore, the green and eco-friendly approach to combat this serious disease in mango is the demand of time. Thus, the biogenic silver NPs could be a better alternative.

Moreover, the fresh solvent was used to check the anti-fungal activity of silver nitrate (10.0 mM). It exhibited 2.65±0.35 mm ZOI (Fig. 9). Also other studies reported significant lower anti-fungal activity of silver nitrate against phytopathogens (Niakan *et al*., 2019) [20].

In the present study aqueous biogenic silver NPs exhibited potent anti-candida activity against *C. gloeosporioides* growth with inhibiting areas of 4.74±0.13 mm, 6.04±0.19 mm, 8.29±0.06 mm and 9.12±0.30 mm at 50, 100, 150 and 200 ppm concentrations, respectively (Fig. 9). Nagaraju *et al*., (2020) [19] studied with Carbendazim (1%) significantly inhibited C. gloeosporioides fungus growth with an inhibition zone of 45% which was more than inhibition zone of the as-synthesized aqueous silver NPs with spherical in shape and diameter range of 19 to 24 nm at highest 100 microL dose. Thus, It can be concluded that an NPs morphology and dose-dependent anti-fungal mechanism and results were obtained. Meanwhile, among different antimicrobial agents, silver NPs interact with the fungal cell outer layer and hinder the fungal cell cycle by proton pump destructing and lead fungal surface protein denaturation (Du *et al*., 2012) [5]. Moreover, silver NPs disturb protein-lipid bi-layer or membrane permeability followed by rupturing the cell membrane. Further, silver NPs also trigger off the conglomerate of silver ions, and efflux of intracellular ions, blocking respiration, and metabolism activity by disrupting the electron transport system (Nisar *et al*. 2019) [21]. Rao *et al*. (2016) [27] observed potent antimicrobial activity against fungi with enhancing doses of Ag NPs synthesized from *Diospyros paniculata* root extract.

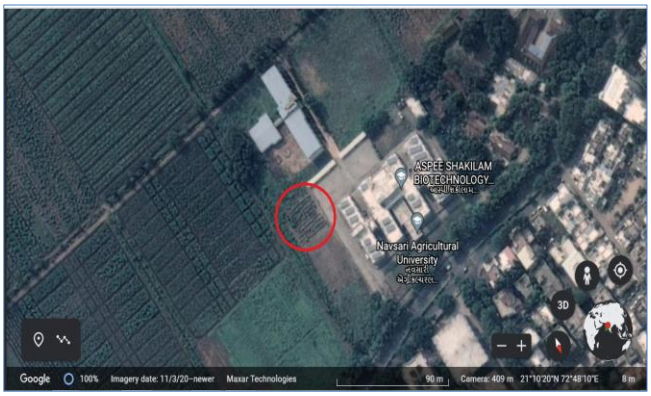

Fig 1: Virtual three dimensional geomapping* of Mango orchard for source of leaf extract at Panas Village, Navsari Agricultural University, Surat, India. (*Retrieved from Superimposing satellite Landsat 7 and 8 images via Google Earth Maxar Technologies)

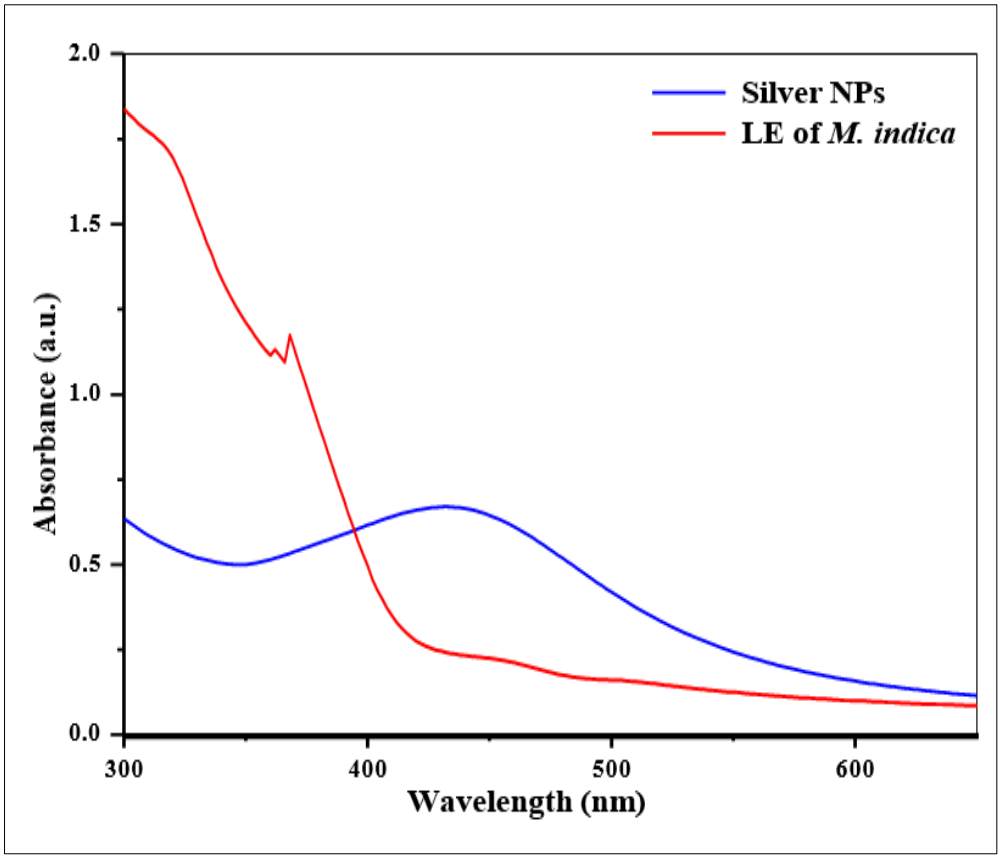

Fig 2: UV-Visible Spectroscopy of biogenic synthesized silver NPs at pH 6.0 and fresh leaf extract of *M. indica*

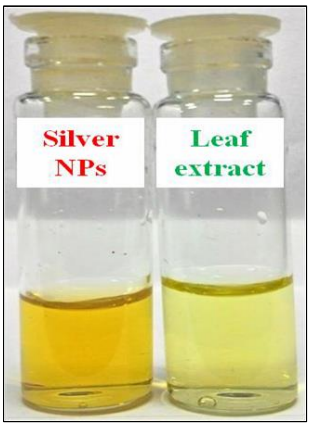

Fig 3: Day light pictures of as-synthesized silver NPs and fresh Leaf extract of *M. indica*

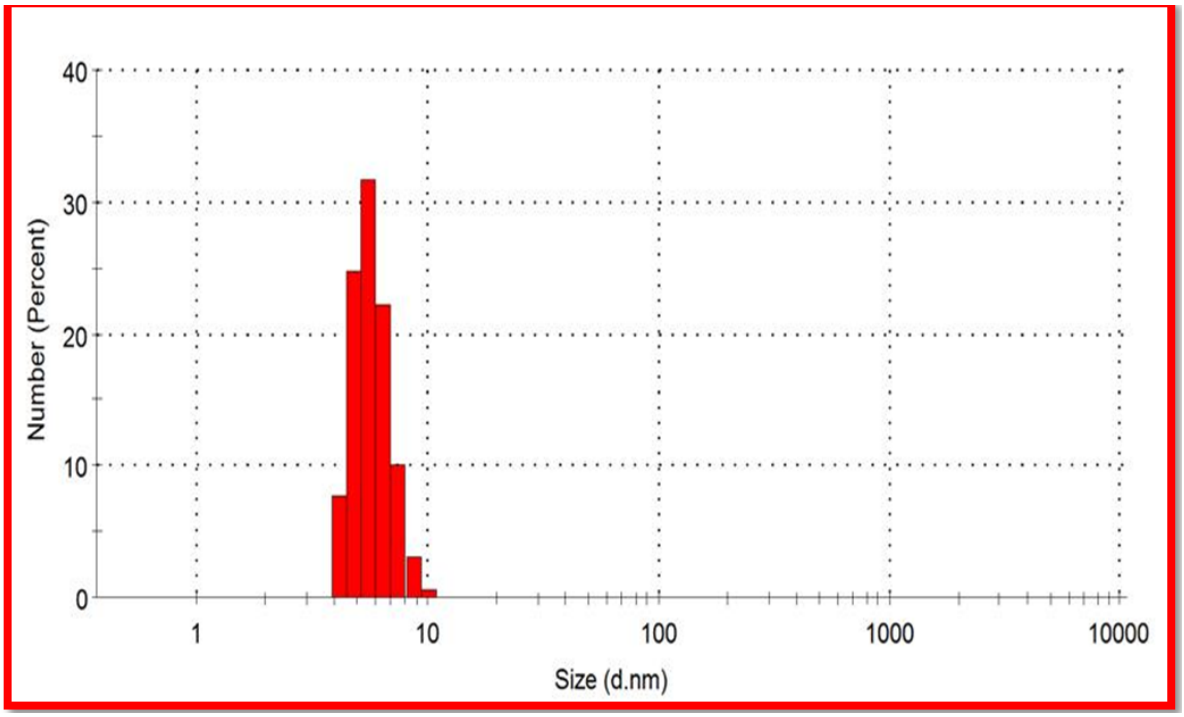

Fig 4: DLS analysis of silver NPs

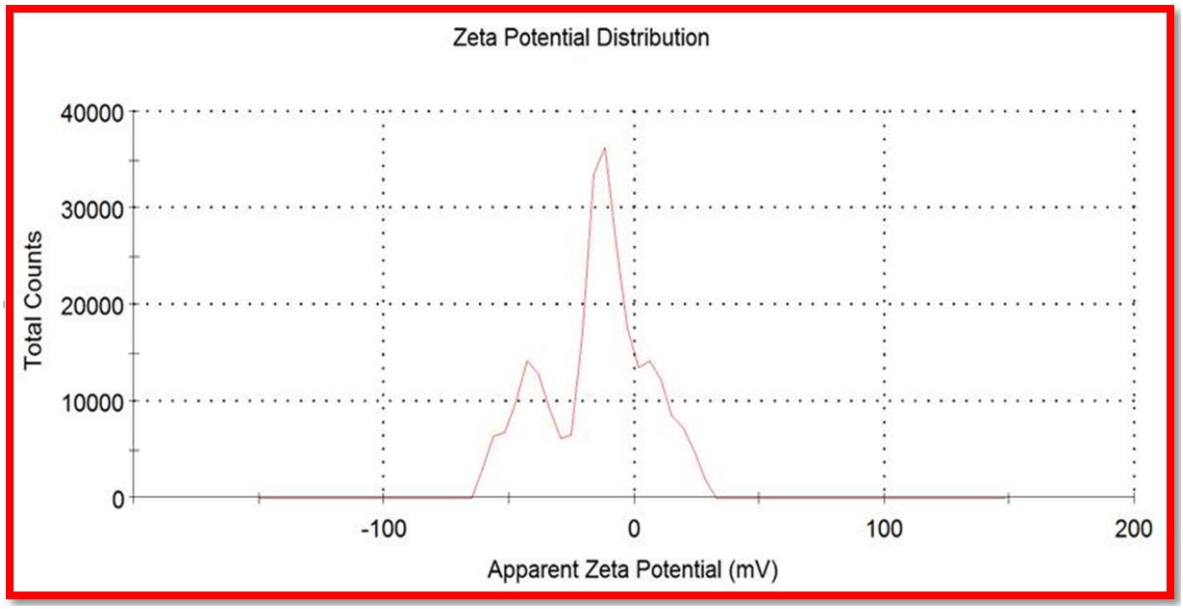

Fig 5: Zeta potential analysis of silver NPs

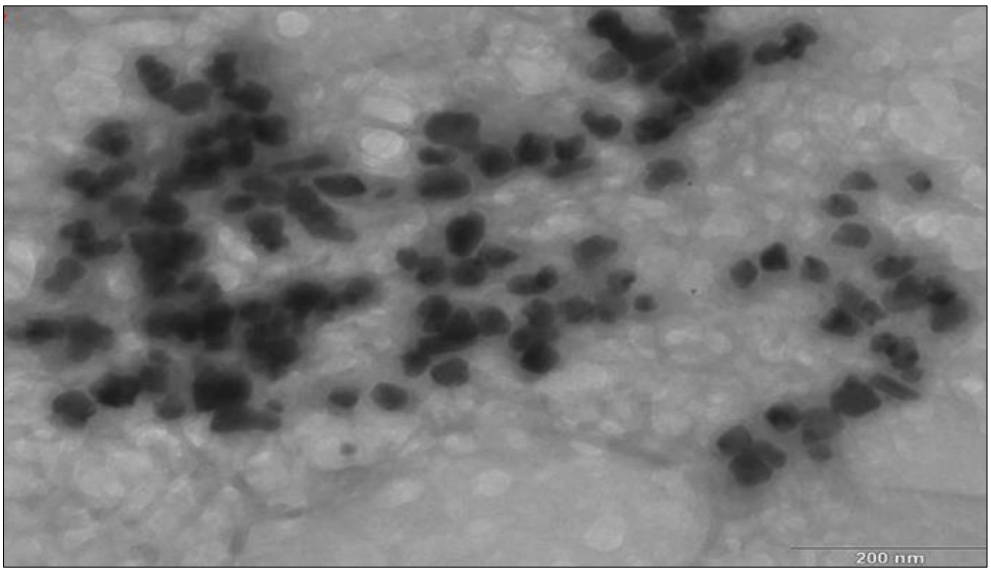

Fig 6: TEM image of silver NPs at 10.0 mM AgNO3 concentration (scale 200 nm)

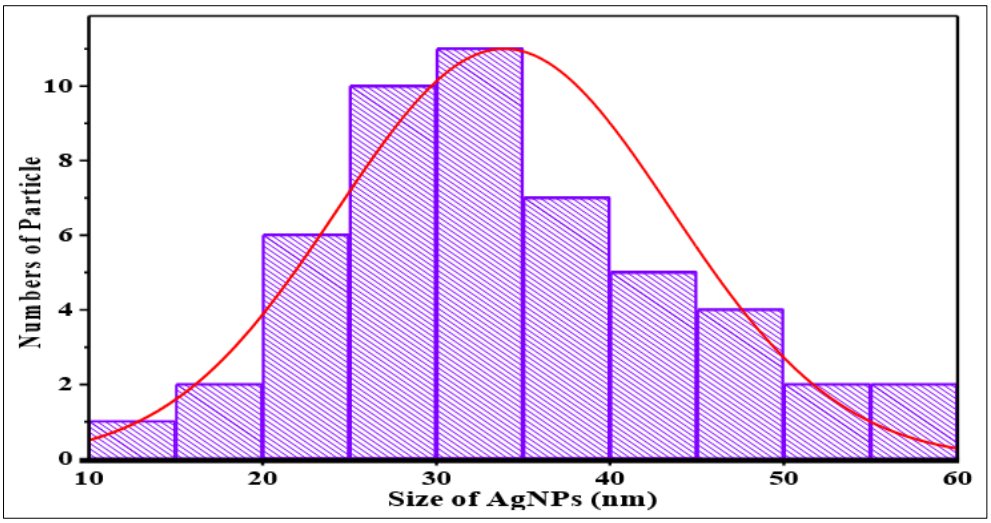

Fig 7: Particles size distribution with histogram of silver NPs

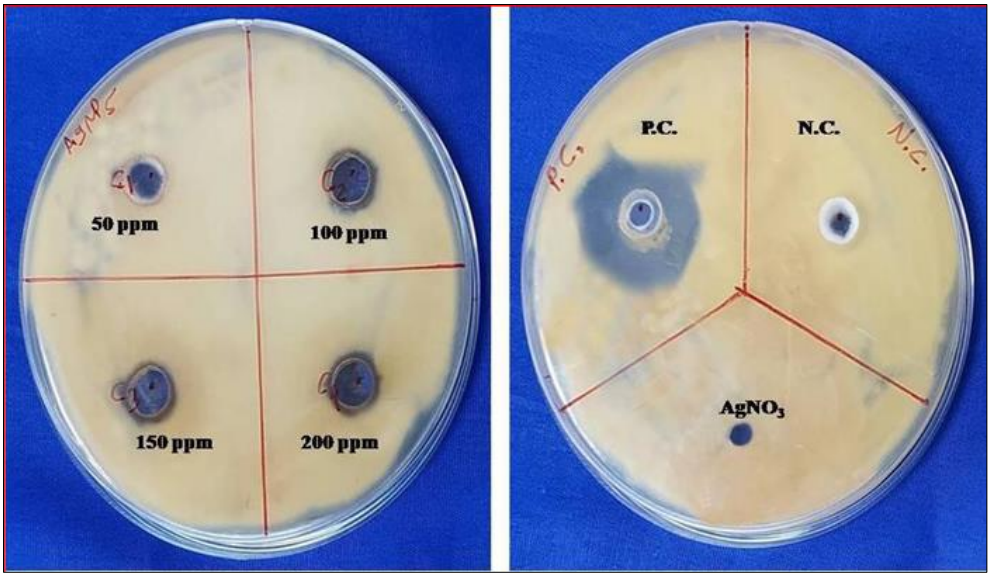

Fig 8: *In-vitro* antifungal activity of synthesized silver NPs with controls and solvent

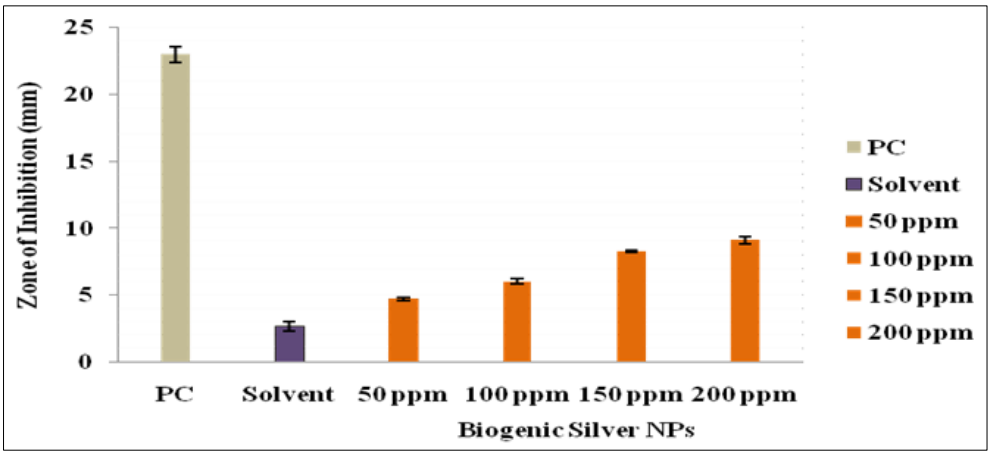

Fig 9: Zone of Inhibition of biogenic synthesized silver NPs with positive controls (PC) and solvent

4. Conclusions

The overall result indicated that the biogenic synthesis of silver NPs can be better, cost effective, eco-friendly and alternative approach for the control of mango anthracnose disease. The higher dose (200 ppm) is advisable to use for antifungal activity. However, the technology need to be scale up, assessed the outcomes in field and *in-situ* condition along with evaluated at multi-trial and multi-location research.

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