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EVALUATION OF GREEN SYNTHESIZED SILVER NANOPARTICLES AGAINST BACTERIAL PATHOGENIC STRAINS OF PLANTS

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Abstract

Aqueous leaf extract of Hyppophae rhamnoides leaves was used to create silver nanoparticles as a reducing and capping agent. Techniques like the UV-Vis spectrophotometer, scanning electron microscopy (SEM), X-ray diffraction (XRD) analysis, and fourier transform infrared spectroscopy (FTIR) analysis were used to characterize the synthesized nanoparticles. Transmission electron microscopy (TEM) provides size distribution specifying sizes of silver nanoparticles ranging from 13 nm to 34 nm in diameter. The shape of the nanoparticles was amorphous, ranging from spherical to rectangular. The absorption bands at 2526, 2340, 2159 and 2030 cm^{-1} are related to the carbonyl group (C=O), confirming more biomolecule involvement with C=O functional groups. The peaks 1869, 1845 and 1829 cm^{-1} are associated with aldehyde (C=O) and alkenes (C=C) functional groups containing compounds. The peak at 668 cm^{-1} depicted the presence of an aromatic ring. Phytochemical profiling of green synthesized AgNPs revealed the incidence of phenols, terpenoids, flavonoids, and phytosterols being involved in successfully reducing and capping nanoparticles. Maximum antibacterial activity was found in higher concentrations of AgNPs (0.35mg/100ul) against *Ralstonia solanacearum* and *Pseudomonas syringae.* Green synthesis is fast, cost-effective, non-hazardous, and environment-friendly compared to its chemical synthesis counterpart. AgNPs can be used as a source of antibiotics in the future.

Key words: Pathogenic bacteria; Plant bacterial strains, Green synthesis, Plant extract, Silver nanoparticles.

Introduction

Nanotechnology is an emerging field of the modern era that deals with synthesizing, planning, and operating metal-based nanoparticle structures with dimensions of 1 to 100 nm in size (Ahmad *et al*., 2016). The researchers are attracted due to its avant-garde nature, broad size range, and diverse chemical, physical and biological nature in bulk. Nanoparticles' novel and superior quality enable them to use broadly in medicine and physical sciences (Ahmad *et al*., 2016). Currently, efforts have been underway to formulate eco-friendly procedures to fulfill the purpose of nanoparticle production with preferred sizes and morphologies to enhance their applications in biomedical sciences. Green processes are gaining much attention and are pre-requisite to avoid environmental issues (Thuesombat *et al*., 2014). The most commercialized nonmaterial is silver, with a production of 500 tons/ year; its production is expected to increase in the next few years (Larue *et al*., 2014). Apart from its vital role in engineering, it has also been involved in antimicrobial activities (El-Chaghaby & Ahmad, 2011). Conventionally silver is used as an antimicrobial agent; this property of silver makes it the best to use as a nanoparticle (Ashokkumar *et al*., 2013). The nanomaterials have applications in medicines due to specificity and are mainly confined to diagnosis and therapeutic processes (Mukherjee *et al*., 2014).

Chemically synthesized silver nanoparticles (AgNPs) are laborious, leaving toxic environmental effects. In contrast to chemical synthesis, green synthesis is fast, cost-effective, non-hazardous, and environmentally friendly (Mittal *et al*., 2014). Therefore, scientists attempt to devise the appropriate biological methods for synthesizing silver nanoparticles. Over the few decades, the use of plants, bacteria, algae, fungi, yeast, and honey for the synthesis of nanoparticles has already been reported in the literature (Torres *et al*., 2012). The majority of nanoparticle synthesis schemes entail the application of toxic compounds having dangerous outcomes (Ahmed *et al*., 2014). In this regard plant, extract-based synthesis involving natural plant compounds could be well thought-out for nanotechnology (Ahmed *et al*., 2014; Kharissova *et al*., 2013). Plant extracts hold phytochemicals such as terpenoids, phenols, flavonoids, and dihydric phenols, displaying a reduced capability for metal salt to produce nanoparticles (Jacob *et al*., 2011).

Sea buckthorn (*Hyppophae rhamnoides*), is reported to be found in Himalayan region and exhibits potential medicinal properties. A deciduous shrub with tiny yellow to orange-red berries, sea buckthorn is widely distributed worldwide but is particularly common in temperate regions of South Asia, India, China, Central Asia, and Europe. The species thrives in the sub-Himalayan region of India and other regions with harsh weather (Madawala *et al*., 2018). Sea buckthorn has different medicinal properties, such as flavanol, alkaloids, tannins, terpenoids, catechin, phospholipid, and unsaturated fatty acids. These ingredients supplement good health and medicinal use such as diabetes, ulcer, inflammation, cardiovascular disease and immune system disorders (Nawaz *et al*., 2018).

To combat the threat of drug resistance, scientists are working around the world to create novel antibiotics and antimicrobials that prevent resistance from developing and use fewer traditional antibiotics. The common plant disease known as bacterial wilt is brought on by the soil-borne bacterium *Ralstonia solanacearum*. *Pseudomonas syringae* is an opportunistic pathogen that targets a range of woody plants, particularly those injured by frost. Current work aims at the synthesis of silver nanoparticles by using the leaf extract of Sea buckthorn (*Hyppophae rhamnoides*) and study the antibacterial potential of synthesized nanoparticles.

Materials and Methods

Materials and Methods: The young leaves were taken from plants grown in Pakistan's Gilgit region. The collected plant specimens were identified using the Flora of Pakistan (https://www.eflora.com). World flora online (http://www.worldfloraonline.org/) was used to locate the right scientific name, and APG IV 2016 was used to determine the right family name. Combining Hyppophae rhamnoides leaf extract with silver nitrate ($AgNO_3$), the solution created a plant extract-mediated production of silver nanoparticles. The entire process took a few minutes to complete at room temperature. UV-Vis spectrophotometry, XRD, SEM, TEM, and FTIR techniques were used to depict AgNPs. The conversion of AgNO3 to AgNPs was seen by capturing the UV-Vis spectrum. Water sanitized and added AgNPs were then ultrasonically processed for five to ten minutes. The UV-Vis spectrum was captured at wavelengths between 300 and 700 nm. The Shimadzu XRD-6000 was used to determine the AgNPs' crystallinity. Powdered samples on XRD in the 5^o–50^o at a 2θ angle were employed to examine the diffraction pattern. At a magnification of 10 K and a BI of 10.0, we examined the morphology of the AgNPs using a SIGMA model MIRA3 TE SEM. A thin film was produced on the copper grid when the AgNPs suspension, prepared in water, was dropped onto it. After using blotting paper to remove any excess solution, the film was allowed to dry under a mercury lamp for five minutes. Scanning through an internal electron beam captured images of the sample surfaces. Using a TEM, specifically a JEOL JEM-1400 operating at 100kV with a 25 K magnification, the size range of the AgNPs was identified. The diameters of the smallest to largest nanoparticles were measured to determine the AgNPs size range. The FTIR analysis of AgNPs was done to recognize the biomolecule(s) responsible for reducing and capping Ag ions, ultimately forming nanoparticles.

Phytochemical screening: The Phytochemical screening of Sea buckthorn leaf extract and synthesized AgNPs was executed by using the standard biochemical method of Fransworth (1996).

Analysis of antibacterial activity: Antibacterial analysis of the green synthesized AgNPs was investigated alongside plant pathogenic gram-negative bacteria *Ralstonia solanacearum* and *Pseudomonas syringae*. The pathogenic bacteria were obtained from the central laboratory at the Institute of Biochemistry & Biotechnology, PMAS-Arid Agriculture University, Rawalpindi. Different concentrations of AgNPs (0.05mg/100ul, 0.15mg/100ul, 0.25mg/100ul, and 0.35mg/100ul) were prepared by dissolving dried powder (w/mg) in distilled water. These Bacterial colonies were developed in Mueller-Hinton agar medium incubated at 37^oC for 24 hrs. The 100 ml of inoculum from newly refined bacterial colonies was selected and spread on separate plates containing Mueller-Hinton agar medium using the disc diffusion method (Kourmouli *et al*., 2018). Then the plates were laden with 0.05 mg/100ul, 0.15 mg/100ul, 0.25mg/100ul and 0.35mg/100ul of green synthesized AgNPs with respective controls and nurtured at 37^oC for 24 hrs. The zone of inhibition (ZI) was observed in Petri plates and the diameter was calculated in millimeters.

Statistical Analysis

Zones of inhibition for *Ralstonia solanacearum* and *Pseudomonas syringae* at concentrations 0.05, 0.15, 0.25, and 0.35 mg/100ul were recorded and compared in Excel-2010.

Results

Plant extract and AgNPs analysis: Table 1 represents the analysis of Sea buckthorn plant extract and green synthesized AgNPs. Carbohydrates were present in the plant extract but not in AgNPs. Phenolics and flavonoids were present in both the plant extract and AgNPs. Terpenoids and phytosterols were also present in both the plant extract and AgNPs. The plant extract contained volatile oil, but it was absent in AgNPs. Vitamins, amino acids, and fatty acids were present in the plant extract but not detected in AgNPs. Overall, the results suggest that the plant extract contains a variety of Phyto-constituents, including carbohydrates, phenolics, flavonoids, terpenoids, phytosterols, volatile oil, vitamins, amino acids, and fatty acids. While AgNPs contained some of the same Phyto-constituents as the plant extract, some were absent, including carbohydrates, volatile oil, vitamins, amino acids, and fatty acids. Notably, the absence of these Phyto-constituents in AgNPs could be due to the nature of the synthesis process used to produce them.

Table 1. Phytochemical profiling of *H. rhamnoides* leaves plant extract and synthesized AgNPs.

Phyto-constituents	Screening	
	Plant extract	AgNPs
Carbohydrates	+	--
Phenolics	+	+
Flavonoids	+	+
Terpenoids	+	+
Phytosterols	+	+
Volatile oil	+	--
Vitamins	+	--
Amino acid	+	--
Fatty acid	+	+

Table 2. Zone of inhibition (mm) showed by AgNPs synthesized *H. rhamnoides* leaves extract.

Conc.	*Ralstonia solanacearum*			*Pseudomonas syringae*		
(mg/100µl)	Control	Extract	AgNPs	Control	Extract	AgNPs
0.05	5	2	11	4	3	9
0.15	8	6	13	7	5	12
0.25	11	9	16	10	8	15
0.35	14	12	21	13	10	19

Characterization of silver nanoparticles (AgNPs): Silver nanoparticles portrayed by UV-Vis spectrometry, XRD, SEM, TEM and FTIR analysis as one technique is not much enough to elaborate the exact picture of AgNPs (Fig. 1). The UV spectrum yielded variable peak formation on respective wavelengths. Figure 1 (a) depicts and confirms AgNPs presence through UV-visible spectra. The crystalline nature of AgNPs was depicted by XRD analysis (Fig. 1b). This technique confirms that silver nanoparticles possessed a face-centered cubic structure. Silver nanoparticles of variable shapes and sizes were obtained when observed from SEM and TEM analysis (Fig. 1c, d). TEM analysis provides a better vision of the exact size of each nanoparticle. The TEM analysis provides size distribution specifying the diverse sizes of silver nanoparticles ranging from 13 nm to 34 nm in diameter (Fig. 1d). The FTIR spectra of silver nanoparticles synthesized from *H. rhamnoides* plant leaves extract showed spectra measured in the wavelength range 500–4000 cm^{-1} at 1 cm^{-1} resolution (Fig. 1e).

Analysis of antibacterial activity: Antibacterial activities of green synthesized AgNPs against two plant pathogenic bacterial strains have been presented (Table 2; Figs. 2-3). The inhibition zone measured in millimeters revealed the antibacterial activity of AgNPs. For *Ralstonia solanacearum*, growth inhibition increased as the concentration of the treatments increased. The Control had the lowest inhibition, with the bacterial growth reaching up to 14 at the highest concentration tested (0.35 mg/100µl). The Extract treatment showed moderate inhibition, with bacterial growth reaching 12 at the highest concentration tested. The AgNPs treatment had the highest inhibition, with bacterial growth reaching 21 at the highest concentration tested. For *Pseudomonas syringae*, the growth inhibition also increased as the concentration of the treatments increased. The Control had the lowest inhibition, with the bacterial growth reaching up to 13 at the highest concentration tested. The Extract treatment showed moderate inhibition, with bacterial growth reaching 10 at the highest concentration tested. The AgNPs treatment had the highest inhibition, with bacterial growth reaching 19 at the highest concentration tested. Overall, the results suggest that AgNPs treatment had the strongest inhibitory effect on both *Ralstonia solanacearum* and *Pseudomonas syringae*, followed by the Extract treatment, while the Control showed the lowest inhibition. Maximum antibacterial activity was found in higher concentrations of AgNPs (0.35mg/100ul) against *Ralstonia solanacearum* and *Pseudomonas syringae* (Table 1; Figs. 2-3).

Discussion

Green synthesis is preferable to other synthesis methods. The leaf extract of Sea buckthorn (Hyppophae rhamnoides) acts as a reducing agent to synthesize AgNPs from silver salt. The plant extract has the potential to coat and encapsulate metal ions to form particle-like entities. The chemical analysis of H. rhamnoides leaves extract showed the existence of carbohydrates, phenolics, flavonoids, terpenoids, steroids, volatile oil, organic acids, vitamins, amino acids, and fatty acids as active constituents. Phytochemical profiling of green synthesized AgNPs revealed the incidence of phenols, terpenoids, flavonoids, and phytosterols being involved in successfully reducing and capping nanoparticles. Rauwel *et al*. (2015) stated that plant extracts have some potent ingredients involved in nanoparticle synthesis. Similar results were also reported by Zayed *et al*., (2012). Plant-mediated coating shields silver ions and diminishes their toxic effect inside the recipient cell. Yin *et al*., (2012) and Yasur & Rani (2013) previously explained the protective activity of plant extract in the form of nanoparticles. The reason behind nanoparticle synthesis is the mutual intermingling between plant metabolites and metal ions to form stable particles (Shah *et al*., 2015). The green production of AgNPs was completed within a few minutes. Chanda (2014) also reported that the formation of AgNPs occurs in a few minutes using plant extract.

Iravani (2011) also stated the formation of the variable peak of AgNPs. The shape of the nanoparticles was amorphous, ranging from spherical to rectangular. The shape variation is due to synthesized particles' thick and condensed nature. Ocwieja & Adamczyk (2014) also reported the amorphous shaped images of AgNPs. The absorption bands at 2526, 2340, 2159, and 2030 cm^{-1} are related to carbonyl group (C=O) and amide (C≡N) stretching, confirming more involvement of biomolecules having C=O and few with C≡N functional groups. The peaks 1869, 1845 and 1829 cm^{-1} are associated with aldehyde (C=O) and alkenes (C=C) functional groups containing compounds. The peaks at 1541 and 1559 cm^{-1} are associated with C=C stretching. The peak at 668 cm^{-1} depicted the presence of an aromatic ring. The peak for the silver was found at 419 cm^{1} (Fig. 1e). According to Veeraputhiran (2013) the carbonyl group had a stronger ability to bind with metals and act as capping and stabilizing agents. Similarly, Zia *et al*., (2016) reported the involvement of carbonyl group-containing compounds, such as flavonoids and terpenoids from *Cydonia oblonga* seed extract behind reducing and capping silver metal to form silver nanoparticles.

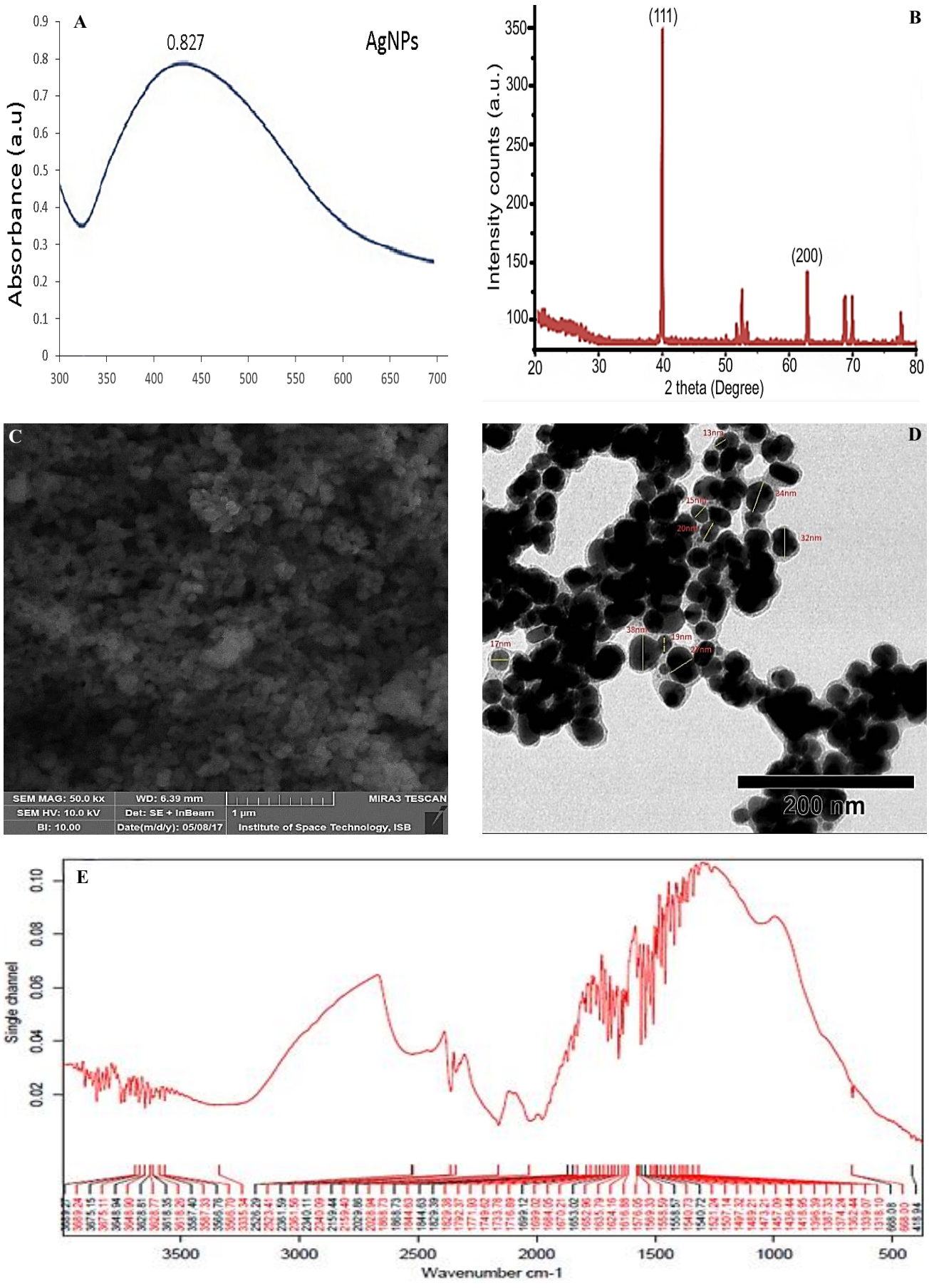

Fig. 1. Characterization of silver nanoparticles (AgNPs) by various techniques (A) UV-Visible absorption spectrum of synthesized AgNPs (B) XRD pattern of synthesized AgNPs (C) SEM micrograph of synthesized AgNPs (D) TEM images of synthesized AgNPs (E) FTIR analysis of synthesized AgNPs.

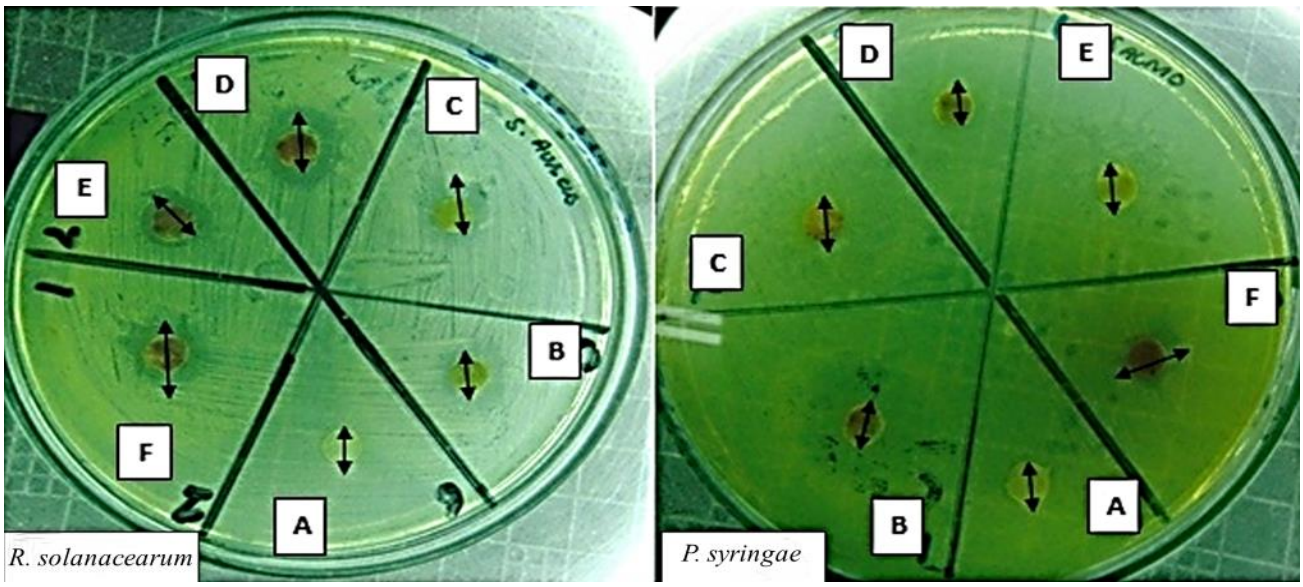

Fig. 2. Inhibition zone of *Ralstonia solanacearum* and *Pseudomonas syringae* (A=Control; B= Extract; C= 0.05 mg/ 100ul; D= 0.15 mg/ 100ul; E= 0.25 mg/ 100ul; F= 0.35 mg/ 100ul of AgNPs).

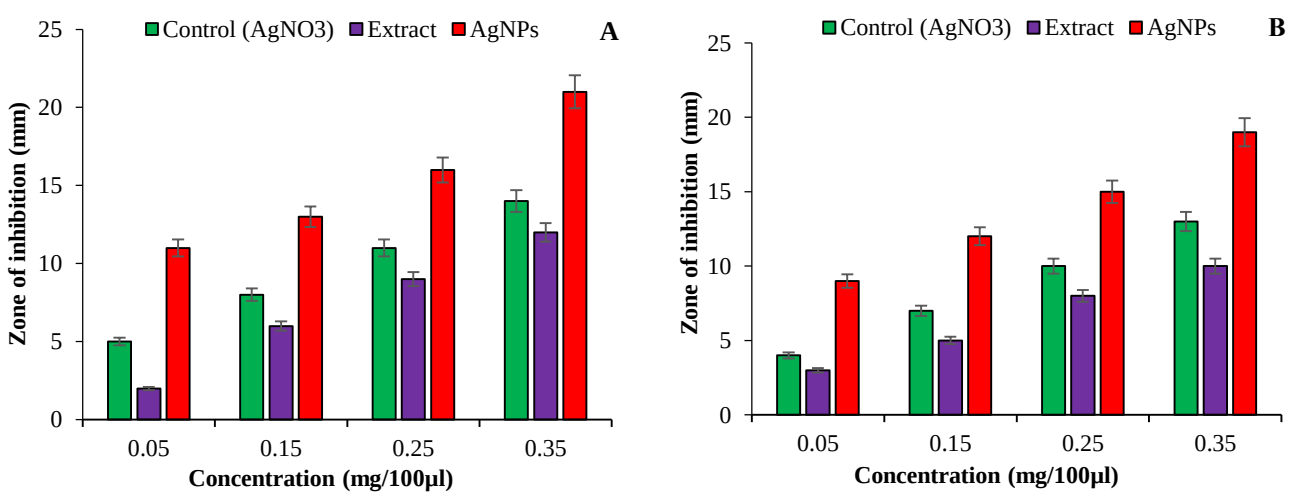

Fig. 3. Inhibition zone of (A) *Ralstonia solanacearum* and (B) *Pseudomonas syringae.*

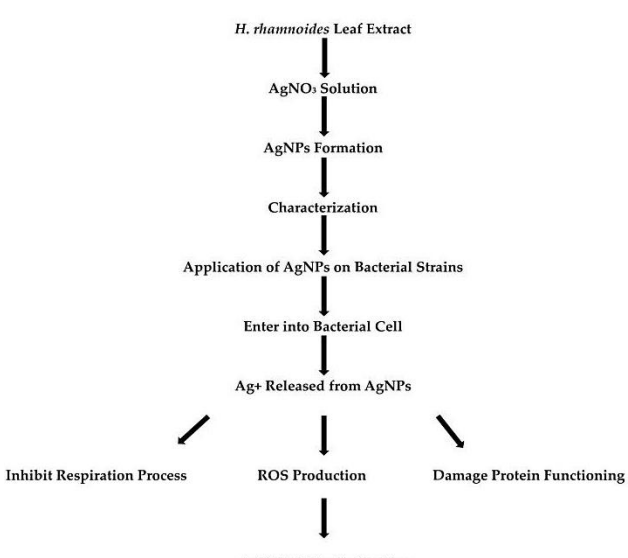

Fig. 4. Possible Mechanism of Antibacterial Activity of *H. rhamnoides* Mediated AgNPs.

In the present work, the maximum zone of inhibition (19 and 21mm) was noticed against *Ralstonia solanacearum* and *Pseudomonas syringae*. The results agree with Ghosh *et al*., (2012), who examined antibacterial activity of plant-based AgNPs in different concentrations against bacterial pathogens. The theoretical approach is projected for the antibacterial activities of phyto-synthesized AgNPs (Fig. 4). Small-sized AgNPs penetration ability is assumed to be much more inside bacterial cell membrane than large particles. These small particles release minute silver ions, which prevent DNA replication and protein formation by combining sulfur and phosphorus and ultimately cause cell death. According to Matsumura *et al*., (2003), these metal ions disorder respiration activity by disrupting the thiol group of NADH dehydrogenase enzymes. Similarly, Kim *et al*., (2007) explained that free radical released by AgNPs persuades cell death through oxidative stress.

Conclusion

The utilization of leaf extract of *Hyppophae rhamnoides* for the synthesis of stable silver nanoparticles was reported in this study. The synthesis's efficiency was exhibited in the context of biomolecules involved in the reduction of silver ions and increased constancy of the manufactured nanoparticles. Silver nanoparticles showed remarkable effectiveness against both *Ralstonia solanacearum* and *Pseudomonas syringae*. Moreover, green synthesis proves to be the eco-friendly and economical approach for NPs production, which might act as the best antibiotic agent in the future.

Conflict of interest

There is no issue between all co-authors regarding publication.

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