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Green fabrication of silver nanoformulation against plant pathogens and as eco-friendly surface disinfectant

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Abstract

In this manuscript we have shown the simple, one pot green synthesis of silver nanoparticles using neem leaves followed by their analytical characterization using UV-visible spectroscopy and Transmission Electron Microscopy (TEM). The as obtained silver nanoparticles were then formulated with different concentrations of Hydrogen peroxide (H_2O_2) and checked against different plant pathogens such as *Xanthomonas*, *Alternaria* and *Fusarium.* The combination of silver nanoparticles and H_2O_2 proved to be more fatal against above mentioned phytopathogens as compare to their nascent counterparts based on observed zone of inhibitions. Interestingly silver nanoparticles alone showed better activity as surface disinfectant and proved to be more potent in killing surface microorganisms as compare to traditional hydrogen peroxide.

Keywords: Silver nanoparticles, neem, hydrogen per oxide, anti-bacterial, anti-fungal, disinfectant.

Introduction

Nanotechnology is one of the most emerging transdisciplinary field in today's scientific world (Taniguchi, *1974*).The word 'Nano' is derived from Greek word which means 'dwarf' or 'very small' and Spanish word named as 'Nino' (Dowling, *2004*). Due to their extremely small particle size ranges from 1 to 100nm that is 1nm = 10^{-9}m (Taylor, *2001*). These Nanoparticles are synthesized and stabilized by different methods like physical, chemical, and biological (Iravani *et al., 2014*). Physical and chemical methods involve high pressure, temperature, hazardous chemicals which are toxic to humans, plants and environment. Synthesis of nanoparticles through biological systems by using Fungi, Bacteria, Actinomycetes and Algae etc. occurs at ambient temperature and pressure (Singh *et al., 2016*; Pantidose and Horsfall, *2014*). Green synthesis of nanoparticles has several advantages such as stability of nanomaterials, scalability, rapidness, simplicity and cost effectiveness (Zanjage and Khan, *2021*; Torresdey *et al., 2003*). In recent years the development of greenor plant based methods for the synthesis of metal nanoparticles has become a major focus of researchers. Different kindof plants such as *Jatropha curcas*, *Acalypha indica*, and *Alfalfa* etc.(Pentidos and Horsfall, *2014*; Bar *et al., 2009*; Krishnakunj *et al., 2010*; Torredey *etal., 2002*) have been used for the synthesis of different nanoparticles. Among plants leaf extract of '*Azadirachta indica'(Neem)* has been ideal choice for the synthesis of various nanoparticles, recently silver nanoparticles synthesized using neem leaves have been shown to

possess not only photocatalytic activity against organic dyes such as Congo red and Potassium permanganate but also antibacterial properties against both gram positive and gram negative bacteria. (Zanjage and Khan, *2021*). It has been reported that the flavonoids and terpenoids present in neem extract may act as reducing agents during synthesis (Roy *et al., 2017*).

In Maharashtra state of India, *Xanthomonas spp* has been major causative agent of bacterial leaf spot in crops such as chilly and pomegranate causing 52% weight loss (Chapke *et al.,2020).* It also infects tomatoes (Dougherty, 1978) and many citrus fruits like Lemon, Orange, Lime, etc. (Md. Nurul Islam *et al., 2019*). Another phytopathogen *Fusarium,* is filamentous fungus widely distributed in plants and soils, causing *Pokkah Boeng* disease of Sugarcane (Vishwakarma *et al., 2013*) it also causes dry rot, crown rot, head blight and scab on cereal grains. *Alternaria spp.* is another economical phytopathogen which is responsible for causing diseases in pomegranate. Since India is the largest producer of pomegranate and Maharashtra is the leading state covering about 68.7% area of cultivation it is important to look for remedies against this phyto pathogen. Phytopathogens are responsible about 10-40% losses in productivity, quality and yields of food crops (Rajwade *et al., 2020*) The increasing challenge to control these phytopathogens require not only the discovery of new biopesticides but also the development of new alternatives.

In this manuscript we have synthesized silver nanoparticles using neem extract and these nanoparticles were formulated along with hydrogen peroxide to obtain a stable nano-peroxide formulation which was used for inhibition of strong phytopathogens such as *Xanthomonas spp*, *Alternaria* and *Fusarium.* Moreover silver nanoparticles also shown to possess surface disinfectant activity against normal flora and has been shown more potent in killing them as compare to hydrogen peroxide.

Materials and Methods

Materials:

Silver nitrate ($AgNO_3$) was procured from Merck and served as the precursor salt in the preparation of 10^{-3} M stock solution in distilled water. Neem leaves were collected from nearby tree. Hydrogen peroxide purchased from Merck & served as activity enhancer in the different concentrations.

Methods

Biological synthesis of silver nanoparticles:

Synthesis of silver nanoparticles were carried out by previously described method with slight modification (Zanjage and Khan, *2021*).The synthesis of silver nanoparticles was carried out by collecting fresh neem leaves from neem tree located at Bafna farm, Pune. 20 gm of neem leaves were weighed & washed thoroughly using RO water for 2-3 times. The washed neem leaves were then suspended in 300ml of distilled water in 500ml Erlenmeyer flask. The neem extract was prepared by autoclaving the flask at 121^0C for 20 min. As soon as the sterilization process was over the flask was taken out 100 ml neem extract was filtered and added to previously prepared 1mM silver nitrate (900ml) precursor solution in warm condition. As soon as the neem extract was added to the silver nitrate solution the color of the silver nitrate was changed from colorless to dark brown indicating the spontaneous synthesis of silver nanoparticles just after mixing, which was later confirmed by UV-Visible Spectroscopic measurements.

Characterization of silver nanoparticles:

UV-Visible spectroscopy:

The bio reduction of $AgNO_3$ in solution was monitored by periodic sampling of aliquots (3 ml) of the aqueous component and measuring the UV-Visible spectra of the solution. Due to the high optical density of the nanoparticle solution, up to 3 times dilution was often required before the analysis. UV-visible spectra of these aliquots were monitored as function of time of reaction using Shimadzu 1800 series (Double beam) UV-visible spectrophotometer operated at a resolution of 1nm.

Transmission Electron Microscopy (TEM):

Samples for TEM analysis were prepared by drop coating biosynthesized AgNPs solution on carbon-coated copper TEM grids. The film on the TEM grid was allowed to stand for 2 minutes, after which the extra solution was removed and the grid was allowed to dry prior to measurement. TEM measurements were performed on a FEI Technai G2 system operated at an accelerating voltage of 200 kV at room temperature.

Antibacterial & Antifungal activity:

The bacterial culture of *Xanthomonas* & fungal plant pathogens *Alternaria spp*. & *Fusarium spp.* were used for the antimicrobial & antifungal activity respectively. These cultures were isolated from soil of Bafna Farm, Pune and maintained in our research lab. The antibacterial activity of AgNPs, only H_2O_2 & AgNPs + $H_2O_2$was evaluated by well diffusion method (Magaldi *et al., 2004*). The bacterial cultures were inoculated in Nutrient broth & Fungal cultures were incubated in PDB broth (potato Dextrose Broth)at 37˚C for 24 -48 h & 27˚C for 48-72 respectively. From the actively growing bacterial & Fungal culture broth, 0.1 ml of suspension was overlaid on the surface of sterile nutrient agar plates. Awell with a diameter of 6-8mm is punched aseptically with a sterile cork borer and 100 µl of Ag NPs (200µg/ ml), hydrogen peroxide (0.5,2 & 4 %) & combination of Ag NPs & hydrogen peroxide (0.5,2 & 4 %) was added into the well. Plates with AgNPs alone & Hydrogen peroxide alone served as control. Bacterial plates were incubated for 24- 48 hours and fungal plates were incubated at 28-30˚C for 48-72 hours and zones of inhibition were observed.

Disinfectant Activity of Silver Nanoparticles:

For this experiment water & different concentrations of hydrogen peroxide served as a control. The swab was taken from floor from one of our microbiological lab and added into water, 0.3 % , 0.5 % , 1 % and 2 % H_2O_2 solution and silver nanoparticle solution (1 mg / 5ml). All the samples were properly vortexed for 5 min and 100 µl of each sample was spread separately on sterile NA plates. The plate samples were observed at 0 h , 12 h and 24 h and microbial colonies were recorded for each sample.

Results and Discussion

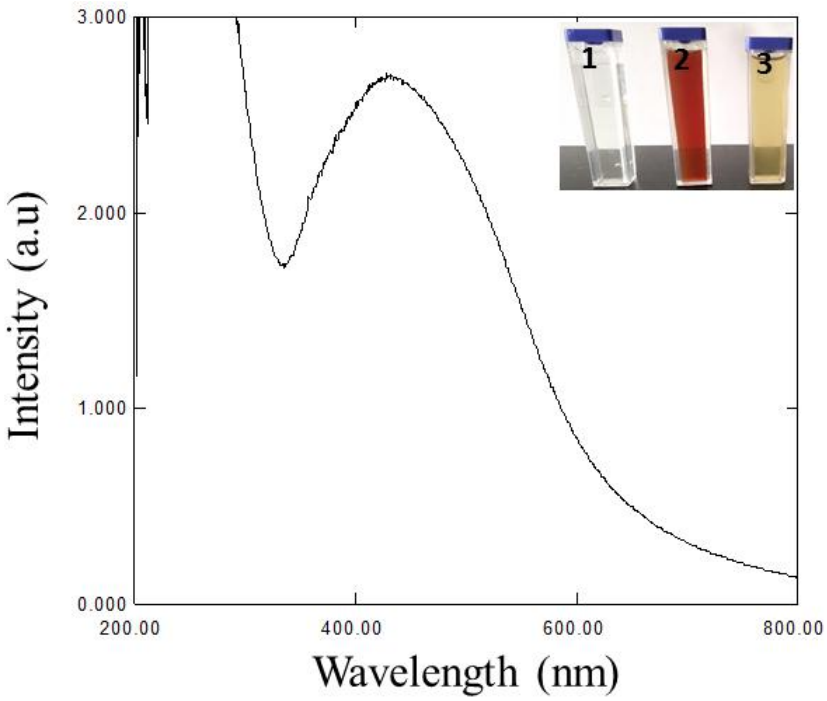

Figure 1: UV-visible spectrophotometric analysis of silver nanoparticles synthesized by neem extract as a function of time. Inset shows silver nitrate precursor solution (vial 1), as synthesized silver nanoparticles (vial 2) and neem extract (vial 3).

Figure 1 represents the UV-visible spectroscopic analysis of Neem extract mediated synthesis of silver nanoparticles as a function of time. From the figure synthesis of silver nanoparticles can be confirmed from the color change and occurrence of sharp SPR band at 430 nm.The change in the colour from colourless to dark brown indicates the formation of silver nanoparticles. Inset in Figure 1 silver nitrate precursor solution, synthesized silver nanoparticles (vial 2) and neem extract (vial 3). The brown colour

arises because of the excitation of surface Plasmon vibrations in the silver nanoparticles (Mulvany, *1996*). After filtration, it was observed that the filtrate solution retained the brown colour hence it can be said that the synthesis and reduction of the Ag+ ions takes place extracellularly. It was observed that the surface plasmon band centered at ~430 nm that corresponds to the surface plasmon resonance of silver nanoparticles (Mulvany, *1996*). Different types of plant molecules such as flavonoids, terpenoids, alkaloids etc. are reported for the reduction and stabilization of silver nanoparticles (Thirumurugan *et al., 2016*; Resmi *et al., 2014*).

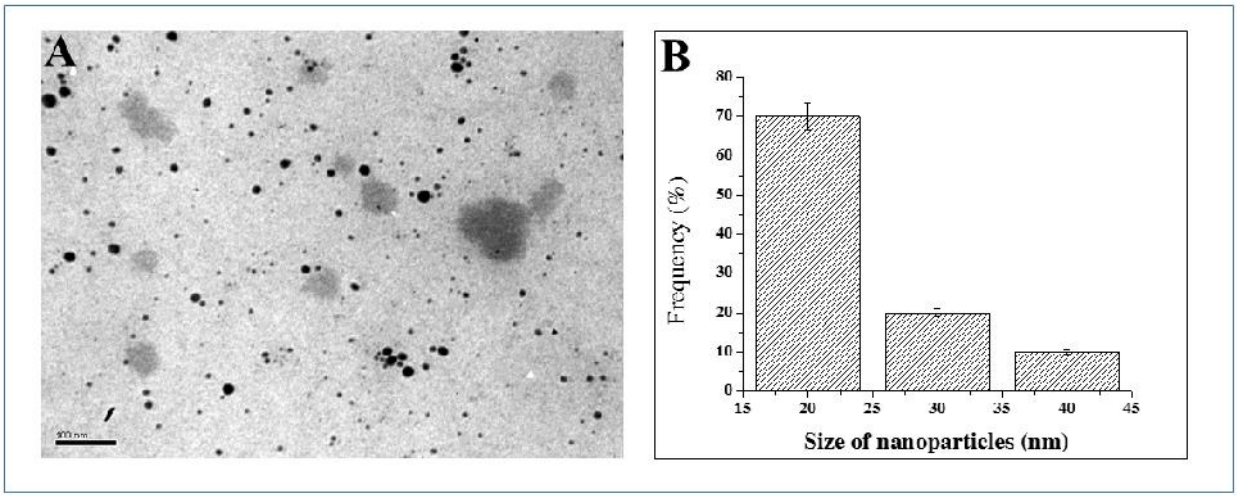

Figure 2: Transmission Electron Microscopic image of silver nanoparticles (A), Particle size distribution of as synthesized silver nanoparticle(B).

Figure 2A. represents the transmission electron microscopic (TEM) image of neem extract mediated synthesis of silver nanoparticles. The particles are mostly monodispersed in shape. These nanoparticles are stabilized by stabilizing molecules present in the neem extract (Thirumurugan *et al., 2016*; Resmi *et al., 2014*). These molecules capped the as synthesized silver nanoparticles and render them monodisperse in solution and prevent their aggregation. Particle size distribution analysis of silver nanoparticles (Figure 2B) confirmed that the nanoparticles are in the range of 20-40 nm with an average size of 20nm.

Figure 3.A. represents antibacterial activity of silver nanoparticles, H_2O_2 & formulation of silver nanoparticles and H_2O_2 against *Xanthomonas spp* (gram negative bacteria). Panel 1 in Figure 3A shows three plates of silver nanoparticles (200µg/ml, plate 1), H_2O_2 (0.5%, plate 2) and Nps+H_2O_2 (0.5%, plate 3). Panel 2 in Figure 3A shows three plates of silver nanoparticles (200µg/ml, plate 4), H_2O_2 (2%, plate 5) and Nps+H_2O_2 (2%, plate 6). Panel 3 in Figure 3A shows three plates of silver nanoparticles (200µg/ml, plate 7), H_2O_2 (4%, plate 8) and Nps+H_2O_2 (4%, plate 9). The zone of inhibitions has been given in Table No. 1. From the Figure 3A and Table No 1 it is clear that formulation of silver nanoparticles and hydrogen per oxide in different concentrations is certainly more potent and active against *Xanthomonas spp*. as compared to nanoparticle and hydrogen per oxide alone. The combined effect of AgNPs&H_2O_2has been reported to be more effective & long lasting. (Mahmoud *et al., 2019*).Figure 3B represents antifungal activity of silver nanoparticles,H_2O_2 & formulation of silver nanoparticles and H_2O_2 against *Alternaria spp*. Panel 1 in Figure 3B shows three plates of silver nanoparticles (200µg/ml, plate 1), H_2O_2 (0.5%, plate 2) and Nps+H_2O_2 (0.5%, plate 3). Panel 2 in Figure 3B shows three plates of silver nanoparticles (200µg/ml, plate 4), H_2O_2 (2%, plate 5) and Nps+H_2O_2 (2%, plate 6). Panel 3 in Figure 3B shows three plates of silver nanoparticles (200µg/ml, plate 7), H_2O_2 (4%, plate 8) and Nps+H_2O_2 (4%, plate 9). The zone of inhibitions for all plates has been given in Table No. 1. From the Figure 3B and Table No 1 it is clear that formulation of silver nanoparticles and hydrogen per oxide in different concentrations is definitely playing a role in inhibition of *Alternaria spp* fungus as compare to their nascent counter parts. Ahmed I. El- Batal and et al also reported antifungal activity against *Alternaria* using silver & selenium nanoparticles, by Agar overlay or Immersion bioautography method (Abdel-Wahab *et al., 2016*)

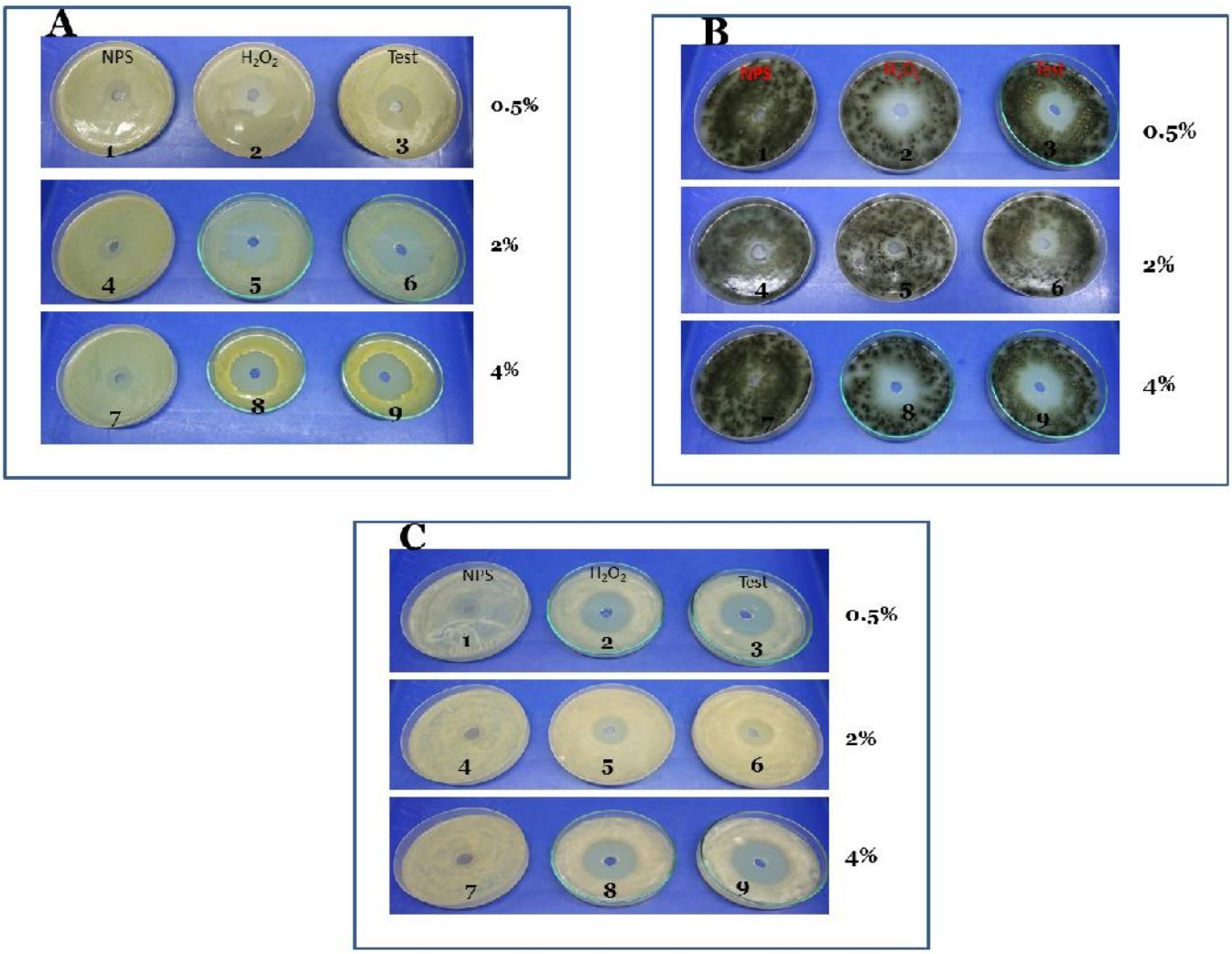

Figure 3: Antimicrobial activity of as synthesized silver nanoparticles against, *Xanthomonas* (A), *Alternaria spp*.(B) and *Fusarium spp* (C).

Figure 3C represents antifungal activity of silver nanoparticles,H_2O_2 & formulation of silver nanoparticles and H_2O_2 against *Fusarium spp*. Panel 1 in Figure 3C shows three plates of silver nanoparticles (200µg/ml, plate 1), H_2O_2 (0.5%, plate 2) and Nps+H_2O_2 (0.5%, plate 3). Panel 2 in Figure 3C shows three plates of silver nanoparticles (200µg/ml, plate 4), H_2O_2 (2%, plate 5) and Nps+H_2O_2 (2%, plate 6). Panel 3 in Figure 3C shows three plates of silver nanoparticles (200µg/ml, plate 7), H_2O_2 (4%, plate 8) and Nps+H_2O_2 (4%, plate 9). The zone of inhibitions for all plates has been given in Table No. 1. From the Figure 3C and Table No 1 it is clear that formulation of silver nanoparticles and hydrogen per oxide in different concentrations is more toxic in preventing *Fusarium* growth as compare to silver nanoparticles and hydrogen per oxide alone. Hydrogen per oxide has long been used for its oxidizing capabilities but its unstable nature has restricted its applications to a greater extent. Silver nanoparticles can be an ideal choice as stabilizing and activating agent, moreover silver nano itself has promising applications as antibacterial and anti-fungal agent. We assume that nascent oxygen from hydrogen peroxide attacks cell walls of microorganisms upon direct contact. Nascent oxygen denatures the cell wall and disrupts cytoplasm stability. This effect is boosted by silver ions that disrupt protein activities associated with reproductive and metabolic systems and deactivates them resulting in rapid cellular degradation and microbial death.

Table No.1. Observed zone of inhibitions of antimicrobial activity of silver nanoparticles,only H_2O_2 and silver nanoparticles + H_2O_2 against for *Xanthomonas, Alternaria and Fusarium*.

Zone of Inhibition in mm			
Samples	***Xanthomonas* spp.**	***Alternaria* spp.**	***Fusarium* spp.**
NPS	23	No zone	No zone
0.5% H_2O_2	35	15	24
0.5% Test	38	23	25
2% H_2O_2	41	26	36
2% Test	45	30	38
4% H_2O_2	40	25	36
4% Test	42	30	40

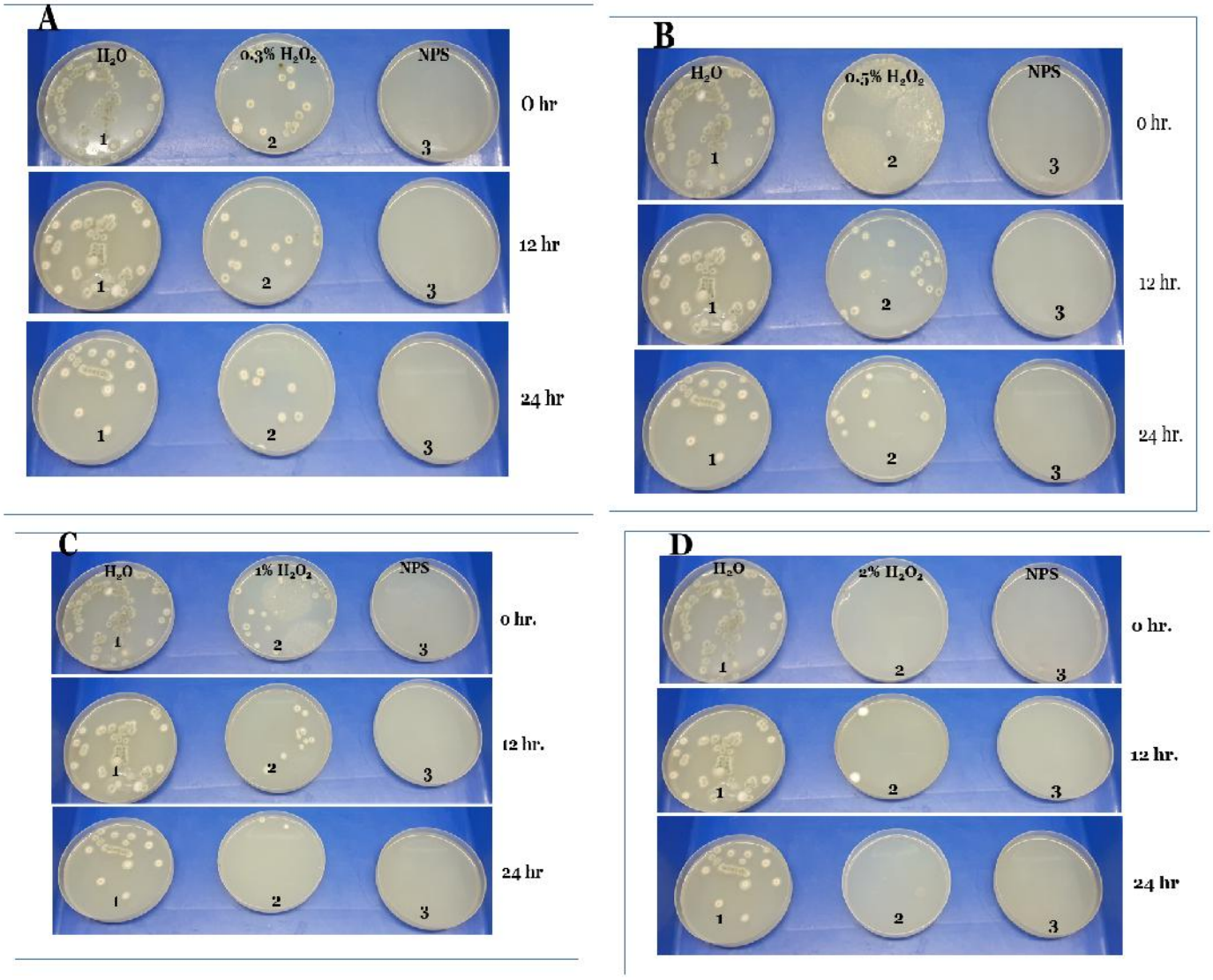

Figure 4: Disinfectant activity of silver Nano particles against general lab contaminants in comparison with H_2O_2 with 0.3 % (A), 0.5 % (B), 1 % (C) and 2 % (D).

Figure 4A,B, C and D represents the disinfectant activity of silver nanoparticles in comparison with hydrogen peroxide with different concentrations viz. 0.3%, 0.5%, 1% and 2% respectively against general lab contaminants at 0h,12h and 24h. Plates 1 & 2 in each panel of the figure were spread with water and H_2O_2 respectively, plate 3 contains silver nanoparticles sample. From the figure 4A, B, C&D, it is clear that the NPS shows better result as a disinfectant against general lab contaminants as compare to traditional hydrogen peroxide disinfectant. It is important to note that silver nanoparticles inhibited the microbial growth from 0h and prevented upto 24h. The no. of colonies counted in each plate has been given in Table no.2. Traditionally hydrogen peroxides, bleach formulations and alcoholic disinfectants are being used in different fields like microbial labs, hospitals and domestic purpose (Bashir *et al., 2011*).These disinfectants may cause allergic reactions to susceptible persons hence eco-friendly, green synthesized silver nanoparticles could be an alternative for disinfectant purpose for cleaning wood and floor surfaces.

Table No.2. Total number of microbial colonies counted in each plate for disinfectant activity of as synthesized silver nanoparticles.

Number of microbial colonies counted in each plate			
Samples	**0 Hours**	**12 Hours**	**24 Hours**
H_2O	84	6	25
0.3% H_2O_2	18	13	8
1mg/5ml NPS	Nil	Nil	Nil
0.5% H_2O_2	8	18	10
1mg/5ml NPS	Nil	Nil	Nil
1% H_2O_2	26	9	2
1mg/5ml NPS	Nil	Nil	Nil
2% H_2O_2	2	9	1
1mg/5ml NPS	Nil	Nil	Nil

Conclusion

In this manuscript, we have synthesized silver nanoparticles using our previously described method with slight modification and characterized them. Further we have formulated as synthesized silver nanoparticles with different concentrations of H_2O_2 in order to check them against phyto pathogens such as *Xanthomonas*, *Alternaria* and *Fusarium*. We found that the prepared formulation was quite effective in inhibiting the afore mentioned phyto pathogens. We have also tested the efficiency of these silver nanoparticles as disinfectant against general microflora in comparison with conventional H_2O_2 and found them to be superior to H_2O_2 in controlling general microflora of the lab. This work has further opened up new doors for applications of ecofriendly silver nanoparticles not only in agriculture sector but also in domestic chores.

Conflict of Interest

The authors have declared no conflict of interest.

Acknowledgments

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