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Antifungal and antioxidant activities of Ag@FeO-NPs@Chitosan preparation by endophyte *Streptomyces aureofaciens*

Haggag, W. M.[1] and Eid, M. M.[2]

[1]Plant Pathology Department, National Research Centre, Dokki, Egypt; [2]Spectroscopy Department, Physics Division, National Research Centre, Dokki, Giza, Egypt.

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Abstract Biological control using beneficial microorganisms is nowdeveloped for reducing the plant pathogens. Nanoparticles with specific functions have been produced and represented an economical alternative to agochemical through the natural methods of nanoparticle formation. Biosynthesis of Ag@FeO-NPs@Chitosan preparation by *Streptomyces aureofaciens* was performed against some soil borne pathogenic fungi i.e. *Fusarium oxysporum* , *F. solani, F. subglutinans, Rhizoctonia solani* and air borne pathogenic fungi *i.e. Botrytis cinerea*, *Colletotrichum gloeosporioides*, *Alternaria solani* and *Aspergillus niger* . To conceive the formation of Ag@FeO-NPs@Chitosan, ultraviolet-visible (UV-Vis) spectroscopy for the biosynthesis of core–shell nanoparticle and fourier transform infrared (FTIR) spectroscopy analysis were done for constructing the structure of nano-composite. Scanning transmission electron microscopy (STEM), and scanning transmission electron microscopy (STEM) micrographs evidenced that the size of synthesized nanoparticles is less than 50 nm. As a result, the data showed that the produced core–shell hemtite @Ag nanoparticles are super-paramagnetic. The chitosan is formed in the outer shell of the formula spontaneously due to the presence of chitin in the fungus cell membrane. *In vitro* bioassay, Ag@FeO-NPs@Chitosan preparation by *Streptomyces* showed distinct antifungal against all plant pathogens, and inhibited the growth and spores germination. Antioxidant activities as reducing power measurement, DPPH (1,1-Diphenyl-2- picrylhydrazyl) radical scavenging activity, nitric oxide scavenging activity, ABTS free radical scavenging activity and total phenolics contents were greatly increased. Synthesized Ag@FeO-NPs@Chitosan preparation by *S. aureofaciens* is demonstrated remarkable potential for using as antifungal compound to combat plant diseases. Chitosan is a good source for the preparation of bionanoparticle materials for industry and applications.

Keywords: Ag@FeO-NPs@Chitosan, *Streptomyces aureofaciens*, Antifungal, Antioxidant

Introduction

The main focus of attention in agricultural production is fungal plant diseases. The majority of disease control is depended on the use of hazardous chemical fungicides. Recent study has revealed the possibility of using biological control to manage plant diseases (Haggag and Ali, 2019).

* **Corresponding Author:** Haggag, W. M.**; Email:**Wafaa_haggag@yahoo.com

Streptomyces strains have been identified as potential biocontrol agents and used as plant growth promoters against a variety of soil and airborne plant pathogens. They can also produce chemical and pharmaceutical compounds that are antifungal, antimicrobial, antiviral, and anti-inflammatory (Kekuda, *et al*., 2010, Mei and Flinn, 2010; Mercado-Blanco and Lugtenberg, 2014, Fei Law *et al.,* 2017). Many secondary metabolites are produced by endophytic actinomycetes. *Streptomyces aureofaciens* promotes plant growth, an is the most important species of *Streptomyces* strains capable of producing antioxidants, extracellular enzymes, and elicit and induced resistance (Haggag and Malaka, 2012). *Streptomyces* has strongly demonstrated as antioxidant activity in a variety of in vitro tests, including reducing power, DPPH (1,1-Diphenyl-2-picrylhydrazyl) radical scavenging activity, nitric oxide scavenging activity, ABTS (2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) free radical scavenging activity, and total phenolics content (Kawahara *et al.,* 2012 and Nanjundan *et al*., 2014).

Nanoparticles (NPs) are one of the most essential tools for generating biocontrol products, having been used in biosensors, diagnostics, targeted medication delivery systems, and biocontrol production. Several researchers have reported on the function of nanoparticles in plant disease management, including their characterizations, impacts, and applications (Lee *et al*., 2014 and Farhat *et al*., 2018). Magnetic NPs have been widely employed in cell sorting, MRI, DNA isolation, medication administration, hyperthermia treatment, and tumor tracking. Magnetite nanocrystals have been reported to be the most widely used magnetic NP in biomedical applications due to their biocompatibility and chemical stability (Yue and Zhang, 2012, Min *et al.,* 2011 and Sureshkumar *et al*., 2016). The use of silver nanoparticles as a pesticide has received a lot of attention (Bhattacharyya *et al.,* 2016). Physiological and chemical changes can occur when nanoparticles are applied to plants, depending on the shape and concentration of the nanoparticles (Tripathi *et al.,* 2017). Through antifungal activity, nanoparticles (NPs) can be employed to directly reduce pathogen infection, resulting in increased crop growth and yield after applying nanoparticles such as Ag, ZnO, Mg, Si, and TiO2 (Liu and Lal, 2015). The antifungal and antioxidant effects of *S. aureofaciens* extract and the Ag@FeO-NPs@Chitosan composition against various economically important phytopathogenic fungi were investigated in this study.

Materials and methods

Nano preparation

Iron (III) chloride-hexahydrate ($FeCl_3\ 6H_2O$), Ammonium iron (II) sulfate hexahydrate ($NH_4)\ 2Fe\ (SO_4)_2\ 6H_2O$ and $AgNO_3$ were purchased from Merck (Germany) and received without any purifications.

Production of microbial biomass

The effective microorganism, *Streptomyces aureofaciens* was used in this study (Haggag and Malaka, 2012)**.** The method has been explained in previous papers (Eid *et al.*, 2018 and , Osman *et al*., 2015), briefly, *S. aureofaciens* was incubated into flasks containing potato dextrose broth were inoculated at 25˚C, under shaking condition (180 rpm) for 72 h for 5 days.

Intracellular synthesis of Ag@ FeO NPs functionalized with chitosan

The structure approach is based on the precipitation of Ag-atoms onto the seeds of FeO NPs in a growth solution containing iron oxide nanoparticles (FeO NPs) and the monitoring of Ag portent reduction (Eid *et al*., 2019). Under purifier circumstances, *Streptomyces* filtrate was carefully isolated. Ag NP seeds were prepared in the following manner with vigorous shaking at 37 ℃, 2 mM AgNO3 was added to 50 ml fungal filtrates. FeO NPs seeds were dissolved in the following solution: 5 mM FeCl3 and 2.5 mM Fe(SO4)2 were combined in 50 ml sterile distilled water in a clean Erlenmeyer flask under vigorous shaking at 80 ℃, with the pH adjusted to 12.5 for the three samples. Both solutions were combined for 72 hours at 37 degrees Celsius under vibration. The core–shell formation was studied using UV/Visible and FTIR spectroscopy after the nanoparticles were washed (Waldron, 1955). Due to the presence of chitin (the deacetylated form of chitosan in the cell wall of the fungus), the functionalization of chitosan active groups on the surface of the nano-composite is accidental in the reaction technique; however, this finding is confirmed through the results and interpretation of the ATR-FTIR data (Casillas *et al.*, 2012).

Characterization of nanoparticles

The optimal conditions for the biogenesis of core–shell nanoparticles were discovered using a UV–visible absorption spectrophotometer (JASCO V-630) with a resolution of 0.2 nm (Park *et al*., 2020). Liquid samples were diluted in distilled water at a 1:4 ratio and scanned at wavelengths ranging from 200 to 800 nm. In order to understand the technique of preparation, the chemical structure of the nano-composite and the cover layer that covers the nanoparticles were analysed using Fourier transform infrared (FTIR) spectroscopy. Diamond cell was used to obtain the transmission spectrum for the MID-Far range. The spectra were captured with a 1 cm1 resolution on a Vertex 70 Bruker Transform Infrared Spectrophotometer (Negrea *et al.*, 2015 and Kumirska *et al*., 2010).

In vitro screening of antifungal properties

Agar well diffusion assay

The Ag@FeO-NPs@Chitosan composition was tested against the isolated plant pathogens (Plant Pathology Department, National Research Centre, Egypt) such as *Fusarium oxysporum*, *Fusarium solani*, *Fusarium subglutinans*, *Rhizoctonia solani*, and air borne pathogenic fungi such as *Botrytis cinerea*, *Colletotrichum gloeosporioides*, *Alternaria solani* (Hongtao *et al*., 2013). Streptomyces cell-free culture was also used as a control treatment.

Colony formation Unit (CFU)

The antifungal activity of Ag@FeO-NPs@Chitosan against plant pathogens was assessed *in vitro* by counting Colonies Forming Units (CFU) on plates (Miller, 1972). After a 10-day incubation period at 25 ℃, fungal spores were collected. Their concentration was adjusted to 10^5 CFU/ml, and they were mixed with successive concentrations of Ag@FeO-NPs@Chitosan (50, 100, and 500 mg/l). After 10-15 days of incubation at 25 ℃, the number of CFUs of pathogens was counted. After five days of culture, *in vitro* germination and sporulation of tested pathogens were recorded using ten duplicates (Bautista-Baos *et al.,* 2000) and compared to media PDA.

Antioxidant assay

Reducing power

The reducing power measurement was determined according to a method of Oyaizu (1986). Absorbance was measured at 700 nm using spectrophotometer.

Diphenyl-1-picryl hydrazyl activity (DPPH) assay

Yen and Wu (1999) described a method for determining the activity of Diphenyl 1 picryl hydrazyl (DPPH) of produced Ag@FeO-NPs@Chitosan. The absorbance was measured using a UV–visible spectrophotometer at 517 nm and calculated using the formula:

$$(\%) = 1 - \frac{\text{Abs Sample}}{\text{Abs Control}} \times 10$$

Nitric oxide (NO) scavenging activity

The scavenging activity of nitric oxide was determined using the Marcocci *et al* (1994) technique. Nitric oxide scavenging was measured at 540 nm with sodium nitrite salt standard solutions.

ABTS free radical scavenging assay

ABTS free radical scavenging assay was done using the method by Zhishen *et al*. (1999). The absorbance was measured at 734 nm by microplate reader.

Total polyphenols

Total polyphenols were determined by the Folin Ciocalteu spectrophotometric method (Folin and Ciocalteu, 1927).

Statistical analysis

The efficiency of all trials was analyzed with ANOVA and means was compared with the least significance difference (LSD) test using SAS statistics.

Results

Synthesis and Characterization of Ag@ FeO NPs

The transmission FTIR (Figure 1 a, b) was utilized to evaluate the molecular structure of the nano-composites, confirmed the iron oxide's formation, and determined the iron oxide's oxidation state. As a result, vibrations at 1617, 1426, 1372, and 1217 cm-1 are attributed to O–H or N–H stretching, amide II stretching vibration, and amide I and III bending vibration in the 1800-400 cm-1 range. Chitin glycogen and the Fe–O stretching vibration band are allocated to the C–O and C–C vibration bands at 1148, 984, and 547 cm-1, respectively. The creation of the Fe3O4 oxidation phase of Fe-O was confirmed by data. The Fe3O4 @Ag samples are seen in Fig 1b. The hysteresis curves indicated no hysteresis, and the coercivity was almost non-existent. As a result, the data showed that the forward and backward magnetization curves overlap.

The results also showed that as the concentration of iron salt increased, the magnetization saturation value increased. As a result, the created core–shell hemtite @Ag nanoparticles were super-paramagnetic, according to the findings. The production of spherical nanoparticles can be seen in HRTEM in Fig. 1c, d. It also showed that the particles are smaller than 50 nanometers. The crystallographic planes of 111, 200, 220 and 311 for the face-centered cubic of the silver crystal are represented by the observed diffraction peaks at 38, 44, 64, and 77 in the Fe3O4/Ag XRD pattern, respectively (JCPDS no. 04-0783). The 220, 311, 511 and 440 planes, which were attributed to reddish crystalline Fe3O4 respectively, which correlated to the peaks at 33, 35, 53, and 64 (JCPDS No. 39-1346).

In vitro screening of antifungal properties

Agar well diffusion assay

At varying doses of Ag@FeO-NPs@Chitosan demonstrated the most action against *F. subglutinans*, *F. oxysporum*, *C, gloeosporioides*, and *R. solani* (Figure 1). *F. subglutinans*, *R. solani*, *F. oxysporum*, and *C. gloeosporioides* were all resistant to the crude cell free culture.

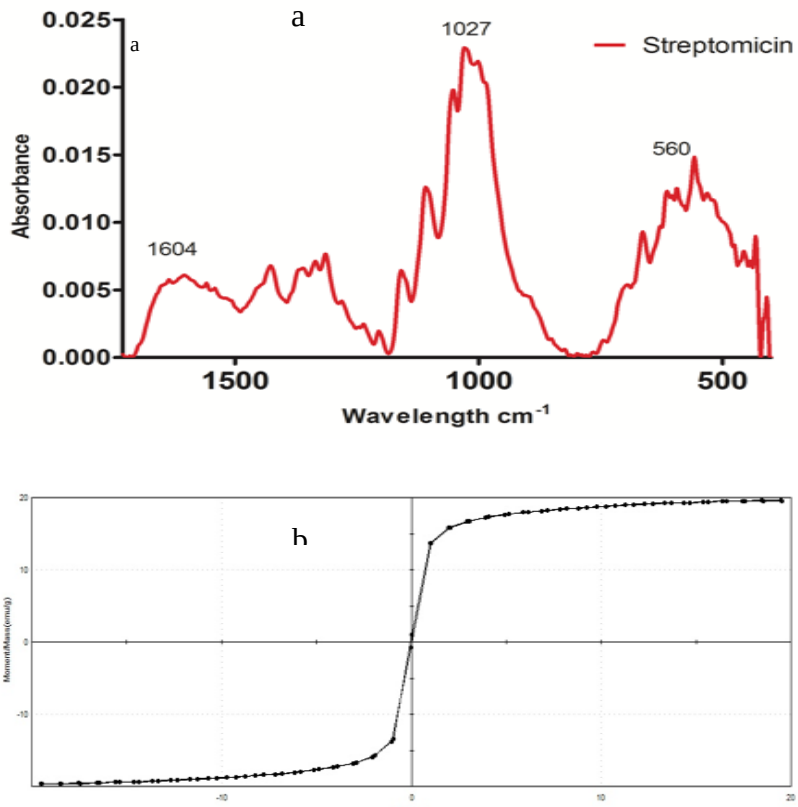
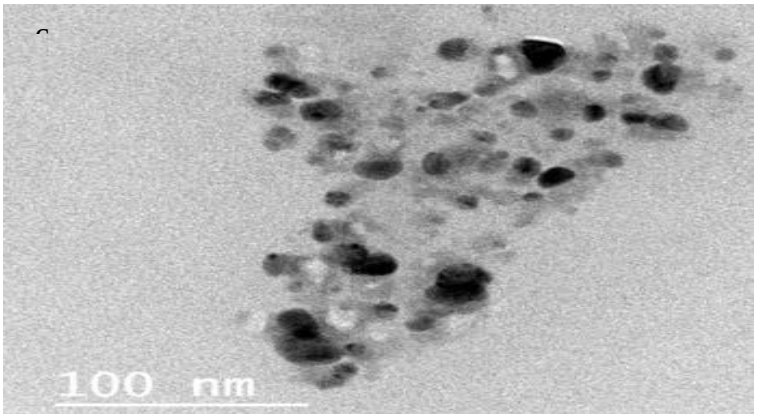


Figure 1. Tthe physico-chemical properties of Fe3O4@Ag@chitosan formed by through the green route using the fungus streptomyces griseus. A) the transmission FTIR in the range mid region (from 1800-400 cm^{-1}), b) the hysteresis curve, c) the HRTEM, d) the XRD

Colonies Forming Units (CFU)

Colonies Forming Units (CFU) counting was used to assess antifungal efficacy based on Ag@FeO-antifungal NPs@Chitosan's activity against plant pathogens (Table 1). At three concentrations, the Ag@FeO-NPs@Chitosan were found to be effective inhibitors of all tested pathogens. *F. subglutinans*, *F. oxysporum*, *C. gloeosporioides*, and *R. solani* were the most effective. *F. subglutinans*, *R. solani*, *F. oxysporum*, and *C. gloeosporioides* were all resistant to the crude cell free culture. *In vitro*

assessments of Ag@FeO-NPs@Chitosan at three concentrations (50, 100, 500 mg/l) and crude cell free culture of *S. aureofaciens* on pathogen germination after 10-15 days incubation at 25 ℃ is shown in Table 2. There were significantly differences (P 0.05) between treatments, indicating excellent pathogen germination inhibition. The Ag@FeO-NPs@Chitosan treatment at a dosage of 500 mg/l resulted in the greatest sporulation reduction. Ag@FeO-NPs@Chitosan 500 mg/l was shown to be the most efficient, and causing total germination inhibition in all plant pathogens except *A. niger* (98.8% inhibition).



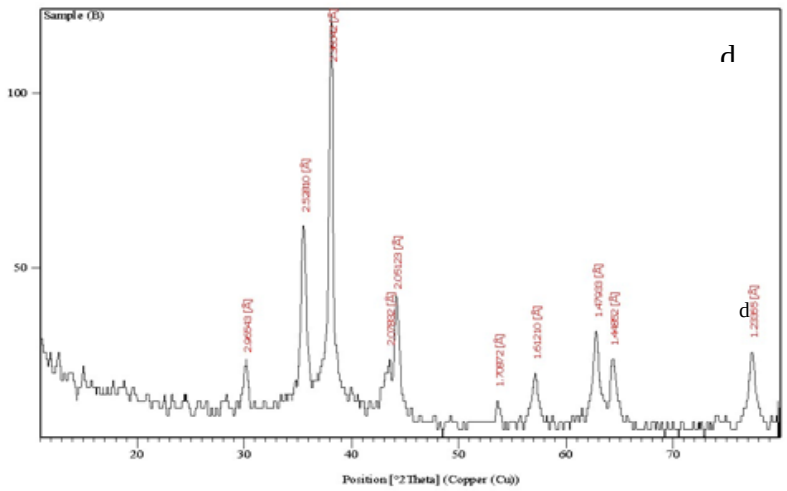


Figure 1(Cont.). The physico-chemical properties of Fe3O4@Ag@chitosan formed by through the green route using the fungus streptomyces griseus. A) the transmission FTIR in the range mid region (from 1800-400 cm^{-1}), b) the hysteresis curve, c) the HRTEM, d) the XRD

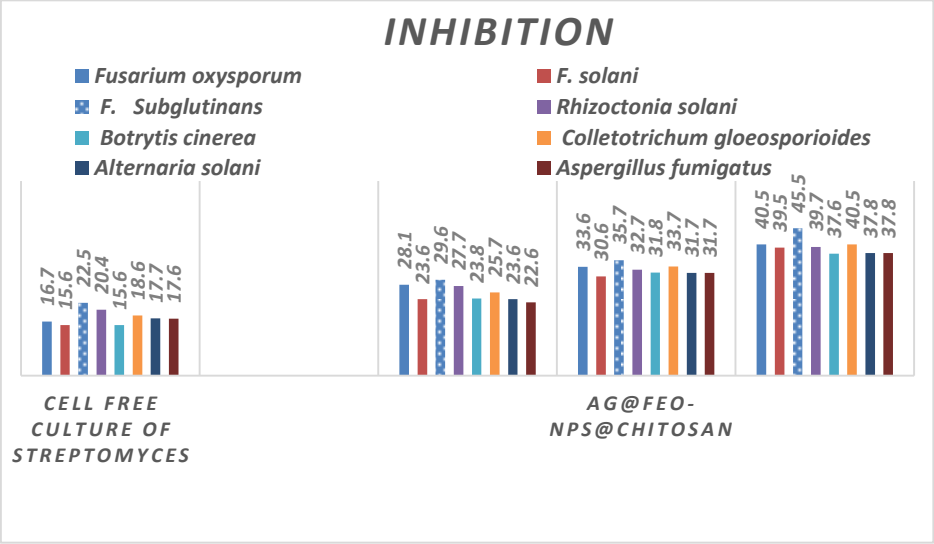


Figure 2. Inhibition zones dimeter of test plant pathogens (mm) with Fe3O4@Ag@chitosan and crude cell free culture of *Streptomyces aureofaciens*

Table 1. Colony formation Unit (CFU) of test plant pathogens with Fe3O4@Ag@chitosan and crude cell free culture of *Streptomyces aureofaciens*

Plant Pathogens	**Cell Free Culture**	**Ag@FeO-NPs@Chitosan**		
		50 mg/l	**100mg/l**	**500 mg/l**
F. oxysporum	$4X10^4$	$2X10^6$	$3X10^9$	$5X10^{10}$
F. solani	$2X10^4$	$4X10^5$	$4X10^7$	$4X10^9$
F. Subglutinans	$6X10^4$	$5X10^5$	$2X10^8$	$6X10^{11}$
R. solani	$5X10^4$	$3X10^6$	$4X10^7$	$4X10^9$
B. cinerea	$5X10^4$	$2X10^5$	$5X10^7$	$3X10^8$
C. gloeosporioides	$6X10^4$	$4X10^6$	$7X10^7$	$3X10^9$
A. solani	$6X10^4$	$5X10^6$	$6X10^7$	$5X10^9$
A. niger	$3X10^3$	$6X10^5$	$4X10^6$	$3X10^8$

Table 2. Inhibition of spores germination of test plant pathogens with Fe3O4@Ag@chitosan and crude cell free culture of *Streptomyces aureofaciens*

Plant Pathogens	**Cell Free* Culture**	**Ag@FeO-NPs@Chitosan**		
		50 mg/l	**100mg/l**	**500 mg/l**
F. oxysporum	30.2b	92.8bc	98.2b	100a
F. solani	29.8b	91.6c	97.8bc	100a
F. subglutinans	35.8a	95.7a	100a	100a
R. solani	30.5b	93.5b	100a	100a
B. cinerea	20.7d	88.6de	96.7c	100a
C. gloeosporioides	32.8b	89.8d	100a	100a
A. solani	23.8c	86.7f	94.8d	100a
A. niger	19.7d	85.7f	91.7e	98.8b
Control	0.0e	0.0g	0.0f	0.0c

*Mean in the same column followed by different letters are statistically different ($P < 0.05$)

Antioxidant assay of** Ag@FeO-NPs@Chitosan extract Concentration of **Streptomyces aureofaciens

Reducing power assay

The Ag@FeO-NPs@Chitosan showed reducing power activities with increasing concentrations of as follows: 1.5 at 50 mg/l, 3.2 at 100 mg/l and 3.2 at 500 mg/l in compared with crude extract of cell free culture (1.0) (Table 3).

Diphenyl-1-picryl hydrazyl activity (DPPH) assay

The DPPH radical is used to imply natural and oxidative scavenging properties. In comparison to crude extract of cell free culture (2.25 1.20), Ag@FeO-NPs@Chitosan demonstrated high scavenging capabilities with increasing concentrations of 11.6 at 1.15 percent at 50 mg/l, 41.6 at 1.27 percent of 100 mg/l, and 82.5 at 1.75 percent of 500 mg/l (Table 3). No scavenging activity: In comparison to crude extract of cell free culture (40.5), Ag@FeO-NPs@Chitosan showed no scavenging capabilities with

increasing concentrations of extracts of 80.5 at 50 mg/l, 90.0 at 100 mg/l, and 96.5 at 500 mg/l (Table 3).

ABTS free radical scavenging activity

In comparison to crude extract of cell free culture (15.5%), the antioxidant capacity of Ag@FeO-NPs@Chitosan displayed scavenging activities with increasing concentrations as follows: 40.6 percent at 50 mg/l, 65.3 percent at 100 mg/l, and 85.5 percent at 500 mg/l (Table 3).

Polyphenol content in total: The Ag@FeO-NPs@Chitosan extract's total phenolic content was determined. In comparison to crude extract of cell free culture was 2.9,mg. The total phenolic compound was determined to be 12.1 mg at 50 mg/l, 75.32. mg at 100 mg/l, and 128.83 mg at 500 mg/l (Table 4).

Table 3. Antioxidant activity of Ag@FeO-NPs@Chitosan and crude cell free culture of *Streptomyces aureofaciens*

Treatment	Conc.	reducing power assay	DPPH radical scavenging activity (%)	nitric oxide scavenging assay	ABTS free radical scavenging assay %
Cell Free Culture	**Crude extract**	1.0	2.25 ± 1.20	40.5	15.5
Ag@FeO-NPs@Chitosan	**50 mg/l**	1.5	11.6 ± 1.15	80.5	40.6
	100 mg/l	3.2	41.6 ± 1.27	90.0	65.3
	500 mg/l	4.5	82.5 ± 1.75	96.5	85.5

Table 4. Total pholyphenol contact of Ag@FeO-NPs@ Chitosan and crude cell free culture of *Streptomyces aureofaciens*

Treatment	Conc.	Total Polyphenol Content (mg)
Cell Free Culture	**Crude extract**	2.9 ±1.2
Ag@FeO-NPs@Chitosan	**50 mg/l**	12.14 ±1.5
	100 mg/l	75.3 ±2.0
	500 mg/l	128.8 ±3.0

Discussion

The use of bio-nanomaterials in plant disease prevention and food production is an under-explored future emphasis (Bhattacharyya *et al.,* 2016 and Farhat *et al.,* 2018). Nanoparticle size and volume are significant characteristics that determine nanoparticle antitumor, antibacterial, and antifungal characterization (Eid *et al.,* 2019). Furthermore, fungi are the most vulnerable to chitosan nanoparticles, which can damage cell membranes and cellular components as well as act as a chelating agent, causing the release of water and nutrients required for fungi growth from cells or the attachment of chitosan nanoparticles to the fungi genome. Chitosan polysaccharide has played an important role in the biocontrol of pathogenic fungus after harvest via films carrying antifungal, antibacterial, or elicitor characteristics that boost plant defences against pathogens, as well as acting as a transporter of a variety of chemicals (Nguyen *et al.,* 2016 and Tripathi *et al.,* 2017).

Because of its electron donating ability, the activities of reducing power, DPPH radical scavenging, and reductive capabilities are linked and may serve as a substantial reflection of antioxidant activity (Kawahara *et al.,* 2012, Lee *et al*., 2014 and Nanjundan *et al*., 2014) and indication of prospective antioxidant. *Streptomyces aureofaciens* produced Ag@FeO-NPs@Chitosan, which showed promising antioxidant activity against DPPH, NO, and hydrogen peroxide radicals. Anti-inflammatory, antioxidant, anticancer, antifungal, and antibacterial effects have all been discovered in phenolic compounds (Kawahara *et al.,* 2012). The presence of phenol hydroxyl groups in phenolics is known to have antifungal action, as is their toxicity against extracellular microbial enzymes. The antibacterial activity of the Ag@FeO-NPs@Chitosan compounds was stronger against all plant pathogens in this study than the crude extract of cell free culture.

To reduce the risk of agro-chemicals, we are developing microbial nano-biofungicides from *S. aureofaciens* for biocontrol of plant diseases such as *F. oxysporum, F.solani,* F. *subglutinans, R. solani,* and airborne pathogenic fungi such as *B. cinerea*, *C.gloeosporioides*, and *Alternaria*. The smallest measured nanoparticle volume of Ag@FeO-NPs@Chitosan that the particle size is less than 50 nm with bioactive components of chitosan solutions showed a greater impact on the surface positive charges of chitosan affecting the nanoparticle stability than crude cell free culture.

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