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Antimicrobial Activity of Nano-silver Particles Produced by Micro Algae

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Nanotechnology became an important and has endless applications in all fields. A warm water extract for the algae *Chroococcus disperses* and *Chlorella vulgaris* was used for silver nitrate reduction. The produced bionanosilver were characterized using electron microscope and the particle size ranged from 25 to 55 nm. The two types of particles were tested against the plant pathogenic bacteria; *Pseudomonas flavescens*, *Erwinia amylovora* in addition to the plant pathogenic Fungi; *fusarium solani, fusarium oxysporium, Rhizoctonia solani, Helminthosprium sp, Alternaria alternata, Sclerotinia sclerotiorum* using the disc diffusion method. The inhibition zones formed by the two types of the nanosilver were compared the generic antibacterial antibiotics; Ampicillin, Gentamycin and streptomycin,. The results showed that a high bacterial activity was obtained by the resultant nanosilver and the inhibition zones were ranged from 35 to 55mm. This activity exceed 5-4 times more than that obtained by the chemical antibiotics. On the other hand, the nanosilver shows high activity against the examined fungi and this activity were higher than the activity of the generic antibiotics 9 times. It can concluded that the produced nanosilver by the algal extract have high activities as antimicrobial activity against the plant pathogenic microbes. A suitable carrier should be examined to approach the resultant pesticide on the infected plants and the disease control will be evaluated.

Keywords: Nanosilver, algal extract and antimicrobial.

Since many years silver had been used as antimicrobial and for that reason, it was used in the past in the storage of drinking water (Silver *et al*., 2006). On the other hand the usage the silver as it is does not make it valuable as gold but the nano-sized silver particles has become more economical especially when used in controlling different plant pathogens (Jo *et al* 2002). In addition, it was reported that algae used as a biofactory for synthesis of metallic nanoparticles. (Singaravelu *et al*. 2007, Rajasulochana *et al*. 2010) synthesized silver bio nanoparticles using the crude extract of the algal strains; *Sargassum wightii, Kappaphycus alvarezii, and Gelidiella acerosa*. Silver nanoparticles were synthesized using microalgae, *Spirulina platensis* (Vivek *et al* 2011 and Govindaraju *et al*, 2008). Moreover two algal species were used in production of AgNPs in different concentrations of silver nitrate (*Mohseniazar., et al 2011*). Kim, J. (2007) reported that silver nanoparticles can modify the microbe activities if these particles used as antibacterial. It was observed that the nanosilver has different physiochemical and biological uniqueness; increased optical, electromagnetic, catalytic properties and antimicrobial activity from the bulk

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materials Choi, *et al* (2009). The nanosilver products able to eliminates about 99% of bacteria and these particles able to kills approximately 650 kinds of harmful germs and virus and kills bacteria in a short time as 30 minutes Emtiazi, *et al*. (2009). We aim in this study to produce bionanosilver using acetone algal extracts of two microalgae. The antimicrobial activity of the obtained nanoparticles was tested against plant pathogenic bacteria such as; *Pseudomonas flavescens , agrobacterium tumefactions*, and *Erwinia* amylovora) and the plant pathogenic Fungi such as; *Fusarium solani, Fusarium oxysporium, Rhizoctonia solani¡ Helminthosprium sp, Alternaria alternaria, Sclerotinia sclerotiorum;* The suitable uses of these bionanoparticles in biological control of a wide range of plant pathogenic microbes.

MATERIALS AND METHODS

The two algal isolates used in this study were *Chroococcus disperses* and *Chlorella vulgaris* were kindly provided by Dr. Mohamed Ismail, faculty of science, Mansoura University, Mansoura, Egypt. Fungal and bacterial isolates were kindly provided from the plant disease department, faculty of agriculture, Alexandria University, Egypt. The fungal isolates are *fusarium solani, fusarium oxysporium, Rhizoctonia solani, Helminthosprium sp, Alternaria alternaria and Sclerotinia sclerotiorum*. Whenever, the bacterial isolates are *Pseudomonas flavescens*, *Agrobacterium tumefactions* and *Erwinia amylovora.*

Algae cultivation, Harvesting, and phytochemical extraction

The algal cultivation was performed by inoculated the algal cells in flasks (250 ml) with 10% of the selected algal cells, incubated at room temperature for 10 hours. After the incubation period the cells were collected by centrifugation at 10000 rpm for 30 minutes. The collected cells were preserved and the supernatant was discarded. The cells were stored at -20 until be used. Algal extraction; about 2gm of algal fresh weight were added to 10 ml of the desired organic solvent (Acetone), mixed well and then the mixture was exposed to Sonication (Cycle 5 min on, 5min off, 1min on, power 100% on Ice). After Sonication the volume was completed into 100ml with worm water were add to the sonicated solution. Then the solution was incubated for 16 hours at 30°C with shaking at 150rpm. Water and methanol extraction were performed according (Singh and Chaudhary. 2010).

Phytochemical determination in the algal extract

About 5gm of dried finely powdered Algae material was taken in a beaker the subjected to extraction using water and methanol in separate steps. The extract was subjected to phytocehmical screening. Detection of alkaloids: The extracts were dissolved individually in dil. Hydrochloric acid (dil. HCl) and filtered. And after that the filtrates were treated with Wagner's reagent (iodine (1.27) and potassium (2g) is dissolved in 5 ml of water and made up to 100 ml with distilled water). Formation of brown/reddish precipitate indicates the presence of alkaloids. Detection of carbohydrates: the product was dissolved in 5 ml distilled water and filtered out. The filtrates were used in order to check for the presence of carbohydrates. Molisch's Test: Two ml of filtrates solution is poured in a tes tube, then two drops of Molisch reagent (a solution of α-napthol in 95% ethanol) is added. The formation of a purple color product at the interface of the two layers; the violet ring at the junction indicates the presence of Carbohydrates. Dtection of glycosides: Extracts were hydrolyzed with dil. HCl, and then subjected to check the presence of glycosides. Modified Borntrager's Test: Extracts were treated with Ferric Chloride solution and immersed in boiling water for about 5 minutes. The mixture was cooled and extracted with equal volumes of benzene. The benzene layer was separated and then treated with ammonia solution. Formation of rose-pink color in the ammonical layer indicates the presence of anthranol glycosides. Detection of saponins: Froth Test: Extracts were diluted with distilled water to 20ml and the last was shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam indicates the presence of saponins. Detection of phytosterols: Salkowski's Test: Extracts were treated with chloroform and filtered out. The filtrates were treated with few drops of Conc. Sulphuric acid, shaken and allowed to stand. Appearance of golden yellow color indicates the presence of triterpenes. Detection of phenols; Ferric Chloride Test: Extracts were treated with 3-4 drops of ferric chloride solution. Formation of bluish black color

indicates the presence of phenols. Detection of tannins: Gelatin Test: 1% gelatin solution containing sodium chloride was added to the extract. Formation of white precipitate indicates the presence of tannins. Detection of flavonoids: Alkaline Reagent Test: Extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow color, which becomes colourless on addition of dilute acid, indicates the presence of flavonoids. Lead acetate Test: Extracts were treated with few drops of lead acetate solution. Formation of yellow colour precipitate indicates the presence of flavonoids. Detection of proteins and aminoacids; Xanthoproteic Test: The extracts were treated with few drops of conc. Nitric acid. Formation of yellow color indicates the presence of proteins. Detection of diterpenes: Copper acetate Test: Extracts were dissolved in water and treated with 3-4 drops of copper acetate solution. Formation of emerald green colour indicates the presence of diterpenes (Tiwari *et al*. 2011).

Biosynthesis of nano-scale Ag particles

For 100 ml of the filtrated extract of the two algal isolates (*Chlorella vulgaris*., *Chroococcus dispersus*) a 0.1 gm of AgNo3 (Sigma Aldresh, USA) was added. The solution was incubated in shekar incubator at 150 rpm for 2 weeks. The turbidity was measured using spectrophotometer at 450 nm along the incubation period. After the two weeks of incubation the nanosilver particles were pelleted using centrifugation at 10.000 rpm for 30 min. The pellet was taken and washed two times using distilled water and then dried in oven at 55°C for 30 min then weighted and dissolves in sterile distilled water.

Morphological characterization (SEM)

The surface morphology of the produced bionanosilver particles observed by helping of a scanning electron microscopy (JEOL JSM-6490, Japan) at an accelerated voltage of 20 kV and a current of 10 µA. The surfaces were vacuum coated with gold for *SEM*. The particle size of the bionanosilver was measured from the SEM image. The average particle size of the bionanosilver was determined by measuring and averaging the particle size of approximately from 25 to 55 nm random particles in each sample using scanning electron microscopy software (SMILE VIEW SOFTWARE developed by JOEL on SEM- Model JEOL JSM-6490) after sputtering by gold.

Antimicrobial activity of the obtained bionanosilver against the plant pathogenic bacteria and fungi

In vitro antimicrobial activities had been examined for plant pathogenic Fungi (*Fusarium solani, Fusarium oxysporium, Rhizoctonia solani, Helminthosprium sp, Alternaria alternaria, Sclerotinia sclerotiorum)* using the agar disk diffusion method according to (Attaie, *et al*., 1987, Murray, 1995). Inhibition zones of growth around the disks were measured after 48 to 96 hours for fungi at 28°C. While in case of bacteria *(Pseudomonas sp, Agrobacterium tumefactions, and Erwinia amylovora)* zones of growth inhibition was measured after 24 hours of incubation at 37°C. The nano-particle activity was compared with some of generic antibiotics (*Ampicillin, Gentamycin* and *streptomycin*).

RESULTS AND DISCUSSION

The results obtained from the present study that the color of the two algal extracts was changed from transparent to dark reddish-yellow due to the production of silver nanoparticles. The dark color means that the reduction of silver nitrate into silver ions in nano-scale by the metabolites of the used algal extract. Phytochemical analysis of the two examined acetone extracts revealed that a

Table 1. Photochemical components of the acetone extract of the two examined algae

Test/ Sample	Acetone extract	
	Chlorella vulgaris	*Chroococcus dispersus*
Alkaloids	-	-
Carbohydrates	+	+
Glycosides	-	-
Flavonoids	+	+
Saponins	+	-
Terpenes	+	-
Steroids	+	-
Phenolics	+	+
Tannins	-	+ + +
Amino acids	+	+
Protein	+	+

(-) = Negative (absent); (+) = Positive (slightly present); (++) = Positive (moderately present).

Carbohydrates, Saponins, Glycosides, Flavonoids, Terpenes, Steroids, Phenolics, Tannins, Amino acids and Proteins are commonly presented in different concentrations (Table 1). Mansuya *et al*. (2010) they found that the aqueous extract of the seaweeds except *Cladophora glomerata* contains *Phytochemical* components which consists of; carbohydrates, Saponins. It well known that flavoniodes, phenols and tannins are antioxidant and they are good antimicrobial compounds (Cox *et al*. 2010). We assume that some or all of these compounds had been loaded on the formed nanosilver particles. The particles contain one or some of these compounds will have efficacy as antimicrobial than those have none.

Characterization of the produced nanoparticles

The produced nanoparticles by the two algal extracts were; spherical and their sizes ranged from 27 to 47 nm in case of *Chlorella vulgaris* and 44 to 55 nm in case of *Chroococcus dispersus* (Fig 1). Rajesh *et al* (2012) used the silver nanoparticles synthesized by *Ulva fasciata* extract and the average size of the reduced bionanoparticles was about 40.05 nm. Similarly, the biosynthesized

Table 2. Mean diameter of growth inhibition zones of bacterial strains treated by the Ag nanoparticles synthesized by the two algal isolates

	Ag Concentration	*Pseudomonas flavescens*	*Agrobacterium tumefactions*	*Erwinia amylovora*
Ag of	1%	-	-	-
Chlorella	25%	-	-	-
Vulgaris	50%	-	-	5mm
	100%	35mm	30mm	40mm
Ag of	1%	-	-	-
Chroococcus	25%	4mm	-	3mm
dispersus	50%	-	-	-
	100%	40mm	50mm	55mm

Table 3. Mean diameter of growth inhibition zones of bacterial strains treated by in the generic antibiotics

Drug	*Pseudomonas*	*Agrobacterium tumefactions*	*Erwinia* amylovora
Ampicillin	-	-	-
Gentamycin	5mm	4mm	-
Streptomycin	9mm	8mm	2mm

Table 4. Mean diameter of growth inhibition zones of fungal strains treated by the produced Ag particles

Nano-particles	Conc.	*Fusarium solani*	*Fusarium oxysporium*	*Helminthosprium sp*	*Sclerotinia sclerotiorum*
Chlorella vulgaris	1%	-	-	-	-
	25%	-	-	-	-
	50%	-	-	3mm	
	100%	25mm	31mm	40mm	43mm
Chroococcus dispersus	1%	-	-	-	-
	25%	-	6mm	-	2mm
	50%	-	-	-	-
	100%	45mm	45mm	50mm	53mm

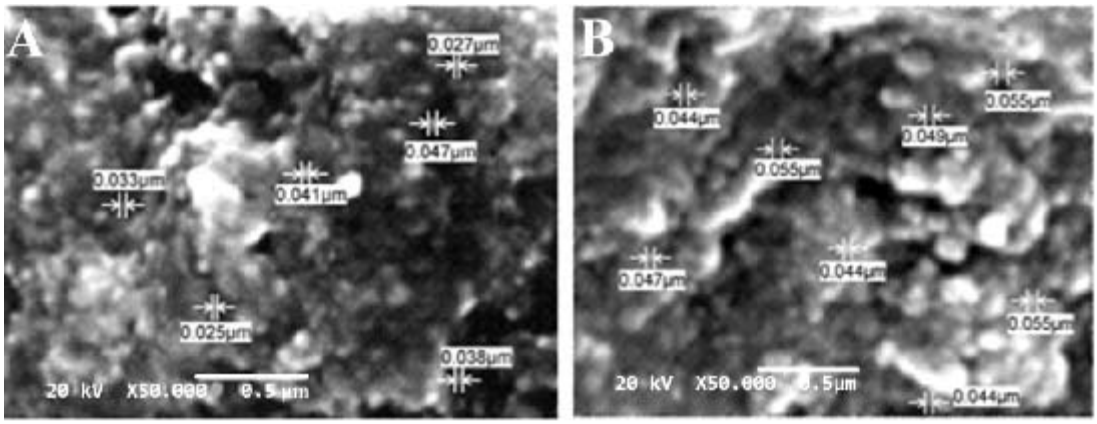

Fig. 1. Scanning electron microscope for the formed bionanosilver. A: The silver nanoparticles formed by the extract of the *Chroococcus disperses*, B: The silver nanoparticles formed by the extract of the *Chlorella vulgaris*

nanoparticle produced by the black tea leaf extract was in size ranged from 10 to 72 nm (Begum *et al.* 2009).

The produced bionanosilver showed high activity against the plant pathogenic bacteria and fungi. This activity was tested against *Fusarium solani, Fusarium oxysporium, Helminthosprium sp, Sclerotinia sclerotiorum* and the results were presented in table (2, 3 and 4) as inhibition zones (El-Aassar *et al*, 2013, Hafaz *et al*, 2011). The inhibition size zones in the treated fungi with the bionanosilver ranged from 25 to 55mm in diameter. On the other hand the generic antibiotics showed inhibition zones ranged from 35mm to 50mm. Scientists synthesized Ag nanoparticles using *Gelidiella acerosa* extract and high activity against the tested fungal isolate (*Mucor indicus, Trichoderma reesei, Fusarium dimerum* and *Humicola insolens*) at a concentration of 50 *µl (Vivek et al. 2011)*.

The produced nanoparticles produed by the *C. dispersus* showed high activity when compared with that obtained by the algal *C. vulgaris* (27 times). The high concentration (100%) of the obtained nanosilver gave high activity with the three examined bacterial strains; P*seudomonas flavescens (35-40), Agrobacterium tumefaction (30-50) and Erwinia amylovora* (by the two algal extract showed activity against *Erwinia amylovora* (40-55mm). The results obtained with the three examined generic antibiotics (Ampicillin, Gentamycin and *Streptomycin*) against the previous three bacterial strains, negative results was observed with ampicillin and a very small inhibition zones when compared with the nanosilver (1/10).

The experimental results concerning the antifungal activity against the tested fungi (Table 3) clearly showed that the two Ag particles produced by the two algal extracts have antifungal activity in high concentration of Ag nanoparticles. The same observation was recorded that the nanoparticles produced by the algal isolate *C. dispersus* gave higest antifungal activity more than that obtained by the algal *C. vulgaris*. Savithramma *et al* (2011) reported that the silver nanoparticles synthesized by *Boswellia ovalifoliolata*, these particles gave antibacterial activity, higher antibacterial activity was observed against, *E. coli* and *Proteus* species; and antifungal activity was observed against *Aspergillus* and *Fusarium*.

CONCLUSION

Bionanosilver could be synthesized by alagal extract using warm water. The produced nanoparticles have biocontrol activity against some of plant pathogenic microbes.

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