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Impact of engineered nano silver on plant parasitic nematode and measurement of DNA damage

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Abstract

Plant parasitic nematodes: *Meloidogyne incognita*, *Pratylenchus penetrans*, and *Tylenchulus semipenetrans* are the most genera which cause economic damage in the agricultural production. Nanotechnology is a promising alternative method in nematode control, especially the green synthesis methods. Present study deals with the effects of Ag engineered nanoparticles (AgENPs) synthesized by using plant extract to evaluate juvenile mortality, egg hatching, morphological change, and DNA damage. Five concentrations of AgENPs were prepared from each AgENPs and were added to300 individuals of nematode separately up to five days. Data approved that, *Meloidogyne incognita* was more affected than *Pratylenchus penetrans* and *Tylenchulus semipenetrans* in all the treatments. *Curcuma comosa* (N1) was the most effective preparation against nematode larvae, followed by Chitosan (N3), *Crotalaria juncea* (N4), and *Cycas circinalis* (N2). These effects were increased proportionally with the nano concentration and the exposure time. While N3 recorded the highest reduction in egg hatchability. Some visual changes were observed on the treated larvae and eggs. Likewise, the most DNA damage for nematode larvae was detected with N1,followed by N4, N3, and N2. As a conclusion, AgENPs may provide a better alternative to chemically synthesized against deferent plant parasitic nematode.

***Keywords*:** AgENPs, Root knot nematodes, juvenile mortality, egg hatching, morphological change, DNA damage, comet assay

Introduction

Plant parasitic nematodes are of the most involved pathogens in plant growth retardations (1,2). Mostly, root-knot nematodes (KRN) are responsible for more than 90% of nematodes damage to plant crops (3) due to their high destructive potential against wide host range and wide geographic distribution (4). Lesion nematodes, *Pratylenchus* sp. consider as the second global important nematodes preceded by *Meloidogyne* in the plant crop damage and the yield loss in several plant production all over the world including Egypt (5). Meanwhile, Citrus nematodes; *Tylenchulus semipenetrans* causes the slow decline disease in citrus trees leading to economic losses in citrus production all over the world (6).

Great interest was directed to find an eco-friendly efficient alternative method in nematode control due to the hazard effects of chemical nematicides on human, plants and non-target living organisms as well as environment[2]. Recently, nanoparticles attracted greater attention because of their successfully applications in management of several pests in plant crops (7), AgENPs is one of the efficient metal nanoparticles which has great

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prominence application as biocides against of some bacteria, fungi, and nematodes (8). This efficiency may be due to the large surface area that increases their activity as antimicrobial agent (9) also, it may involve with the induction of oxidative stress in the targeted cells (10). Moreover, it may be due to their high capacity of volume to surface which enhances their biochemical properties against some pests (11).

Several effective methods are used for the synthesis of nano silver (AgENPs) such as electrochemical reduction (12), heat evaporation (13), photochemical reduction (14), and castly chemical reduction (15) which consider as the most common one. Because of the toxicity and the biological risks of some chemical reduction which used in nano preparation, the looking for an eco-friendly technique is needed to use in synthesis process (16) such as: the using of some microorganisms (17), and the use of some plant materials such as *Curcuma longa* extract (18). The plant mediated synthesis method was simple, cost-effective and ecofriendly, and performed far better than the chemical NPs with most of the parameters, amending the toxic impacts of as treatment (19).The effect of antagonistic plant parts which have nematicidal properties may be due to containing alkaloids, iso-thiocyanates, glucosides, phenolics, fatty acids and thiophenics and diphenyl heptanoids which isolated from turmeric *Curcuma comosa* (2) and *Crotalaria juncea* (20). Chitosan is soluble deacetylated form of Chitin which consider as a nontoxic biodegradable compound to plants or animals (21). The main source of chitosan is the shell of shrimp, crabs, and other marine arthropods (22), This material has a promising suppressive effect on root knot nematode (23). Crotalaria has a suppressive effect on root-knot nematodes and their egg hatching (8, 24) and on *Pratylenchus* penetrans (25), depending on the concentration and the exposure time (26). Its effect may be attributed to producing monocrotaline and pyrrolizidine alkaloids which are toxic to nematodes and jerk their bodies (27). The green silver nanoparticles using *Curcuma longa* as a biological reductive material produces a highly suppressive nano production, due to their content of some flavonoids and alkaloids (18). Moreover, the green synthesis may increase the suppressive effect of Crotalaria, Curcum, and Chitosan when co-exist in the synthesis process (8,20). Some study indicate that, Cycas didn't record any suppressive effect on nematodes, but it has a rich content of flavonoids, and some other phenolic compounds and this may improve the efficiency of the nanoparticles itself (28), and increased the crystalline stability in the solution, reduce the time of nano biosynthesis (29) for these reasons cycas considered more economic, and efficient in synthesis of nano process (28).

Understanding the interaction of AgENPs with biological surfaces is important, as such understanding will facilitate predictions of the further effects of nanoparticles on biological systems are still insufficient (30,31). Over the past decade, the single-cell gel electrophoresis, or comet assay, has become one of the standard methods for assessing DNA damage and has been used to find the genotoxicity of nanoparticles due to its reliability, simplicity, low cost, sensitivity and versatility (32,33). The modified alkaline Comet assay can be used to detect oxidative stress-induced DNA damage and thus may be applicable for in *vivo* genotoxic assessments (34). The use of comet assay technique in other organisms, such as bacteria, plants, fungi or invertebrates, is not limited (35).

As indicated before, chemical reductives which use in nano particles synthesis may cause biological or environmental risks, therefore this research aimed to increase the efficiency of nano silver particles by using different plant materials in the green synthesis of nano production to minimize doses of AgENPs which need to control the plant parasitic nematodes, in addition to estimate their suppressive effect against nematode viability and the measurement of the DNA damage which causes by AgENPs.

MATERIALS AND METHODS

Study area: The study was carried out during 2020-2021 at the Department of Plant Protection, Faculty of Agriculture, Ain Shams University

Nematode cultures:

Tomato plants (*Solanum lycopersicum*) were inoculated with juveniles *Meloidogyne incognita* (Mi) and maintained as astock culture in the greenhouse for the IJs of Mi. Grape plants (*Vitis* sp.) were used as a stock for *Pratylenchus pentrans* culture. 1.1.3. Orange plants (*Citrus* sp.) were used as a stock for *Tylenchulus semipentrans* culture in screenhouse.

Nano materials

Nano materials were obtained from Nano fab, Egypt, which prepared as following

Synthesis of silver nanoparticles

Silver nano particles was prepared using silver nitrate ($AgNO_3$) (from Sigma Aldrich) as follow, 60 ml of 10^{-3} M $AgNO_3$ solution were added to 3 ml of plant extract and left at natural room conditions until reaching a transparent yellow color which turn into a dark brown and this is the indicator to AgNPs formation, the reduction of Ag+ ions was obtained using UV–visible spectral analysis. The dark brown solution was centrifuged to collect the AgNPs then washed three times using deionized water and finally re-suspended in 95 % ethanol (Fisher Scientific). The pH value of synthesis was 9. AgNPs were kept in sonicator.

Preparation of Colloidal Curcumin, Cycas and Crotalaria Nanoparticles

The extract was made using 20 g of fresh leaves of *Curcumin*, *Cycas* and *Crotalaria*. Prior to extract preparation, the plant leaves were cleaned thoroughly using deionized water and then cut into small pieces. The leaves were then added into 125 ml of boiling deionized water, and left to boil for 3 min. The solution was then removed from the heat source and left to cool to ambient temperature (approximately 25° C). The extract was then filtered through a coarse sieve to remove any leaf matter and the resultant filtrate was then refrigerated. The Plant extract was collected and stored at 4° C. Finally, the extract was used for the synthesis of AgENPs. nanosuspension were prepared by solvent-antisolvent precipitation method. Briefly, they was extracted in ethanolic solution with concentration 1.3 % wt/vol under vigorous stirring at 60 C overnight. Then, the mixture was filtered using Whatman filter paper. The extracted filtrate was then subjected to vigorous stirring at heat at 60 C. Then about 30% vol/vol H_2O has been added to ethanolic solution as an anti-solvent and PVP 10 %wt/vol added under vigorous stirring and heat at 60 C for another 3 hr. A milky yellowish nano-colloidal solution has been obtained indicating to formation of *Curcumin*, *Cycas* and *Crotalaria*nano-colloids.

Preparation of Colloidal Chitosan Nanoparticles

Nanoparticles of Chitosan preparedusing ion gelation method as follow, chitosan was dissolved in aqueous soln of acetic acid (1%v/v) under vigorous stirring at 60 ˚C till get a clear solution. Then cross linker agent TPP was added dropwise to the chitosan solution (CS) under vigorous stirring reaching a ratio of 5 CS to 1 TPP, the reaction was left for 30 min after addition, then washed and centrifuged at 8000 rpm for 20 min several times till pH 7. Nanoparticles was kept Freezing Dryer till obtain the nano powder, finally the powder was redispersed into distilled water.

Nanoparticles characterization

After 15 min, the mixture of silver nitrate solution and leaf extract was turned from color less to faint yellow, indicating the formation of AgNPs, and the color became dark brown at 120 min, which mean a high concentration of AgNPs. The completion of the reduction reaction done after180 min, this was confirmed by UV–Vis spectroscopic analysis.

Transmission Electron Microscopy (TEM)

Shape and size of AgENPs were practically obtained using TEM. Specimens for TEM measurements were prepared by placing a drop of colloidal solution on 400 mesh copper grid coated by an amorphous carbon film and evaporating the solvent in air at room temperature. The average diameter of the prepared AgNPs was determined from the diameter of 2-100 nanoparticles found in several arbitrarily chosen areas in enlarged microphotographs.

Nematicidal bioassays

Effects of AgENPs on juvenile mortality:

Infected tomato roots (*Solanumlycopersicum*) with *Meloidogyne incognita* (Mi), grape infected roots with *Pratylenchus pentrans*, and infected citrus roots with *Tylenchulussemipentrans* were washed gently with water and cut into small pieces separately to extract nematodes using mist chamber and the emerging juveniles of nematodes were collected daily. To evaluate the efficacy of each silver nano particles, approximately 300 nematodes in 6 ml of water were added to 6 cm diameter petri dish with 2 ml of solutions containing 0, 2.5, 5.0, 10.0, 20.0, and 40 ppm. of AgENPs/replicate of each silver nano synthesis. Each treatment was replicated five times. Other five replicates were received water instead of AgENPs and kept as a control. All the replicates were incubated at the room temperature (25 °C±2). Healthy and dead nematodes were counted using the light microscope after 1, 2, 3, 4, 5 days of the AgENPs treatments, the corrected mortality percentages were calculated by using Abbott's formula (36) as follows: Corrected Mortality (%) $=\left\{\frac{T-C}{100-C}x100\right\}$.Where, T = percent mortality in the treatment and C = percent mortality in the control.Dead nematodes were defined as the straight bodied nematodes, while the unavailable bodies were considered as the live nematodes.

Effects of AgENPs on egg hatching

Eggs were collected from a 35-day old, infected tomato seedling with *Meloidogyne incognita*in the greenhouse.

The egg masses were obtained from the tomato roots using diluted sodium hypochlorite (37). The experiment was conducted as the same procedure in the previous juveniles. The ratio of hatching inhibition in each treatment was corrected by the same formula[36] as follows:

Haching inhibition (Hi) = $\left\{\frac{T-C}{100-C}x100\right\}$. Where Hi = hatching inhibition, T = hatching inhibition in the treatments and C = hatching inhibition in the control.

Effects of AgENPs on morphological change in juveniles and eggs

The effect of AgENPs on morphological change of nematode juveniles and eggs in each experiment was documented with photo by using LEICA light microscope model DM-500 supplemented with a digital camera LEICA ICC 50 HD with LAS E7 software version 2.1.0 2012.

Genotoxicity assay

The Effects of AgENPs on DNA damage were detected by using alkaline comet assay as following:

Extraction

Nematodefrom each of 5 concentrations of 4 AgENPs compounds were collected and placed in a petri dish with Sörensen buffer (50 mM sodium phosphate, pH 6.8, 0.1 mM ethylene diamine tetra acetic acid (EDTA), 0.5% dimethyl sulfoxide (DMSO) kept on ice. The nematode was repeatedly dipped in the cold Sörensen buffer. The suspension with released nuclei was filtered through a 30 µm disposable filter (Partec, Műnster, Germany) to remove most of the debris and centrifuged at 550 g for 5 min at 4ºC, the protocol described by Galea *et al*., (38)**.**

Preparation of alkaline Comet assay

Microscope slides were coated with 0.5% normal melting agarose and dried at room temperature. Forty µl of the nuclei suspension was mixed with 40 µl of 0.1% low melting agarose, spread on the slide surface and subjected to gel formation for at least 10–15 min on a cooling plate at 4°C. Lysis was carried out in 2.5 M NaCl, 10 mM Na_2EDTA (pH8), 10 mM Tris-HCl (pH8), 1% N-lauroylsarcosine sodium salt, 1% TritonX-100, 10% DMSO for 15 min at 4°C in the dark. Electrophoresis was performed in prepared TAE buffer (pH8) at 0.5, 1, 2 and 5 V/cm for 10 min for nematode nuclei. The slides were dehydrated in 70% and 96% ethanol for 5 min and dried at room temperature. The slides were covered with solution of the fluorescent dye acridine orange (10 µg/ml) (38). Visualization of the stained comets was carried out using a fluorescence microscope (Zeiss Jenamed-2) coupled with a digital camera (Samsung Digimax V50). Three independent experiments were performed, and 50 comets were analyzed per point in each experiment. Damage was detected according to the fragments intensity which migrated during electrophoresis (39,40).

Statistical analysis

The data were analyzed by One-way Analysis of Variance (ANOVA) to determine the significance of differences at $p < 0.05$ level, comparing by Salkind (41).

RESULTS

Morphological Structure of AgENPs

Data in Fig. 1showed that, the green synthesis of AgNPs produced a very small particles which differs in size according to the type of the redactor which use in synthesis, that it was about 15 ± 5 nm (Fig. 1a), 35 ± 8 nm (See Fig. 1b), 50 ± 15 (Fig. 1c),nm and 30 ± 10 nm (Fig. 1d) using *curcumin*, *Cycas*, chitosan and *Crotalaria* was, respectively.

Colloidal Stability Properties

Hydrodynamic particle size of prepared nanoparticles is remarkably increased, agreed with the agglomeration which presented in TEM micrographs. In this regard, *Curcumin* capped AgNPs has a hydrodynamic diameter (H_D) of 52 ± 9.1 nm (Fig. 2a) where the polydispersity index (PdI) is about 0.568. Moreover, the Zeta potential (η) of *Curcumin* capped AgNPs was about -7.7 mV (Fig. 3a). While the hydrodynamic particle size of *Cycas* capped AgNPs was larger than *Curcumin* capped AgNPs of 76.57 ± 29 nm (Fig. 2b). In addition, the polydispersity index (PdI) was about 0.3 while Zeta potential (η) of *Cycas* capped AgNPs exhibits less negatively charged -11 mV (Fig. 3b). Furthermore, Silver capped with chitosan nanoparticles exhibited the highest H_D, the hydrodynamic particle was about 664 ± 90.6 nm (Fig. 2c), and the polydispersity index (PdI) is about 0.155 indicating to their fair dispersion in water and their Zeta potential (η) was about -12.7 mV(Fig. 3c). Finally, the hydrodynamic diameter of *Crotalaria* capped silver nanoparticles was about 160.3 ± 19.2 nm(Fig. 2d) with a polydispersity index (PdI) of 0.870, and the Zeta potential (η) was about -17.9 mV (Fig. 3d).

1. Effect of AgENPs on juvenile mortality

Generally, all the AgENPs *Curcuma comosa*, *Cycascircinalis*, *Chitosan*, and *Crotalaria juncea* were tested against the three investigated nematodes, *Meloidogyne incognita*, *Pratylenchus penetrans*, and *Tylenchulus semipenetrans*. Table (1) revealed that, AgENPs which prepared using N1, was the most effective case than others, followed by N3, N4andN2,which has the lowest effect on the tested nematode genera. These effects were increased proportionally with the nano concentration and the exposure time. However, data in Table (1) demonstrated that, AgENPs which prepared using N1didn't record any significance between the highest three concentrations, (40, 20, and 10 ppm all over the five days. Significant differences between the different concentrations in N3, N4andN2 they have lower effect than N1 on the second infective juveniles (2IJs) of root knot nematodes showed in Table (1).

Tables 2 and 3 appeared that, *Pratylenchus penetrans* was more tolerant than *Meloidogyne incognita*as recorded in Table (3) there was no significance between the highest concentrations of AgENPs after the first, second, and the fourth days. While, in contrast fifteen was considerable differences after the third and the fifth days in case of N1, and between the highest two concentrations (40, and 20 ppm). On the other hand, there was no significance between the same doses after four and five days in the case of N3.

As with *Tylenchulus semipenetrans* (Table 3),showed the same degrees of significance like those of *Pratylenchus penetrans* (Table 2) in the different concentrations of AgENPs within the tested exposure times.

2. Effect of AgENPs on egg hatching of *M. Incognita.*

Data in table (4) demonstrated that, using of AgENPs in different green synthesis caused different levels of hatching and hatching reduction. These reductions were directly proportions to nano concentrations and the exposure time. Generally, the lowest egg hatching was obtained with N3, and this may be due to the chitosan compound in AgENPs suspension followed by N1, N4, and N2which cause the highest hatching value (Table 4). These data agreed with Mercer et. al.,[42] who recorded a higher suppressive effect of chitinase on egg hatching of *M. hapla* and on *Criconemella xenoplax* more than their effects on other stages.

Table (5) indicated that, the highest egg hatching percentage reduction was obtained with N3, and this may be due to the Chitosan compound in AgENPs suspension. Moreover, the highest effect was observed in the high two concentrations (20 ppm and 40 ppm). On the other hand, the reduction ratios out be arranged,N3, N4, N1andN2 respectively. However, no significance between N4andN1was recorded.

3. Effect of AgENPs on morphological changes of *M. incognita* juveniles and eggs:

The treated and untreated juveniles and eggs were photted using the light microscope (LICA) with high magnification Figures from 4to 8. Some visual changes were observed when the different stages were exposed to the green synthesis of AgENPs as follow:

A. Curcuma comosa(N1):it was observed, some swollen in the juvenile body and some disintegration in their cuticle, as well as decay in the internal structures in the treated juveniles (Fig. 4. A) comparing with the untreated one (Fig. 4. B). Furthermore, it was noted some decaying of the embryo in the treated eggs (fig. 8. C).

B. Cycas circinalis **(**N2) Fig. (5-A and 8-A) illustrated malformation, disintegration and shrinkage in the juvenile cuticle and eggshell when treated by AgENPs with N2 comparing with these of untreated juveniles and eggshells. These changes may be due to silver particles into ultrafine nano particles which are rich in phytochemicals such as flavonoids and other polyphenols [18,43].

C. Chitosan (N3): it was shown that there is perforation all over the cuticle of juveniles when treated by AgENPs with N3(Fig.6. A) comparing with these of untreated juveniles (Fig. 6. B), and in the eggshell (Fig.5. B), these data agreed with Mercer *et al*.,(42) who recorded some perforations and rupture in the larval cuticle and the eggshell in the presence of chitin products. Also, our data supported by Bernard *et al.,* (43) who reported an extensive effect on the cuticle of nematodes, by chitin which can enhance the penetration of silver nano particles.

D. Crotalaria juncea (N4): it was observed that, there are burst in juvenile's cuticle when treated by AgENPs with N4 (Fig. 7A) comparing with the untreated juveniles (Fig.7 B) and some dissolving in the eggshell (Fig.8 D).

Regarding of the DNA damage, comet assay was performed on the *M. incognita*, by using the 4 AgENPs (Table 6). Most accurate parameter of DNA damage was DNA percentage in tail because it reflects the total tail intensity and the comet total intensity being not reliable on the tail length. Meanwhile, DNA %, DNA in tail, tail length, tail moment and olive tail moment were presented in Table (6). Generally, using of green synthesis of AgENPs caused some effect on DNA parameters: percentage of DNA, tail length, DNA in tail, olive tail moment, and tail moment, and this effect was inversely correlated with the nano particles concentration and the highest effect was obtaining using 40 ppm. and the lowest was recorded using 2.5 ppm. on the other hand, the damage in DNA parameters were higher with N1, followed by N4, N3, and N2.

DISCUSSION

The obtained data demonstrated that, the green synthesis of AgENPs using *Curcuma comosa, Cycascircinalis, Chitosan,* and *Crotalaria juncea* affected on *Meloidogyne incognita, Pratylenchus penetrans,* and *Tylenchulus semipenetrans* viability and increased the corrected mortality % in all the investigated doses and the highly effects were recorded using the highest dose and the last examine date. N1 was the most effective case than others, followed by N3, N4 which were highly similar. N2, has the lowest effect on the tested nematode genera.

In this respect, Johnson and Prabu (44); Kalaiselvi *et, al.*, (18) stated that, *Cycas circinalis, Curcuma longa* can improve the reduction of silver ions and increased its stability in the solution in addition because of their highly content of flavonoids, and other polyphenols, metallothioneins, phyotchelatins and glutathiones. Moreover, Taha (8) and Goel *et al*.(20) pointed that, the green synthesis may increase the suppressive effect of *Crotalaria*, *Curcum*, and *Chitosan* when use in the synthesis process.

The recorded nematicidal effect of AgENPs using Chitosan is agreed with Lopez-Moya *et. al.*, (21) and Zeng *et. al.*, (22) who attributed this effect to the forming of free radicals from the surface of AgENPs which increase the oxidative stress on the cell membrane**.**

Green synthesis of AgENPs using *Crotalaria* and *Curcuma* showed a high suppressive effect on the nematode viability and these data were explained

by Goel *et. al.*,(20) and Rashid *et al.*, (2) as antagonistic reaction among all elopathic contents of some flavonoids, pyrrolizidine alkaloids and alkaloids which participate in distortion and malformation treated nematodes**.**

Using of AgENPs showed some malformation, proliferation, rupture and burst, on the larval cuticle and eggshell, our data agreed with Chen *et. al*.,(42); Bernard *et. al.*,(43); Mahmoud (45), who recorded some bursts on the cuticle of nematode juveniles by *Crotalaria juncea* these data may attributed to the effect of high content of monocrotaline and pyrrolizidine alkaloids which are toxic to nematodes leading to the malformation (24,27).

The present results of comet analysis of DNA were important in determining the concentration of AgNPs required to apply with plant parasitic nematode; *Meloidogyne incognita*, *Pratylenchus penetrans*, and *Tylenchulus semipenetrans*. The obtained data show that the damage in DNA parameters was increased proportionally with concentration of AgENPs. These data agreed with those obtained by Galea *et.al.*,(46) used comet assay to detect the genotoxicity of environmental pollutants with *C. elegans* nematode. Establishment of protocol for cell dissociation from nematode *C. elegans* to assessment the genotoxicity of the environmental pollutant benzopyrene (BaP) using the alkaline version of comet assay by Imanikia *et al.*,(47). Screening method of toxicity ranking to heavy metals by using adult nematode *C. elegans* was studied by Hunt *et. al.*, (48). Nano silver induces DNA damage, suppresses growth and morphological changes in nematode *C. elegans* (49). Dose-response effects were appeared in nematode *C. elegans* by AgNPs moreover, lethal effects of silver nano particles on *C. elegans* was was recorded (50). The interaction of citrate-coated Ag nano particles (cAgNPs) with the biological surfaces of the nematode *C. elegans* was evaluated by Kim *et al.*, (30) and found that, a clear reduction was evidenced of the reproduction and survival in nematode *C. elegans*, and also noted that, significant interactions of cAgNPs with biological surfaces of *Caenorhabditis elegans*. Burst and severe epidemic edema were exhibited in the exposure groups. The survival ratio of *Caenorhabditis elegans* rapidly decreased with the uptake of nano particles from L4 larval stage (31) moreover, both body size and reproduction rate of *Caenorhabditis elegans* were also reduced after treated by full Erol nano particles, otherwise, ectopic cell corpses was found which caused by apoptotic cell death in adult larval grown with fullerolnano- particles.

Conclusion

This study investigates the green synthesis of nano silver using plants which have nematicidal properties on plant parasitic nematodes such as *Curcuma comosa*, *Crotalaria juncea*, or natural nematicidal degradable compound such as Chitosan, as well as an efficient reductive plant such as *Cycas circinalis*, that can be beneficial in plant parasitic nematode control. This study will help the researchers to investigate an alternative method in nematode control in addition to improv the plant growth using natural materials instead of chemical reductive agents which use in the AgNPs synthesis.

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