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ANTIBACTERIAL ACTIVITY OF SILVER NANOPARTICLES SYNTHESIZED USING *CITRUS SINENSIS* PEEL EXTRACT AGAINST PATHOGENS ISOLATED FROM DISEASED TOMATO (*SOLANUM LYCOPERSICUM* L.)

Onyemaechi Henry OBIAZIKWOR and Hakeem Olalekan SHITTU*

Address (es):
Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Benin, Benin City, Nigeria.

*Corresponding author: onyemaechi.obiazikwor@uniben.edu

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ABSTRACT

Among all the noble nanoparticles, silver nanoparticles have gained boundless interests because of their unique properties such as chemical stability, catalytic and most important antimicrobial activities. This study was carried out to investigate the antibacterial activity of phytosynthesized silver nanoparticles against bacteria pathogens isolated from diseased tomato plant leaves. Silver nanoparticles were synthesized using *Citrus* peel extract and the formation of nanoparticles was monitored using spectrophotometer. Diseased tomato plant leaves were obtained from a farm located at Ovia North-East Local Government Area, Edo State, Nigeria for the isolation of bacteria pathogens. The isolated bacteria include *Pseudomonas* sp. and *Enterobacter* sp. Antibacterial testing using the phytosynthesized silver nanoparticles was carried out via the agar well diffusion method on the test isolates. Zones of inhibition of 10 and 8 mm were obtained for *Enterobacter* and *Pseudomonas* species respectively by 100 µl nanoparticles treatment after 24 hours of incubation. This indicated that the phytosynthesized silver nanoparticles have antibacterial activity against the bacterial pathogens. Further studies should be carried out to determine the mode of action of silver nanoparticles and the potential of the test nanoparticles in plant disease management. The potential of members of the genus, *Enterobacter* as causative agents of plant diseases should be further investigated.

Keywords: Antibacterial, nanoparticles, pathogens, phytosynthesis, silver

INTRODUCTION

Scientists have discovered that substances tend to be more active when broken down to nano scale compared to their bulk counterpart. Nanoparticles are substances in structure ranging from approximately 1 – 100 nm in size (**Ahmed *et al*., 2016**). The emerging field of modern research that deals with synthesis, strategy and manipulation of nanoparticles is known as nanotechnology (**Ahmed *et al*., 2016**). Nanoparticles are synthesized using physical, chemical and biological methods (**Panigrahi, 2013**). There is advancement of biological method (green synthesis) over physical and chemical methods because it is ecofriendly, cost effective and it can easily be scaled up for large production (**Ahmed *et al*., 2016**). The biological method involves the use of living organisms such as bacteria, fungi and plants (**Panigrahi, 2013**). The use of plant extracts offers additional advantages over microorganisms such as ease of maintenance and reduction of cost of isolating microorganisms (**Ahmed *et al*., 2016**). Nanoparticles exhibit unusual physical, chemical and biological activity due to their reduced small sizes (**Rao and Paria, 2013; Mariselvan *et al*., 2014**). Among all the noble nanoparticles, silver nanoparticles have gained boundless interests because of their unique properties such as chemical stability, good conductivity, catalytic and most important antimicrobial activities (**Ahmed *et al*., 2016)**. Due to their medicinal and antimicrobial properties, silver nanoparticles have been incorporated into more than 200 consumer products including clothing, medicine and cosmetics (**Ahmed *et al*., 2016)**. Nanoparticles and nanotechnology have been applied in various disciplines including engineering, medicine and agriculture. In agriculture, they are currently been exploited for plant disease control. Phytopathogens such as bacteria, viruses, fungi cause lots of damages when they infect crop plants, thereby leading to low productivity (**Savay *et al*., 2012**). Different management strategies such as physical, cultural, chemical and biological methods have been adopted for the management of plant diseases (**Agrios, 2005**). These strategies include control methods that eradicate or reduce the pathogen inoculum and they have several limitations; and each pathcogen has a control strategy that works best for it. There is also a growing microbial resistance against antibiotics and development of resistant strains. Scientists are therefore seeking other control measures for plant pathogens in order to reduce crop losses due to pathogen attack. This will ensure food security and contribute to meeting the food demand of the ever-growing human population. Nanoparticles are being considered in this regard. This study was aimed at testing the antibacterial activities of silver nanoparticles synthesized using *Citrus sinensis* peel extract against pathogens isolated from diseased tomato plant leaves.

MATERIALS AND METHODS

Collection of samples

The diseased tomato plant leaves used for this study were obtained from a tomato farm at Ovia North-East Local Government, Edo State, Nigeria. The fresh orange fruits were got from an orange tree growing in an open place around Ishior, Edo State, Benin city, Nigeria.

Isolation of bacteria pathogens from diseased tomato plants

The diseased tomato plant leave samples were prepared by teasing the plant parts into smaller pieces, followed by surface sterilization with alcohol to remove surface contaminants. The pour plate method of inoculation technique was used for the isolation of phytopathogens. Nutrient agar (NA) medium was used and the inoculated plates were incubated at room temperature (28±2 ^{o}C) for 24 hours.

Identification of bacterial isolates

Cultural characteristics were observed on Nutrient agar plates for the identification of the bacterial isolates. The cultural characteristics include: size, shape, surface, opacity, texture, elevation and pigmentation. Pigmentation was determined by visual observation.

Biochemical tests

Biochemical test was also carried out to further identify the bacterial isolates. The tests include: Gram staining, motility test, catalase test, oxidase test, urease test, citrate test, indole test and carbohydrate fermentation tests.

Synthesis and characterization of silver nanoparticles

Silver nanoparticles were synthesized according to the method described by **Kaviya *et al*. (2011)**. *Citrus sinensis* peel extract was prepared by washing fresh pills with double distilled water, followed by cutting into smaller pieces. About 20 grams of the peels were weighed and transferred into a 250 ml beaker containing 200 ml of sterile distilled water and the mixture was then boiled for 2 minutes. The peels were removed by filtering the extract before using it. An aliquot of 3 ml of orange peel extract was added to 40 ml of 1 mM aqueous solution of AgNO3. The resulting solution was mixed thoroughly and incubated for 30 minutes at room temperature (28±2 ^{o}C). A brownish colour appearance indicated the formation of silver nanoparticles (Plate 1). The bioreduction of Ag+ in solution was monitored using UV–visible spectrophotometer. This was carried out by measuring the absorbance at regular intervals (1, 24 and 48 hours after synthesis) within the wavelength of 300-500 nm.

Plate 1 Silver nanoparticles synthesized using *Citrus sinensis* peel extract

Antibacterial testing

The effect of silver nanoparticle on the test bacterial isolates was carried out using the agar well-diffusion method. The pure cultures of bacteria were sub cultured on nutrient broth medium. Each isolate was swabbed uniformly onto the individual petri dishes containing nutrient agar using sterile cotton swabs. Wells of 4 mm diameter were made on nutrient agar plates using cock borer. Using a micropipette, 60, 80 and 100 µl of nanoparticle solution was poured onto each well on all plates. After incubation at room temperature for 24 hours, the different levels of zone of inhibition of bacteria were measured.

Statistical analysis

Each treatment was in three replicates and results were presented as mean ± standard error. The data obtained were subjected to parametric and descriptive statistics using the Statistical Package for the Social Sciences (SPSS), version 20 software. An alpha value of 0.05 was used as the level of significance.

RESULTS

Figure 1 showed the absorbance values of the phytosynthesized silver nanoparticles measured at different wavelengths after 1, 24 and 48 hours of synthesis. The absorbance values were observed to increase with increasing hours of incubation after synthesis. The maximum absorption values were recorded around the wavelength of 415-450 nm.

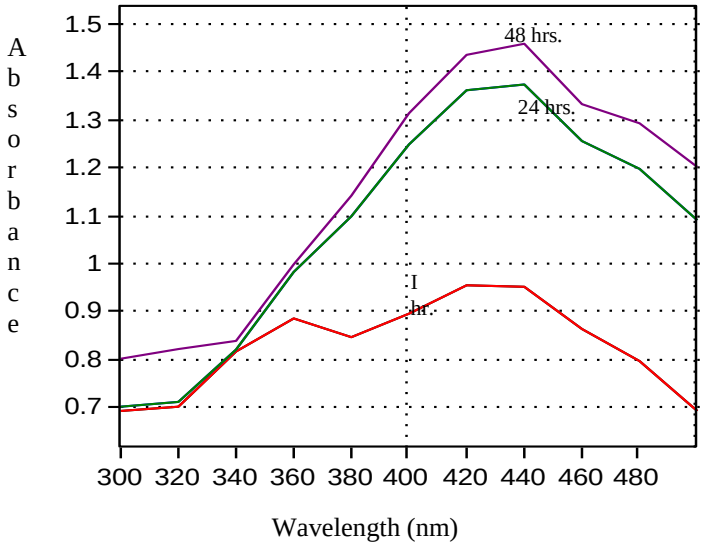

Figure 1 Absorbance values of phytosynthesized silver nanoparticles measured after 1, 24 and 48 hours of synthesis.

In this study, the two bacteria organisms isolated from diseased tomato plant include *Pseudomonas* and *Enterobacter* spp. The biochemical characteristics of the isolated bacteria organisms are shown in Table 1. The results showed that they were both gram negative bacteria. The both isolates had the capacity to metabolize sucrose and glucose and they were both motile, being positive to motility test. *Psedomonas* sp. and *Enterobacter* sp. were both positive to catalase test. The organisms showed some dissimilarity in their biochemical characteristics such as lactose test, urease and oxidase test.

Table 1 Biochemical characteristics of bacteria isolated from diseased tomato plant leaves

Biochemical test	***Pseudomonas* sp.**	***Enterobacter* sp.**
Gram staining	-	-
Lactose	-	+
Sucrose	+	+
Glucose	+	+
H_2S	-	-
Motility	+	+
Urease	+	-
Catalase	+	+
Oxidase	+	-

Legend:
- : negative to test
+ : Positive to test

Table 2 shows the zone of inhibition of silver nanoparticles against *Pseudomonas* and *Enterobacter* species. Silver nanoparticles treatment of 100 µl gave the highest zone of inhibition in both organisms. The highest zone of inhibition (10 mm) of silver nanoparticles was recorded for *Enterobacter* sp. However, there was no significant difference between the zones of inhibition observed for *Enterobacter* sp. subjected to the different amounts of silver nanoparticles, but a significant difference was observed for *Pseudomonas* sp.

Table 2 Zone of inhibition (mm) of silver nanoparticles against *Pseudomonas* and *Enterobacter* species after 24 hours of incubation

Amount of nanoparticles (µl)	***Pseudomonas* sp.**	***Enterobacter* sp.**
60	6.00^{a}±0.00	8.00^{a}±0.50
80	6.00^{a}±0.40	8.00^{a}±0.60
100	8.00^{b}±0.00	10.00^{a}±0.50

Values are presented as means ± standard error; Figures bearing similar superscripts within columns are not significantly different using Duncan's Multiple Range (DMR) test at 0.05 level of significance.

DISCUSSION

The absorbance values of the biosynthesized nanoparticles after 1, 24 and 48 hours of synthesis (Figure 1) showed increase in the various time intervals and the peaks were noticed around 420-445 nm corresponding to surface plasmon resonance of silver nanoparticles. The observation indicated that the reduction of silver ions took place extracellularly. This mechanism of plant assisted reduction has been attributed to the presence of phytochemicals such as terpenoids, flavones, ketones, aldehydes, amines and carboxylic acids in plants **(Panigrahi, 2013)**. Several plant extracts such as *Ocimum teniflorum*, *Solanum tricobatum*, *Syzygium cumin*, *Centella assiatica* and *Citrus sinensis* have been reported for the synthesis of silver nanoparticles **(Logeswari *et al.*, 2015)**. The plant extracts were reported to act as reducing and capping agents, thereby reducing the silver ion (Ag^{+}) to metallic silver (Ag^{o}).

In this study, the two bacteria organisms isolated from the diseased tomato plants include *Pseudomonas* sp. and *Enterobacter* sp. (table 1). Most *Pseudomonas* sp. infect plants, few infect animals or human **(Agrios, 2005)**. For example, *Pseudomonas syriingae*, pv. tomato causes bacteria speck in tomato. The association of *Pseudomonas* sp. with the diseased tomato plant showed that it could be responsible for the symptom observed. However, it is also possible that the organism was just present as a secondary pathogen. *Enterobacter* sp. was also isolated from the diseased tomato plants. This organism seems not to have been reported to cause disease in plants, but several infections such as skin and soft

tissue infections in human have been attributed to *Enterobacter* species **(Grimont and Patrick, 2006).** There is the possibility that mutation might have taken place in *Enterobacter* species, thus, making it pathogenic to plants. However, the possibilities of members of this genus, "*Enterobacter*" as diseases causing agents in crop plants should be further investigated. The biochemical characteristics of the bacterial isolates showed that they were both negative to Gram reaction. This has been attributed to the physical and chemical properties of their cell walls which have a thinner layer of peptidoglycan compared to Gram positive bacteria. Both *Pseudomonas* and *Enterobacter* species are positive to catalase test because they have the capacity to secrete the enzyme catalase which is capable of destroying hydrogen peroxide to release oxygen gas and water.

Table 2 shows the zone of inhibition (mm) of silver nanoparticles against *Pseudomonas* sp. and *Enterobacter* sp. An aliquot of 100 µl of silver nanoparticles gave a larger zone of inhibition (8 mm±0.00 for *Pseudomonas* sp. and 10 mm±0.50 for *Enterobacter* sp.) compared to 80 and 60 µl treatments. This suggested that the nanoparticles were more effective in the control of *Enterobacter* sp. compared to *Pseudomonas* sp. However, Duncan Multiple Range (DMR) test reveals that there was no significant difference (at 0.05 level of significance) in the zone of inhibition observed for *Enterobacter* sp. subjected to the various amounts of silver nanoparticles (60, 80 and 100 µl). There was a significant difference in the zone of inhibition recorded for *Pseudomonas* sp. subjected to different amounts of silver nanoparticles (60, 80 and 100 µl). An aliquot of 100 µl of silver nanoparticle was more effective in the control of *Pseudomonas* sp. with 8.00±0.00 zone of inhibition recorded. The antibacterial activity of silver nanoparticles reported in this study agrees with previous studies that have reported the antimicrobial activity of silver nanoparticles **(Balakumaran *et al*., 2016; Logeswari *et al*., 2015; Kumarasamyraja and Jganathan, 2013; and Suna *et al*., 2014, Obiazikwor and Shittu, 2018)**. The higher zone of inhibition observed for *Enterobacter* compared to *Pseudomonas* could be attributed to the differences in the test organisms.

CONCLUSION

The findings from this study indicated that the phytosynthesized silver nanoparticles have antibacterial activity against the test pathogens. Further studies should be carried out to determine the mode of action of silver nanoparticles. The possibility of members of the genus, *Enterobacter* as causative agent of plant diseases should be further investigated.

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