Research Article

Application of Biosynthesized Silver Nanoparticles for the Control of Land Snail *Eobania vermiculata* and Some Plant Pathogenic Fungi

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The land snail *Eobania vermiculata* is an important crop pest causing considerable damage in agriculture. The aim of the present work is to evaluate the possibilities of using silver nanoparticles (AgNPs) to control the land snail. The AgNPs have been synthesized biologically using white radish (*Raphanus sativus* var. *aegyptiacus*). The biosynthesis was regularly monitored by UV-Vis spectroscopy. X-ray diffraction spectra revealed peaks of crystalline nature of AgNPs and the transmission electron micrographs further confirmed the size of the synthesized nanoparticles ranging from 6 to 38 nm. The exposure of the snails and soil matrix to AgNPs in a laboratory experiment reduced the activity and the viability of the land snail (20% of AgNPs treated snails died) as well as the frequency of fungal population in the surrounding soil. Moreover histology and ultrastructure alterations have been found in both kidney and the digestive gland of AgNPs treated land snails. The synergistic effect of synthesized AgNPs as antifungal was evaluated and clearly revealed that AgNPs can be effectively used against various plant pathogenic fungi. The present study results may open a new avenue to use the snail as bioindicator organism of environmental pollution.

1. Introduction

The synthesis of metallic nanoparticles is an active area of academia and, more importantly, in nanotechnology. Metallic nanoparticles have drawn a lot of attention due to their unusual physical and chemical properties, which largely differ from their bulk properties [1, 2]. The modification of properties was observed due to size effects, modifying the catalytic, electronic, and optical properties of the nanoparticles [3–5]. In the last years, biosynthesis of nanoparticles have received considerable attention due to the growing need to develop clean, nontoxic chemicals, environmentally benign solvents, and renewable materials [6, 7]. Biological processes that are based on bacteria, fungi, bioderived chemicals, and plant extracts are extensively investigated due their eco-friendly protocol and better morphological control [8–10].

Recently, plant (leaf, flower, seed, tuber, and bark) extract mediated biological process for the synthesis of

silver nanoparticles has been extensively explored and compared to other bioinspired processes [11–13]. Haverkamp and Marshall, 2009 [14], have demonstrated the uptake and conversion of metal salts like AgNO_3 , $\text{Na}_3\text{Ag}(\text{S}_2\text{O}_3)_2$, and $\text{Ag}(\text{NH}_3)_2\text{NO}_3$ to metal silver nanoparticles when treated with *Brassica juncea*. Present research has prompted further exploration in the use of plant extracts for the synthesis of silver nanoparticles from white radish extract.

Radish (family Brassicaceae) is widely grown all over the world and commonly seen as a small-rooted, short-season vegetable [15]. Different parts of radish including roots, seeds, and leaves are used for medicinal purposes [16].

Land snails are considered as crop pest which cause damage to agricultural crops or decrease their quality and also may transmit parasites to humans, animals, and plants [17]. The nanotechnology-based pesticides differ from the other synthetic pesticide in surface character and size. Nanomaterials could be useful in biological researches and

applications due to their size which is similar to that of most biological molecules [18] so they can diffuse through cell membranes [19]. Nanomaterials have a toxic effect on gastropods [20]. El-Hommossany and El-Sherbibni, 2011 [21], used nanomaterials to control the freshwater snail *Biomphalaria alexandrina* by reducing its fertility. Jo et al., 2013 [22], used silver nanoparticles as a safe and eco-friendly pesticide. The digestive gland is the main organ for detoxification, nutrient absorption, and metabolism and heavy metals cause alteration of its tissue [23, 24]. Also, kidney of mollusca plays a vital role in metal detoxification and reabsorption [25, 26].

Synthetic chemical fungicides are widely used in conventional agriculture to control plant diseases. Environmental hazards caused by excessive use of pesticides pose health problems as modern society is becoming more health-conscious [27]. Therefore, scientists in the agricultural field are searching for alternative eco-friendly and less capital intensive approaches to control plant diseases. As an alternative to chemically manufactured pesticides, use of silver nanoparticles as antimicrobial agents has become more common as technological advances make their production more economical [28]. So, the objective of this study was to evaluate the possibilities of using biosynthesized silver nanoparticles to control the land snail and plant pathogenic fungi.

2. Materials and Methods

2.1. Biological Synthesis of Silver Nanoparticles. Fresh leaves (5 grams) of white radish (*Raphanus sativus* var. *aegyptiacus*) were washed and boiled in 100 mL distilled water for 5 min. Further, the extract was filtered with Whatman number 1 filter paper and stored at 4°C for further experiments [29]. The AR grade silver nitrate (AgNO_3) was purchased from Sigma-Aldrich Chemicals. Aqueous solution of AgNO_3 (1 mM) was added dropwise into 50 mL of plant leaf extract. The mixture was incubated for 18 hours at room temperature. Control without the AgNO_3 was also kept at the same conditions.

2.2. Characterization of Silver Nanoparticles. The synthesized silver nanoparticles were characterized by ultraviolet-visible spectroscopy, transmission electron microscopy, and X-ray diffraction. Concentration of silver in the solutions was assayed using atomic absorption spectrophotometer (AAS).

2.3. Ultraviolet-Visible Spectroscopy (UV-Vis). The bioreduction of silver nitrate solution and formation of silver nanoparticles by white radish leaf extract were scanned in the 300–900 nm wavelength range using a double beam spectrophotometer (Perkin-Elmer lambda 750 spectrophotometer) [30]. A strong absorption of electromagnetic waves was exhibited by metal nanoparticles in the visible range due to the surface plasmon resonance. The stability of stored biologically synthesized AgNPs was also performed by UV-Vis spectral analysis.

2.4. Transmission Electron Microscope (TEM). The silver nanoparticles formed by the white radish leaf extract were

prepared by placing a drop of synthesized AgNPs on a negative carbon coated copper grids and dried in air. The shape and size of the AgNPs were analyzed and TEM micrographs of the sample were taken using the JEOL TEM 100 CXII (Electron Microscope Unit, Assiut University, Egypt).

2.5. X-Ray Diffraction (XRD). The formation of silver nanoparticles was checked by X-ray diffraction technique using an X-ray diffractometer (Shimadzu XD-3A).

2.6. Snail Experiments. The land snails (*Eobania vermiculata*) were collected from the Assiut University Farm. The snails were acclimatized for several weeks under laboratory conditions (25–28°C with a 12 h light : 12 h darkness). Snails fed on lettuce and were kept in plastic containers full with soil. Two groups of ten snails were used for the main experiment. One group was used as a control (untreated snails). Snails of the second group and their food were exposed with an AgNPs solution (30 ppm) for five successive days. Every day snails were examined and died animals were eliminated. At day 5, after four hours of treatment, five snails of each group were dissected. The soil under the snails was collected and examined for the mycological diversity.

2.7. Histological Studies. To detect the histological and structural alteration in AgNPs treated snails relative to the untreated snails, digestive glands and kidneys were taken from both groups and fixed in Kahle's solution. Paraffin sections (6–7 microns) were prepared and slides were stained with haematoxylin and eosin and examined with light microscope. The other was stained with periodic acid Schiff (PAS). Sections were oxidized in 1% periodic acid for 5 minutes, washed in running tap water for 3 minutes, and then stained in Schiff reagent for 15 minutes. Sections were washed for 10 minutes in running tap water, dehydrated, and then mounted according to Pearse, 1972 [31].

2.8. Preparation for Transmission Electron Microscopy. Tissues were fixed in 2% glutaraldehyde, postfixed in osmium tetroxide, and dehydrated in an ascending ethanol. The samples embedded in Epon 812 according to protocol of the Electron Microscope Unit, Assiut University. Ultrathin sections (60–90 nm) were cut with an ultramicrotome (Reichert Ultracut s), contrasted with uranyl acetate and lead citrate, and examined with a transmission electron microscope (JEOL TEM 100 CXII) at 80 Kv and photographed.

2.9. Energy Dispersive X-Ray (EDX) Spectra. To detect the element composition and the existence of AgNPs in the complex tissues of both digestive gland and kidney, JEOL JSM-5400 L.V. scanning electron microscope (SEM) equipped with a Tracor Northern 5200 energy dispersive (EDX) analysis system was used. Thin film of the sample was prepared on a holder and then allowed to dry by putting it under a mercury lamp.

2.10. Isolation and Identification of Soil Fungi. The fungal community structure of control and treated soil under

the snails were examined by the serial dilution method on potato dextrose agar (PDA) medium. After 7 days of incubation at 25°C, the colony forming units were counted and expressed as CFU/g of soil. All the fungal isolates were morphologically identified.

2.11. Assessment of Antifungal Assay In Vitro. Four pathogenic fungi were isolated and identified as *Fusarium graminearum* (cause head blight of wheat), *Fusarium oxysporum* (cause tomato wilt), *Fusarium solani* (cause potato rot), and *Penicillium expansum* (cause apple rot). Antifungal activity of silver nanoparticles synthesized by white radish was performed by the agar dilution method. The agar medium was supplemented with two concentrations of biogenic AgNPs (15 & 30 ppm). A disc (1.5 cm) of mycelial growth of the tested fungi, taken from the edge of 6-day-old fungal culture, was placed in the center of each plate. The plates with the inoculums were then incubated at 25°C. The efficacy of AgNPs treatment was evaluated after 8 days by measuring the radial growth of fungal colonies [32]:

$$\text{Inhibition rate (\%)} = \frac{R - r}{R}, \quad (1)$$

“where R is the radial growth of fungal hyphae on the control plate and r is the radial growth of fungal hyphae on the plate supplements with AgNPs”

2.12. Statistical Analysis. Statistical analysis was performed by SPSS software. Paired-sample t -tests have been used to determine if there are significant differences between treated and untreated snails. Obtained values of $P < 0.05$ were considered significant.

3. Results and Discussion

There are several physical and chemical methods for synthesis of metallic nanoparticles [33]. However, development of simple and eco-friendly biological systems would help in the synthesis and application of metallic nanoparticles. The feature of using plants for the synthesis of silver nanoparticle is that they are easily available and safe to handle and possess a broad variability of metabolites that may help in reduction. The leaves extract of white radish (*Raphanus sativus* var. *aegyptiacus*) was found to be suitable plant source for the green synthesis of silver nanoparticles. The plant extract was incubated with silver ion (1 mg). Rapid appearance of a yellowish-brown colour in the reaction mixture suggested the formation of colloidal silver nanoparticles [34]. The synthesis process of silver nanoparticles was quite fast and formed within 10 minutes of silver ion. The colour noted by visual observation increased in intensity giving brown colour after 24 h of incubation. This increase in intensity could be due to the formation of more nanoparticles.

The UV-Visible absorption spectra of the aqueous component of radish leaves extract were measured in the range 300–900 nm, using a double beam UV-Vis spectrophotometer. Figure 1 shows a strong broad absorption band located between 405 and 430 nm for silver nanoparticles prepared by

FIGURE 1: UV-Visible spectrum of silver nanoparticles synthesized by radish leaf extract.

FIGURE 2: TEM micrograph of silver nanoparticles synthesized by radish leaf extract.

radish leaves extract. The silver nanoparticles band remaining around 420 nm indicates that the particles were well dispersed without aggregation. This peak, assigned to a surface plasmon resonance (SPR) typical of silver nanoparticles, is well-documented for various metal nanoparticles with sizes from 2 to 100 nm [35, 36]. Figure 2 shows TEM micrograph of silver nanoparticles. TEM analysis is performed to examine the size and shape of synthesized AgNPs. There is a variation in particle sizes from 6 to 38 nm with an average size approximately 21 nm. Most particles are smaller than 15 nm. TEM micrograph clearly reveals that these particles were separated and are not aggregated. Most synthesized AgNPs were spherical.

The XRD pattern of silver nanoparticles powder is shown in Figure 3. The XRD analysis confirmed the presence of Ag nanocrystals and no extra diffraction peaks of oxidation or other crystalline phases present, indicating that the

FIGURE 3: XRD pattern of crystalline silver nanoparticles synthesized by radish leaf extract.

pure silver nanoparticles are crystalline in nature. The XRD pattern shows four diffraction peaks at 2θ values equal to 38.02° , 46.12° , 64.6° , and 77.26° due to reflection from the crystallographic (111), (200), (220), and (311) planes of face-centered cubic silver, respectively. The obtained data was matched with the Joint Committee on Powder Diffraction Standards (JCPDS files number 03-0921).

3.1. Snail Experiment. After characterization of biosynthesized silver nanoparticles (AgNPs) using leaf extract of white radish (*Raphanus sativus* var. *aegyptiacus*), the effect of AgNPs on land snails was studied. The activity of treated snails decreased and two out of ten snails died. Gastropods have the ability to accumulate metals in their tissues [37] especially the digestive gland and the Kidney [38]. So they are used as target organs to study the response of their tissues to metals absorbed [39, 40].

The digestive gland of molluscs is the organ responsible for metabolism, absorption, and detoxification [38]. So, the digestive gland was used in this study to illustrate the effect of AgNPs. The hazard effect of NPs is due to their high reactivity, large surface area, and small size [39].

3.1.1. Digestive Gland

(1) Light Microscopy

(a) Untreated Snails (Control Group). Examination of sections of untreated snails showed that the digestive gland consists of many tubules. Each tubule is lined by four types of cells laying on a basement membrane. These cells are digestive cell (the most common cell type), calcium cell, excretory cell, and thin cell (sometimes could not be distinguished). In such tubules the different cell types are arranged around a narrow lumen (Figure 4(a)). The same results were obtained when studying the digestive gland of *Eobania vermiculata* and *Monacha cartusiana* [41, 42].

(b) Treated Active Snails Group. In treated active snails, the size and the lumen of many digestive tubules increased and the cells lining the tubules are irregularly arranged. The apical part of some cells separated to form blebs which are signs of cell death (Figure 4(b)) [43].

(c) Treated Inactive Snails. The feeding capacity of these snails decreased. The snails lose their activity and stay in the bottom of the container. The excretory cells (ex.c.) increased in number (Figure 4(c)). Some cells changed in their morphology. Hemocytes infiltration was detected. Many of the epithelial cells became vacuolated (Figure 4(d)).

An increase in the calcium cells number, swelling, and abnormal apices of the digestive cells in *Marisa cornuarietis* treated with copper and lithium was reported [37]. Also, the vacuolization in the digestive cells was recorded by Ünü et al., 2005 [44], in the study of the effect of Thiodan on *Lymnaea stagnalis*.

In the study of the effect of Pt (platinum) on the tissues of the digestive gland of *Marisa cornuarietis* many alterations were recorded such as the irregular shape of cells (cells became flattened) dilatation of the intertubular spaces, enlarged tubule lumen, destruction of tubules, necrosis of digestive, and basophilic cells and vacuoles increased in number [45].

(2) Electron Microscopy. Transmission electron microscopy of the digestive gland of inactive treated snails showed a decrease in the size of calcium cells in comparison with the untreated snails (Figures 5(a) and 5(b)). Also, the size and number of calcium granules decreased in the inactive treated snails (Figure 5(b)). The structure of the mitochondria of the digestive cells appeared small and spherical of moderate electron density (Figure 6(a)), while in the inactive treated snails become vacuolated with the presence of small electron dense particles in the cytoplasm and homogenous light electron dense granules with variable size (g) (Figure 6(b)). Excretory cells had large vacuoles that occupied most of the cell (Figure 6(a)). The presence of altered compartments in the digestive gland of *Marisa cornuarietis* treated with copper and lithium was reported [45].

In the present study the nucleus of the cell situated peripherally and is surrounded by small amounts of cytoplasm containing cell organelles and the nuclear chromatin condensed against the nuclear envelope (Figure 7). These results are signs of cell death [43]. The damage of the digestive gland may alter its functions of absorption, digestion, excretion, and secretion.

3.1.2. Kidney

(1) Light Microscopy

(a) Untreated Snails (Control Group). Light microscopic study of untreated kidney showed that the epithelial cells have central nuclei. Some epithelial cells contain large apical vacuole and their cytoplasm faintly was stained with Periodic

FIGURE 4: (a–d) Light micrographs: (a) the digestive gland showing a digestive tubule lined with four types of cells: digestive cell (d.c.), calcium cell (c.c.), excretory cell (ex.c.), and thin cell (t.c.). Narrow lumen (l). (b) The digestive gland of active treated snails showing presence of cellular blebs (arrows) with wildness of the lumen of the digestive tubules. (c) The digestive gland of inactive treated snails showing increases of the number of the excretory cells (ex.c.). (d) The digestive gland of inactive treated snails showing hemocytic infiltration (h) and vacuolation of the epithelial cells (v) (scale bar = 20 μ m).

FIGURE 5: (a, b) TEM micrographs: (a) the normal calcium cells of untreated snails (g = granules). (b) The calcium cells showing decrease in the size in the inactive treated snails with decrease in size and number of calcium granules (g.).

acid-Schiff stain (PAS); the other cells with no vacuoles and their cytoplasm and granules are deeply stained with PAS (Figures 8(a) and 8(b)). The two types of epithelial cells of molluscan kidney have a great role in accumulation of metals, excretion and may have the ability of reabsorption [26].

(b) *Treated Inactive Snails.* Many pyknotic peripheral nuclei were detected (Figure 8(c)). Most of the epithelial cells of the kidney of treated snails contain large vacuole and the cytoplasm was faintly stained with PAS (Figure 8(d)). The presence of granules that stained with PAS in the kidney cells

FIGURE 6: (a, b) TEM micrographs: (a) the digestive gland of untreated snails showing digestive cell (d.c.) containing small spherical mitochondria (arrows), excretory cell (ex.c.), and nucleus (n). (b) The digestive cells containing vacuolated mitochondria, small electron dense particles and homogenous light electron dense variable size granules (g), and nucleus (n).

FIGURE 7: TEM micrograph: excretory cells with peripheral cytoplasm (c), large vacuole (v), peripheral nucleus (n), aggregation of organelles beside the nucleus (o), and nucleus (n).

also detected in *Mya arenaria* that collected from polluted sediments [46].

(2) *Electron Microscopy*. In the untreated kidney the organelles in the epithelial cells present mostly in the lower portion of the cell where the situation of the nucleus is in the upper portion (Figure 9(a)). While the epithelial cells of inactive treated snails contain a spherical nucleus and marked vacuolization of cytoplasm with marked decreases of the cell organelles (Figure 9(b)).

The alteration in the structure of the kidney was also reported in the study that made evaluating the effects of the sublethal exposure to cadmium on the kidney of the *Littorina littorea* [40]. Vacuolization of the epithelial cells detected and

aggregation of organelles may be due to alteration in the excretory activity due to exposure to NPs.

3.2. The EDX Analysis. The EDX analysis (energy dispersive X-ray analysis) is an analytical method for complex samples; it is very useful tool to identify the existence of AgNPs. It was performed and represented in Figures 10 and 11 to investigate the elemental composition of the sample. The EDX analysis exhibits various intense peaks associated with Ag atoms peaks, Na, Mg, Al, P, S, Cl, K, Ca, Fe, Cu, and Zn. The accumulation of Ag was detected only in treated kidney and digestive gland. The concentration of K in the digestive gland significantly increased ($P = 0.05$), the concentration of Ca in the kidney decreased ($P = 0.02$), and the concentration of Fe in the kidney increased ($P = 0.04$).

The result obtained from the elemental spectra demonstrated the accumulation of Ag in both digestive gland and kidney tissues. It also revealed an alteration in the element composition of these organs which mean alteration in their function [47].

3.3. Soil Fungi. The results of our present investigation depict biologically synthesized AgNPs induced decrease in frequency of fungal population in treated soil. Data in Table 1 revealed that 13 species belonging to 8 genera were isolated from untreated soil under the snails, whereas 10 species belonging to 7 genera were isolated from soil treated with AgNPs. The total counts of soil fungi were 105.7×10^3 CFU per g dry soil, of which untreated soil was the richest in the fungal population giving rise to 66.7×10^3 CFU/g dry soil. Remarkably, the genera *Aspergillus*, *Penicillium*, and *Trichoderma* were the most common genera. Complete inhibition of *Aspergillus fumigatus*, *Cochliobolus specifier*, and *Fusarium solani* was noted with treated soil, while comprising

FIGURE 8: (a–d) Light micrographs: (a) kidney of untreated snail showing (I) epithelial cells with large apical vacuole, (II) cells with no vacuoles, and central nuclei (n). (b) Kidney of untreated snail showing (I) cells with faint PAS staining, (II) cells with deeply stained cytoplasm, and granules. (c) Kidney of treated snail showing pyknotic peripheral nuclei (arrows). (d) Kidney of treated snail showing (I) cells with greatly decreased number of cells, (II) cells with deeply stained cytoplasm, and granules increased in number (scale bar = 20 μm).

FIGURE 9: (a, b) TEM micrographs: (a) untreated kidney showing: (I) cell contain large vacuole and (O) the cytoplasm, (II) cell with aggregated organelles (arrows). (b) Epithelial cells of treated kidney with small amount of organelles aggregated within the vacuolated cytoplasm (arrow).

4.8%, 3.4%, and 1.2% of total fungi isolated from untreated soil.

In the present study the biosynthesized silver nanoparticles showed excellent fungicide and that the green metal silver nanoparticles could be efficacy control pathogenic soil fungi. Fungicides have broad spectrum antimicrobial activity,

but it is reasonable to state that the smaller particles having the larger surface area available for interaction will give more fungicidal effect than the larger particles. It has been suggested that the toxicity of AgNPs is primarily due to the free silver ions released by the NPs [48]. While antimicrobial properties of ionic silver have long been known, the

FIGURE 10: (a, b) EDX Photographs: (a) untreated digestive gland. (b) Treated digestive gland.

FIGURE 11: (a, b) EDX Photographs: (a) untreated kidney. (b) Treated kidney.

TABLE 1: Colony forming unit (CFU) of fungal species isolated from soil, amended with or without AgNPs.

Fungal species	CFU/g soil $\times 10^3$	
	Without AgNPs	With AgNPs
<i>Aspergillus flavus</i>	8.4	3.6
<i>Aspergillus fumigatus</i>	3.2	0
<i>Aspergillus japonicus</i>	3.5	2.1
<i>Aspergillus niger</i>	18.5	12.6
<i>Cladosporium cladosporioides</i>	5.1	4.7
<i>Cochliobolus spicifer</i>	2.3	0
<i>Epicoccum nigrum</i>	1.2	0.5
<i>Fusarium oxysporum</i>	1.6	1.2
<i>Fusarium solani</i>	0.8	0
<i>Penicillium funiculosum</i>	6.7	5.3
<i>Penicillium duclauxii</i>	5.4	1.3
<i>Rhizopus stolonifer</i>	3.9	1.9
<i>Trichoderma harzianum</i>	6.1	5.8

complexities encountered in various environments combined with the unique properties and behaviors of AgNPs (and nanoparticles in general) complicate estimations of the fate of released AgNPs and their organismal interactions [49, 50].

3.4. Antifungal Assay In Vitro. The inhibitory effect of biologically synthesized AgNPs of different concentrations (15

and 30 ppm) was analyzed in PDA (Figure 12). All tested pathogenic fungi were inhibited to various extents by AgNPs. The lowest level of inhibition was observed against *Fusarium oxysporum* on PDA treated with a 15 ppm concentration of AgNPs, while the highest level of inhibition was observed against *Fusarium solani* treated with a 30 ppm concentration of AgNPs (91%). The results clearly demonstrated that biologically synthesized AgNPs are hopeful antifungal agents against the plant pathogenic fungi. The use of silver nanoparticles as antimicrobial agents has become more common as technological advances make their production more economical.

One of the potential applications in which AgNPs can be utilized is in management of plant diseases. Since AgNPs display multiple modes of inhibitory action to microorganisms [51], they may be used for controlling various plant pathogens in a relatively safe way compared to synthetic fungicides [51]. The antifungal mechanism of AgNPs may be due to the fact that the formation of free radicals produced from the nanoparticles could disturb the membrane lipids and then finally spoil the membrane functions [52, 53]. Stoimenov et al. [54] and Sondi and Salopek-Sondi [55] have depicted a new finding that the membrane could be deteriorated by the formation of pits on the surface of the cell wall membrane of microorganisms. The formation of pits on the membrane leads to increase in the permeability and irregular transport that result in the death of the cells. So, the green-synthesized

FIGURE 12: Inhibitory rate (%) of biologically synthesized AgNPs on some plant pathogenic fungi.

silver nanoparticle is a good source, which is easily produced and extensively useful in agricultural applications.

Conflict of Interests

The authors declare that they have no competing interests.

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