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Synthesis and coating of nanosilver by vanillic acid and its effects on *Dunaliella salina* Teod.

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A B S T R A C T

Plant phenolics have high reducing capacity which can be exploited in the synthesis of nanomaterials. In the present study, phytoreductant vanillic acid is used to produce and coat silver nanoparticles. The effects of Ag nanoparticles on the unicellular green algae *D. Salina* were then investigated. Under optimum pH and temperature, silver ions were reduced to silver metal by vanillic acid. The absorption spectra of the silver nanoparticles showed a maximum band of 410 nm, which is characteristic of the surface plasmon resonance of silver nanoparticles. Dynamic light scattering (DLS) showed a narrow distribution size with an average of 52 nm. High concentrations of Ag nanoparticles reduced growth, total carotenoids, chlorophyll content, phenolics and antioxidant activity of the algae. Based on these results, phytoreductant vanillic acid can be used for synthesis and coating of nanosilver. Due to the projected increase in quantities and types of nanomaterials which leads to their elevated release into the environment and also because of the toxicity of nanomaterials, an urgent need to evaluate the impacts of nano-sized particles on the environment and living organisms is felt.

Key words: Green Chemistry, Phenolic compounds, Ag nanoparticles, *Dunaliella salina*

INTRODUCTION

Nanoscale-sized particles, such as metals and metal-oxide nanoparticles, quantum dots and carbon nanotubes have attracted the attention of many scientists because of their unique optical, electronic, magnetic and chemical properties. As a result, applications in different areas such as medicine, optics, biological sensors and electronic devices have been found [1-4]. Various methods have been used to synthesize nanostructured metals [5]. Utilizing chemical reducing agents, such as hydrazine and sodium borohydride, has been

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employed extensively [3, 6]. Recently, there has been growing interest in developing environmentally friendly processes of synthesizing nanoparticles without using or generating substances hazardous to the human health and the environment [5, 7]. Biological methods for the synthesis of the metallic nanoparticle using microorganisms and plant extracts have been considered as possible eco-friendly alternatives to chemical synthesis [8-11]. In microbial and plant extracts, the reduction of metal ions and coating of synthesized nanometals could be due to different metabolites, particularly different redox active metabolites. Therefore, mechanisms of reduction and coating are not clear. Synthesis of metallic nanoparticles using pure plant primary and secondary metabolites as reductants has attracted considerable research interest [12-14]. Using gallic acid as a reducing and coating agent, it was possible to synthesized silver nanoparticles with different sizes by varying some reaction parameters [15]. Accordingly, the oxidation of phenol groups to quinoid compound was responsible for both reduction and stabilization of silver nanoparticles [15]. Together with glucose, gelatine as a natural biopolymer with good biocompatibility and biodegradability have also been used for silver nanoparticle synthesis [16]. The obtained nanoparticles were uniform and had a narrow particle size distribution with an average diameter of 5 nm.

Although antimicrobial effects of silver nanoparticles are well established [17, 18], there is growing concern regarding the biological and environmental impact of nanomaterials including silver nanoparticles. Nanosilver has concentration- and size- dependent cytotoxicity [19]. The production of reactive oxygen species (ROS) and the release of silver ions have been suggested as the mechanisms of cytotoxicity [20].

Plant phenolics constitute a large class of natural antioxidants. They are multifunctional and can act as reducing agents, hydrogen donating antioxidants and singlet oxygen quenchers [21]. In the phenolic acid class, vanillic acid has intermediate antioxidant activity which may be used as a reducing agent for nanometal production. In this study, we report the process of synthesis and coating of silver nanoparticles by vanillic acid. The obtained nanoparticles were characterized by dynamic light scattering (DLS) and ultraviolet-visible (UV- Vis) absorption spectroscopy. Also, in this study, the cytotoxicity of synthesized nanoparticles was investigated using unicellular green algae *Dunaliella salina*. Changes in growth, pigment contents and antioxidant capacity as affected by the synthesized nanosilver are reported.

MATERIALS AND METHODS

Synthesis and characterization of nanosilver: reen synthesis of silver nanoparticles was carried out using phytoreductant vanillic acid (Sigma-Aldrich, 99%). First, 200 mg vanillic acid was dissolved in 5 mL ethanol and the volume was increased to 100 mL using distilled water. Then, in a 15 mL vial containing 2 mL 10 mM silver nitrate (Sigma-Aldrich), 10.0 mL vanillic acid stock solution was added and thoroughly mixed. The pH of the solution was immediately adjusted to 10.0 by 1.0 M NaOH. The colour of the solution changed from colourless to yellow and finally to dark brown indicating silver nanoparticle formation. Since silver nanoparticles show characteristic surface plasmon resonance, the UV-Vis absorption spectra of the synthesized particles were recorded over the range of 300

to 800 nm using a Shimadzu UV-160 A spectrophotometer. The silver nanoparticles were further characterized by dynamic light scattering (DLS) using HORIBA LB- 550, which determines particle size distribution. The median diameter of nanoparticles is reported (Fig. 4).

Algal strain and culture condition: *Dunaliella salina*, previously isolated from Maharlu salt lake Shiraz, Iran, and identified based on rDNA ITS sequences (NCBI accession no. KC477401) was used to evaluate the cytotoxicity of silver nanoparticles. 250 mL glass flasks containing 100 mL of culture medium as described by Ben-Amotz et al. [22] were added to an algal suspension to give the initial cell density of 10^5 cells mL^{-1}. The cultures were kept in a growth chamber under a continuous and constant light intensity of 100 µmol m^{-2} s^{-1} at 21± 2º C. At mid-logarithmic growth phase, algal cells were exposed to 0.0, 25, 50,100 and 200 µM of nanosilver and the cultures were incubated as above for 1, 3 and 10 days. At indicated time intervals, the samples were taken for growth, chlorophyll, carotenoids, phenolics and antioxidant capacity determinations.

Measurements: Cell counting was preformed using a hemocytometer under a light microscope. Total chlorophyll and total carotenoids were extracted using 80% acetone and quantitative determinations were carried out as described by Lichtenthaler and Buschmann [23].

Total phenolics were extracted and measured according to Hajimahmoodi et al. [24]. In brief, 200 µL standard solution or sample was mixed with 1.5 mL Folin-Ciocalteu reagent previously diluted tenfold with distilled water. After 5 min, 1.5 mL 6% (w/v) sodium bicarbonate solution was added to the solution. The mixture was incubated for 90 minutes at room temperature and the absorbance was measured at 750 nm. The standard curve was constructed using gallic acid standard solutions (25-125 µg mL^{-1} in 50% methanol). The results are presented as pg gallic acid equivalent $cell^{-1}$.

Antioxidant activity measurements were done as described by Thaipong et al. [25]. In brief, the FRAP reagent contained 2.5 mL 10 mM TPTZ (2, 4, 6-tripyridyl-S-triazine) solution in 40 mM HCl, 2.5 mL of 20 mM $FeCl_3$ and 25 mL of 300 mM acetate buffer pH 3.6. It was freshly prepared and warmed at 37°C. 150 µL standard solution or sample was mixed with 2850 µL of the FRAP reagent and kept in a dark place for 30 min. The absorbance of the produced ferrous tripyridyltriazine complex was recorded at 593 nm. The standard curve was linear between 25 -800 µM Trolox. Additional dilution was made in case the measured FRAP value was over the linear range of the standard curve. The antioxidant activities are expressed as pg Trolox equivalent $cell^{-1}$ (pg TE $cell^{-1}$).

RESULTS

Silver nanoparticles properties: The production of silver nanoparticles due to reduction of silver ions by vanillic acid (Fig. 1) was followed by colour change and UV-Vis spectroscopy. The colour of the reaction mixture changed gradually from transparent to yellow and then to brown indicating the formation of silver nanoparticles (Fig. 2). In UV-Vis spectra, the synthesized silver nanoparticles displayed a surface plasmon resonance

peak at 410 nm which confirms the production of silver nanoparticles (Fig. 3). Particle size distribution of the nanosilver analyzed by dynamic light scattering showed a peak centered at 52 nm (Fig. 4).

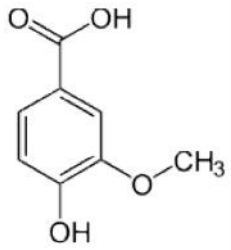

Figure 1. Structure of vanillic acid (4-hydroxy-3-methoxy benzoic acid).

Figure 2. Photographs of solutions of silver nitrate (1.7 mM) before (A) and after (B) exposure to the vanillic acid.

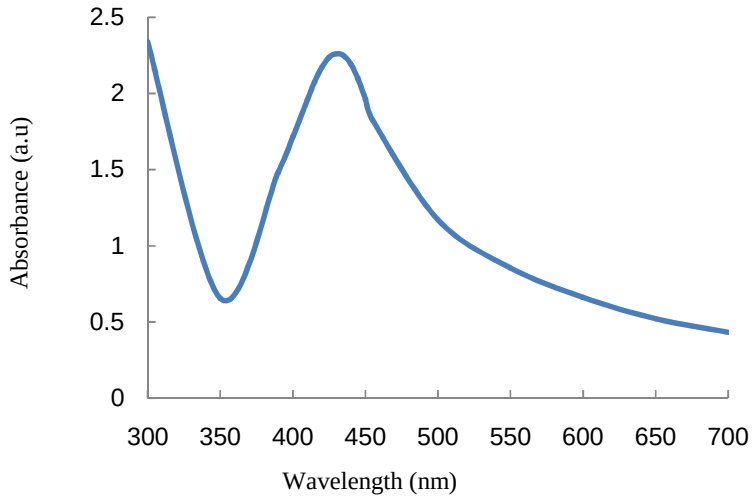

Figure 3. The UV-Vis absorption spectra of the silver nanoparticles synthesized by vanillic acid.

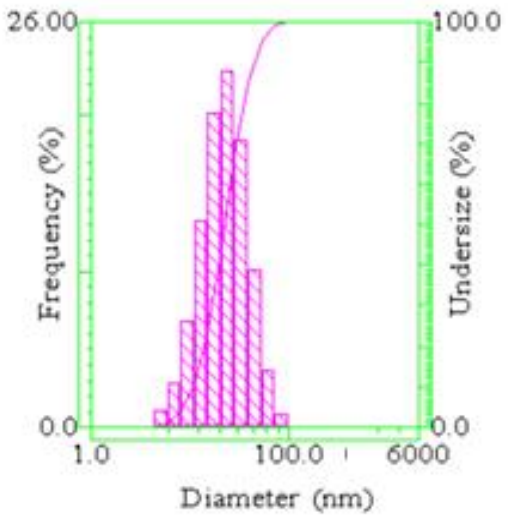

Figure 4. Particle size distribution of synthesized silver nanoparticles analyzed by Dynamic light scattering (DLS) method.

Responses of *D. salina* to silver nanoparticles: In the absence of Ag NPs (control), growth and reproduction, expressed as the number of cell mL^{-1} of algal culture, increased over 10 days of the experimental period (Table 1). At 25 and 50 µM AgNPs, the increase in cell number, determined at 1, 3 or 10 days after the start of experiment, was not significantly ($P< 0.05$) different from that of the control. At 200 µM AgNPs, the cell number decreased significantly ($P< 0.05$) at all time intervals (1, 3 and 10 days) tested compared to the control.

Table 1. Cell number ($mL^{-1}\times10^4$) as affected by different concentrations of silver nanoparticles.

	Ag NPs (µM)				
Time (Day)	0	25	50	100	200
1	151.12±4.5	151.00±6.3	141.00±8.2	134.20±4.9	104.20±4.5*
3	220.83±3.1	219.80±2.0	210.80±6.0	189.20±4.0*	120.30±3.8*
10	298.70±4.8	298.50±7.9	266.20±8.0	92.50±10.0*	110.30±9.5*

Values are mean ± SE, n=3. In each row, significant differences at $P < 0.05$ are shown by asterisks.

Chlorophyll content in control cultures gradually increased over the experimental period (Table 2). At 25 and 50 µM AgNPs, changes in the chlorophyll content were not significantly different from the control, while at 100 and 200 µM, chlorophyll content significantly reduced after 10 days of exposure to nanosilver.

Table 2. Effects of silver nanoparticles on chlorophyll content (µg mL^{-1}).

	Ag NPs (µM)				
Time (Day)	0	25	50	100	200
1	3.11±0.12	3.24±0.22	3.34±0.15	3.30±0.11	3.07±0.18
3	4.18±0.14	4.39±0.17	4.70±0.15	4.73±0.09	4.12±0.27
10	4.19±0.21	4.00±0.19	4.16±0.24	2.51±0.09*	0.73±0.20*

Values are mean ± SE, n=3. In each row, significant differences at $P < 0.05$ are shown by asterisks.

Compared to chlorophyll, the carotenoids content was more susceptible to nanosilver (Table 3). One day after the beginning of the experiment, the carotenoids content significantly decreased at 200 µM nanosilver, while after ten days, the decrease in the carotenoids content was even significant at 50 µM AgNPs.

Table 3. Effects of silver nanoparticles on carotenoids content (µg mL^{-1}).

	Ag NPs (µM)				
Time (Day)	0	25	50	100	200
1	2.09+±0.14	2.06±0.20	1.82±0.15	1.86±0.15	1.43±0.13*
3	4.62±0.23	4.00±0.11	3.83±0.10	2.68±0.07*	1.50±0.23*
10	8.95±0.30	8.88±0.35	4.91±0.40*	4.21±0.21*	0.92±0.06*

Values are mean ± SE, n=3. In each row, significant differences at $P < 0.05$ are shown by asterisks.

Total phenolic compounds, expressed as gallic acid equivalent (GAE pg $cell^{-1}$), and antioxidant capacity, reported as trolox equivalent (TE pg $cell^{-1}$), also significantly decreased at 100 and 200 µM nanosilver (Table 4).

Table 4. Antioxidant activity (pg TE $cell^{-1}$) and total phenolic content (pg GAE, $cell^{-1}$) of *D. salina* as affected by different concentrations of silver nanoparticles.

	Ag NPs (µM)				
	0	25	50	100	200
Total Phenolic Content (pg GAE, cell -1)	3.68+±0.12	3.37±0.10	3.34±0.14	2.67±0.11*	1.61±0.09*
Trolox Equivalent (pg TE cell -1)	11.03±0.78	10.92±0.42	10.85±0.34	9.70±0.22*	3.66±0.11*

Values are mean ± SE, n=3. In each row, significant differences at $P < 0.05$ are shown by asterisks.

DISCUSSION

Various plant extracts have been used as environmentally friendly and safe reducing agents for silver nanoparticles synthesis [26, 27], but the capacity of their reducing

constituents for the production of metal nanoparticles has not been investigated for many natural products [13]. Plant phenolics are strong antioxidants with high reducing capacity [28] which can be used for nanosilver synthesis [15]. In the phenolic acid class, vanillic acid shows intermediate antioxidant activity equivalent to 1.43 mM trolox [21]. It has been reported that vanillic acid scavenges cellular reaction oxygen species in H_2O_2- treated BNLCL2 cells [29]. Due to the electron donating ability of vanillic acid, silver ions are reduced to nano scale-sized silver particles. The quinoid compound produced due to the oxidation of the phenol group in phenolics can be adsorbed on the surface of nanoparticles, accounting for their suspension stabilization [30].

Silver nanoparticles are one of the most widely used nonmaterials [31]. Since they can be easily transported into an aquatic environment [32], their environmental impact on aquatic ecosystems needs to be investigated. In the present study, the content of the growth pigments and the antioxidant capacity of *D. salina* significantly reduced at high concentrations (about 100 µM) of nanosilver. At 10 mg L^{-1} nanosilver, 88 and 96% reduction in viable cells was observed for *Chlorella vulgaris* and *Dunaliella tertiolecta*, respectively. Excessive reduction in chlorophyll content and increased lipid peroxidation were also reported in these species [31]. Silver nanoparticles caused gill pathology and mortality in zebrafish with LD_{50} of 7.2 mg L^{-1}. Nanosilver also caused morphological abnormalities in zebrafish larvae [33]. As indicated by Wang et al [34], the projected increase in quantities and types of nanomaterials for industrial and consumer products could lead to significant release of nanomaterials into the environment. An urgent need is thus felt to evaluate the impact of nanomaterials on the environment, on organisms and on human health.

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