Making good use of the byproducts of cultivation: green synthesis and antibacterial effects of silver nanoparticles using the leaf extract of blueberry

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Revised: 8 August 2017 / Accepted: 15 August 2017 / Published online: 29 August 2017
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Abstract In this study, we made an effort to use blueberry leaves extract as a reducing and a capping agent to biosynthesize silver nanoparticles (AgNPs). The successful formation of AgNPs was confirmed with UV–visible spectroscopy, transmission electron microscopy, Fourier transform infrared spectroscopy and X-ray diffraction. Furthermore, their antimicrobial activities against four kinds of multi drug resistant clinical pathogens and aquatic pathogens were investigated. The results showed that as-prepared AgNPs exhibited broad-spectrum antibacterial activities. The antibacterial effects of the AgNPs based on the structural damage and DNA degradation of the tested bacteria were also studied. The green synthesis of AgNPs using blueberry leaf extract, which provided a new idea on the use of the byproducts of blueberry harvesting.

Keywords Blueberry leaf · Green biosynthesis · Silver nanoparticles · Antibacterial activity · Clinical pathogen · Aquatic pathogen

Introduction

As promising antimicrobial agents, silver nanoparticles (AgNPs) have received much attention in the past decade (Abbai et al. 2016). When the AgNPs are synthesized by the conventional methods, the use of toxic chemicals as reducing agent and stabilizing or capping agents is unavoidable (Velusamy et al. 2015). To synthesize AgNPs in an environmentally friendly manner, biological systems including yeast, fungi, bacteria, and plant have been widely investigated (Soman and Ray 2016).

Plant extracts are potentially exciting biological agents for the synthesis of metal nanoparticles (Dubey et al. 2010; Velmurugan et al. 2014). Since plant extracts are rich in secondary metabolites, such as phenolic compounds, alkaloids, proteins, flavonoids and saccharides, there is no need to use any reductants, capping agents, or templates for the formation of stable nanoparticles. To date, several plant species such as *Ocimum sanctum* (Rao et al. 2013), *Ziziphus oenoplia* (Soman and Ray 2016), *Dillenia indica* (Singh et al. 2013), *Azadirachta indica* (Velusamy et al. 2015), *Malva parviflora* (Zayed et al. 2012) and *Pongamia pinnata* (Priya et al. 2016) have been reported to be used to synthesize AgNPs. In our previous studies, we reported the biosynthesis of AgNPs with the aid of *Gynostemma pentaphyllum* leaf and *Ipomoea batatas* root extract (Wang et al. 2016a, b). Compared with that prepared by conventional methods, the AgNPs prepared using biological materials have better dispersion, smaller size and higher permeability, which are favoured by the cosmetic, textile,

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Electronic supplementary material The online version of this article (doi:10.1007/s13197-017-2815-1) contains supplementary material, which is available to authorized users.

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medical and food industries (Patil et al. 2012). Therefore, it is necessary to have available, low-cost and efficient biological materials to synthesize AgNPs in a large-scale to satisfy the increasing demand.

Blueberry (*Vaccinium* spp.) fruits are well known as “superfood” for their high content of bioactive natural products, including phenolic compounds, flavonoids, tannins and anthocyanins (Liu et al. 2015; Song et al. 2016). Blueberry leaves are the main byproducts of harvesting, and tons of blueberry leaves are wasted each year. The composition and beneficial properties of blueberry leaves have attracted the interests of scientists in the past few years, and the blueberry leaves have been viewed as a potential source of antioxidant and natural antimicrobial agent. Recent studies suggested that the bioactive metabolites in blueberry leaves were similar to those of the fruits or even richer and higher (Ferlemi and Lamari 2016). For example, Harris et al. (2007) identified ten different compounds in the leaf extract of lowbush blueberry (*V. angustifolium*). Among them, chlorogenic acid was the most abundant phenolic compounds, and its content was 30 times higher than that of the fruits. Previously, we also reported that the vitamin C content of blueberry leaves were much higher than that of fruits (Liu et al. 2015). Moreover, highbush blueberry (*V. corymbosum*) leaf extract was reported to act as an antimicrobial agent, especially against *Salmonella typhimurium* and *Enterococcus faecalis* (Pervin et al. 2013). Therefore, blueberry leaves may be utilized as a source of bioactive natural products for use as food supplements, nutraceuticals or antimicrobial agents.

In this study, we tried to utilize the blueberry leaves, the byproducts of planting industry, to synthesize AgNPs. The biosynthesized AgNPs were thoroughly characterized by UV–visible spectroscopy (UV–Vis), transmission electron microscopy (TEM), X-ray diffraction (XRD) and Fourier transform infrared (FTIR). The as-prepared AgNPs exhibited superior antibacterial activities against clinical multidrug resistant pathogens and aquatic pathogens. Moreover, the antibacterial effects of the AgNPs based on the structural damage and DNA degradation of the tested bacteria were investigated.

Materials and methods

Materials

Fresh leaves of blueberry *V. corymbosum* were collected from an organic blueberry farm in Yantai, Shandong, China (37°31'N, 121°21'E) and washed thoroughly with double distilled water. After the leaves were dried, they were stored at 4 °C before the extraction.

Preparation of blueberry leaf extract

Dried leaves (5.0 g) were cut into small pieces and then extracted at 80 °C for 1 h with 100 mL of distilled water. The obtained extract was cooled down and filtered through Whatman No. 1 filter paper, and further centrifuged at 3000 rpm for 10 min. The supernatant was collected and stored at 4 °C for further use.

Biosynthesis of AgNPs

The obtained leaf extract (2 mL) was added into 20 mL of 1 mM AgNO₃ solution at 90 °C with constant stirring. In order to optimize the reaction time for the synthesis of AgNPs, color change of the solution was recorded within 60 min.

Characterization of AgNPs

The biosynthesized AgNPs were monitored with UV–Vis (Shimadzu, UV-2550) in the range of 200–800 nm. The shape and size of AgNPs were analyzed with TEM (JEM 2100) at an acceleration voltage of 200 kV. The XRD analysis of the synthesized material was performed on an X-ray diffractometer (Rigaku, D/max-2500VPC) with Ni-filtered Cu K α radiation. The size of the AgNPs was calculated according to the following Scherer's equation (Miri et al. 2015),

$$D = k\lambda / \beta \cos \theta$$

where D is the average crystal size, k is the Scherer coefficient (0.89), λ is the X-ray wave length ($\lambda = 1.5406$ Å), θ is Bragg's angle (2θ), β is the full width at half maximum (FWHM) in radians. FTIR spectra of vacuum-dried leaf extract and AgNPs were recorded on a spectrophotometer (Nicolet, Nexus 670) in the diffuse reflectance mode in KBr pellets.

Antimicrobial tests

Pathogens

Eight pathogenic bacteria, including four clinical pathogens [*Escherichia coli* (ATCC 25922); *Staphylococcus aureus* (ATCC 25923); *Pseudomonas aeruginosa* (ATCC 27853); *Bacillus subtilis* (ATCC 21332)] and four aquatic pathogens [*Vibrio anguillarum* (ATCC 19264); *Vibrio alginolyticus* (ATCC 17749); *Aeromonas punctata* (ATCC 15467); *Vibrio parahaemolyticus* (ATCC 17802)] were used as model strains for antibacterial test.

Inhibition zone test

As described previously, the cup-plate assay was used to study the antibacterial activity of the prepared AgNPs (Wang et al. 2016a, b). The four tested clinical pathogenic bacteria were incubated into Luria–Bertani (LB) medium (Code NO. A500859, Sangon Biotech, China) and shaken at 37 °C, and the other aquatic pathogens were cultured at 28 °C in 2216E culture medium (Code NO. HB0132-1, Hopebio Biotech, China). After 12 h of culture, the bacterial suspension was adjusted to 10^6 CFU per mL^{-1} , and each sample of 100 μL was spread uniformly onto agar plates. The plates for clinical pathogens were incubated overnight at 37 °C, while for aquatic bacteria at 28 °C. The inhibition zones were measured to evaluate the antibacterial performance. Mean values $\pm$ SD of three replicates were reported, and significant differences were defined as $P < 0.05$ in Duncan's analysis.

Minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) test

For the MIC and MBC measurement, the standard tube double dilution method was performed. *Escherichia coli* was chosen as the model organism and the culture was incubated overnight at 37 °C. The bacterial suspension was adjusted to 10^6 – 10^7 CFU per mL^{-1} , and then the same volume of the prepared AgNPs with different concentration was added. The final concentration of AgNPs in each tube was obtained as follows: 65.0, 32.5, 16.3, 8.2, 4.1, 2.0, 1.0 and 0.5 μg per mL^{-1} . The LB medium was used as negative control, and 10^6 – 10^7 CFU per mL^{-1} bacterial suspension as positive control. Bacterial growth was observed after 24 h of incubation at 37 °C. The lowest concentration of the prepared AgNPs at which no bacterial growth was observed in the culture suspensions was defined as the MIC. The above culture suspensions without bacterial growth were further spread on the LB agar plate, and cultured for an additional 24 h, respectively. The lowest concentration at which no growth was visually observed on the plate was regarded as MBC. For the accuracy of results, all concentration values were the average ones from three independently experiments.

Bacteriostatic kinetic test

Escherichia coli was selected as index bacteria and the normal saline was used as control. The bacterial suspension (10^6 – 10^7 CFU per mL^{-1}) was mixed with the AgNPs solution at 10 and 20 μg per mL^{-1} , and incubated overnight at 37 °C. Bacterial growth rates were measured and

Fig. 1 **a** Time dependent of UV–Vis spectra recorded during the formation of AgNPs at different time intervals and **b** TEM images of the biosynthesized AgNPs

monitored with the optical density at 600 nm (OD_{600}) on a spectrophotometer.

Antimicrobial effect of AgNPs

TEM analysis was carried out to examine effects of AgNPs on the morphology of *E. coli*. The *E. coli* was incubated at 37 °C overnight and then treated with 20 μg per mL^{-1} AgNPs. After 3 h of treatment, the cells were collected by centrifugation at 8000 rpm for 10 min. The pellet was washed with phosphate buffer saline (PBS) solution three times, and then fixed with 2.5% glutaraldehyde for 2 h. After being washed with PBS, 20 μL samples were placed on the carbon coated copper grids for TEM analysis.

Additionally, the bacterial suspension (10^6 – 10^7 CFU per mL^{-1}) was incubated with the 5, 10 and 20 μg per mL^{-1} AgNPs solution at 37 °C for 3 h, respectively. The genomic DNA samples of *E. coli* were extracted using the Easy

Fig. 2 The FTIR spectrum of the blueberry leaf extract and the synthesized AgNPs samples

Fig. 3 XRD spectrum of the biosynthesized AgNPs with leaf extract of blueberries

pure bacteria genomic DNA isolation kit (TransGen Biotech, China). The isolated DNA samples of the same volume were analyzed using 1% agarose gel electrophoresis. Non-treated bacteria were used as control.

Results and discussion

Biosynthesis of AgNPs

When the leaf extract of blueberry was added into the AgNO_3 solution, the color of the reaction mixtures changed from light yellow to yellow brown, and then to brick red at the end of experiment, which was due to the excitation of surface plasmon of the formed AgNPs (Malhotra et al. 2013) (Supplementary material Fig. S1).

Fig. 4 Antibacterial activities of the biosynthesized AgNPs against clinical and aquatic pathogens. 1–3 represented the biosynthesized AgNPs, normal saline and blueberry leaf extract, respectively

Characterization of AgNPs

The AgNPs formation was further clarified by UV–Vis at different time points after reaction. As shown in Fig. 1a, a weak peak occurred within 30 min of reaction, indicating AgNPs began to form. The height of the peak increased along the reaction and a more significant reaction curve was observed after 60 min of reaction. The characteristic surface plasmon resonance (SPR) peak of the as-prepared AgNPs was at about 434 nm, which confirmed the reduction of silver ions to silver metal (Yilmaz et al. 2011).

The synthesis of AgNPs from AgNO_3 requires the reduction of silver ion to silver. Analytical studies have shown that the leaves of *V. corymbosum* are rich in various phenolic compounds, such as phenolic acids and esters, flavonols, anthocyanins, and procyanidins, etc. (Deng et al. 2014; Sahu et al. 2013). Previously, we also found the high levels of vitamin C and *myo*-inositol in the leaves of *V. corymbosum* (Liu et al. 2015; Song et al. 2016). The above results suggested that these components in the blueberry leaves extract had the potential to rapidly biosynthesize AgNPs.

TEM images were used to observe the morphology and size of the as-prepared AgNPs. As shown in Fig. 1b, most of the AgNPs were spherical, well-dispersed. The diameters of these particles were 10–30 nm in diameter with an average of 20 nm. Because the combination of AgNPs to the bacteria depends on the interaction of their available surface area, the spherical particles with smaller size will have more antimicrobial effects (Sahu et al. 2013; Yilmaz et al. 2011). Therefore, the biosynthesized AgNPs with wasted blueberry leaf extract might be ideal antimicrobial agents.

The FTIR analyses were carried out to elucidate the possible biomolecules present in the leaf extract of blueberry which were responsible for the formation and stability of AgNPs. As shown in Fig. 2, the leaf extract of blueberry had absorption peaks at 3419, 2919, 2850, 1613, 1382 and 1069 cm^{-1} . The strong and wide band at 3419 cm^{-1} was referred as the strong stretching vibrations of O–H functional group. The corresponding peaks at 2919 and 2850 cm^{-1} were due to the C–H vibrational mode, while those at 1613 and 1382 cm^{-1} to the stretching vibrations of cyclohexene and alkane, respectively. The band observed at 1069 cm^{-1} could be assigned to the C–O stretching vibration of phenols. The FTIR spectrum of the biosynthesized AgNPs shared the similar absorption peaks with that of the blueberry leaf extract, supporting that the

phenolic compounds and proteins in the leaf extract of blueberry facilitated the synthesis of AgNPs.

The XRD pattern of the as-prepared AgNPs was shown in Fig. 3. There were four distinct diffraction peaks at $2\theta = 38.2^\circ$, 44.4° , 64.5° and 77.5° , which corresponded to the (111), (200), (220) and (311) planes of the cubic Ag crystal (JCPDS No. 04-0783), respectively, indicating the formation of metallic AgNPs. The unpredicted peaks (46.1° , 57.4° and 71.6°) might be due to other organic compounds in the leaf extract (Huang et al. 2007; Noginov et al. 2006). The average crystallite size of the AgNPs calculated according to the Scherer's equation was about 14.6 nm, which further confirmed the result from TEM analysis.

Antibacterial activity of the biosynthesized AgNPs

The antibacterial activity of the biosynthesized AgNPs was carried out according to the cup-plate assay as described previously (Wang et al. 2016a, b). As shown in Fig. 4, the synthesized AgNPs showed excellent antimicrobial activity against gram-negative *E. coli*, *P. Aeruginosa*, *V. alginolyticus*, *V. anguillarum*, *V. Parahaemolyticus*, *A. punctata* and gram-positive *S. aureus*, *B. subtilis*. Although the blueberry leaf extract was antibacterially active (Pervin et al. 2013), the as-prepared AgNPs exhibited remarkably higher antibacterial activity. And the control of normal saline did not show any inhibition indicating that the procedure was correctly executed. As shown in Supplementary material Table S1, the diameter of the inhibitory zone for the 8 bacteria was in the range of 11.2–14.8 mm. It has been reported that the AgNPs synthesized from various plants such as *Glycyrrhiza glabra*, *Balsamodendron mukul*, *Berberis aristata*, *Acorus calamus* and *Psoralea corylifolia* had antimicrobial activities, and the diameters of inhibition zones usually were not more than 8 mm, which were smaller than that of the as-prepared AgNPs from blueberry leaves (Prashanth et al. 2001; Kaviya et al. 2011). Particularly, in previous studies the antibacterial analyses of the biosynthesized AgNPs were mainly focused on clinical pathogens. Here, we also analyzed the antibacterial activities of the as-prepared AgNPs against the aquatic pathogens. Among the four tested aquatic pathogenic bacteria, *V. alginolyticus* showed the largest inhibition zone at 14.8 mm. These results indicated that the AgNPs synthesized from blueberry leaf extract had promising application in aquaculture.

During the minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) tests, gradiently diluted AgNPs were incubated with equal volumes of *E. coli* suspensions. After 24 h of incubation, the MIC value was defined as $8.2 \mu\text{g per mL}^{-1}$. The as-obtained culture suspensions were spread on the LB agar

plates for additional 24 h, and the MBC value was $16.3 \mu\text{g per mL}^{-1}$ (Table 1).

Furthermore, the growth kinetics of *E. coli* treated with the as-prepared AgNPs was studied (Supplementary material Fig. S2). With treatment of $10 \mu\text{g per mL}^{-1}$ AgNPs, there was a growth delay of *E. coli*, followed by gradual growth. When *E. coli* was treated with $20 \mu\text{g per mL}^{-1}$ AgNPs, *E. coli* was completely inhibited as the OD_{600} was almost the constant. However, in the negative control of normal saline, the OD_{600} increased rapidly during the bacteria log phase, indicating the reproduction of *E. coli*. These results further confirmed the powerful antibacterial activity of as-prepared AgNPs from the blueberry leaf extract.

Antibacterial effect of the biosynthesized AgNPs

As shown in Fig. 5a, the untreated *E. coli* cells showed a regular outlined outer cell wall, and the plasma membrane lied closely to the outer wall and the periplasmic space was thin and had a uniform appearance. However, after the incubation with the AgNPs, the outer cell wall and the plasma membrane of *E. coli* cells became wrinkled, and the periplasmic space was hyperhydrated. These results were probably due to the electrostatic interaction between the positively charged nanoparticles and the negatively charged membrane, which further disrupted the integrity of bacterial membrane and resulted in cell death (Soman and Ray 2016).

Recently, Ramamurthy et al. (2013) reported the effects of AgNPs on *E. coli*, which could penetrate into the cells and cause DNA damage and cell death. To examine whether the as-prepared AgNPs could cause the bacterial DNA damage, the agarose gel electrophoresis of the genomic DNA from the *E. coli* cells was performed. The *E. coli* cells were treated with different concentrations of AgNPs, and the untreated were used as control. As shown in Fig. 5b, the DNA sample from the untreated cells showed a single band, indicating the genomic DNA remained intact.

Table 1 MIC and MBC tests of the biosynthesized AgNPs

Concentration of Ag ($\mu\text{g/mL}$)	24 h	48 h
65.0	–	–
32.5	–	–
16.3	–	–
8.2	–	+
4.1	+	+
2.0	+	+
1.0	+	+
0.5	+	+

Fig. 5 **a** TEM micrographs of *E. coli* treated with 20 μg/mL AgNPs for 3 h and **b** the gel electrophoresis analyses of genomic DNA samples from the *E. coli* cells treated with different concentrations AgNPs. M, Trans 15 K DNA Marker; 1 DNA sample from the *E. coli* cells untreated; 2–4, DNA samples from the *E. coli* cells treated with 5, 10 and 20 μg/mL AgNPs, respectively

In contrast, the DNA samples from the cells treated with AgNPs had a weaker band of genomic DNA and smearing bands of small DNA fragments, indicating that the genomic DNA had degraded. Additionally, with the increase of the AgNPs concentration, the genomic DNA was degraded more significantly. Our results suggested that the biosynthesized AgNPs could cause DNA damage of *E. coli* cells.

Conclusion

In this study, spherical and well-dispersed AgNPs with an average diameter of 20 nm were successfully biosynthesized from the leaf extract of blueberry. The characteristic SPR peak of the as-prepared AgNPs was at about 434 nm. The FTIR spectrum of the biosynthesized AgNPs shared the similar absorption peaks with that of the blueberry leaf extract, supporting that the phenolic compounds and proteins in the leaf extract of blueberry facilitated the synthesis of AgNPs. Particularly, the as-prepared AgNPs exhibited superior antimicrobial activities against eight types of clinical and aquatic pathogens. And the best antibacterial activity of the as-prepared AgNPs was against an ocean pathogenic bacterium, *V. alginolyticus*. Besides, the disrupted plasma membrane and the degraded genomic DNA of the *E. coli* cells treated with AgNPs suggested that the as-prepared AgNPs could cause cell damages. To the best of our knowledge, this is the first report that the blueberry leaf extract could be used as highly efficient reducing agent in the synthesis of AgNPs, which opened a new door for the use of wasted by-product of blueberry harvesting.

Acknowledgements This work was supported by the National Natural Science Foundation of China (Nos. 31570649; 31470661; 31400447), Key Research and Development Program of Shandong Province (No. 2016GNC111016), the Project of Shandong Province Higher Educational Science and Technology Program (No. J15LE03) and the Key Research and Development Program of Yantai (No. 2016ZH059).

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