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RESEARCH ARTICLE

Comparison of ethanolic extracts of phytoestrogenic *Dendrolobium lanceolatum* and non-phytoestrogenic *Raphanus sativus* to mediate green syntheses of silver nanoparticles

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Abstract: Green synthesis of silver nanoparticles (AgNPs) mediated by plant extracts has drawn many research interests due to its simple, cost-effective, and eco-friendly approach. However, the extracts derived from phytoestrogenic plants that produce high phenolic-based compounds exhibiting the estrogenic activity have not yet investigated. This work reported the comparison of ethanolic extracts derived from phytoestogenic *Dendrolobium lanceolatum* and non-phytoestrogenic *Raphanus sativus* to facilitate the green synthesis of AgNPs. The total phenolic content and the reducing activity of *D. lanceolatum* extract were significantly higher than those of *R. sativus* extract. In addition, the formation of AgNPs could detect in the reaction using *D. lanceolatum* extract, but not *R. sativus* extract, as determined by the characteristic surface plasmon resonance peak of AgNPs at 416 nm. The synthesized AgNPs were spherical with an average diameter of 74.60±17.11 nm, which their face-centered cubic structure of silver was confirmed by X-ray diffraction analysis. Moreover, the synthesized AgNPs exhibited the antibacterial activity against both Gram-negative *Escherichia coli* and Gram-positive *Staphylococcus aureus*. The results of this work, thus, suggested the potential uses of phytoestrogenic plants as a good source of reducing and stabilizing agents for the production of AgNPs and other metallic nanoparticles.

Keywords: antibacterial activity, phenolic content, plant extract, reducing activity, silver nanoparticles

1 Introduction

Silver nanoparticles (AgNPs) have received many research interests of the scientific community due to their remarkable antibacterial property, which their applications can be seen in various daily commercial products such as textiles, personal cares and food storages.[1] In general, mass production of AgNPs is obtained by using chemical and physical synthesis approaches, which have some drawbacks on a use of hazardous solvents, a generation of toxic by-products and a requirement of high energy.[2] Green synthesis of AgNPs, therefore, has drawn a lot of interests as an alternative eco-friendly and cost-effective approach. A green synthesis refers to a method that reduces or eliminates a use or generation of hazardous substances in reaction processes. In general, it involves the use of less dangerous and low environmental-toxic capping substances, reducing agents and solvents. By this method, the use of natural biomolecules as an alternative reducing and capping agents has received increasing interest for green production of AgNPs. These natural biomolecules include polysaccharides,[3] proteins,[4] and phytochemicals.[5] In addition, green synthesis of nanoparticles can be carried out by living organisms such as bacteria, yeasts and fungi.[6]

In the past few years, plant extracts have been received many research interests for green production of AgNPs, due to the simple and cost-effective process. Phytochemicals and biomolecules in plant extracts can facilitate the formation and stabilization of zero valence silver.[7] Many works reported on the uses of plant extracts derived from many species and various parts of plants for the green synthesis of AgNPs including leaf, latex, stem, root and fruit of edible, ornamental and medicinal plants. The examples were holy basil (*Ocimum sanctum*),[8] garlic (*Allium sativum*),[9] bamboo (*Bambusa arundinacea* and *Bambusa nutans*),[10] *Acalypha hispida*,[11] *Verbesina encelioides*,[12] red ball snake gourd (*Trichosanthes tricuspidata*)[13] and bitter melon (*Mo-*

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mordica charantia).[14] The phytochemicals in these plant extracts, such as alkaloids, tannins, phenolics, saponins, terpenoids, proteins, vitamins and polysaccharides, were proposed to serve as reducing agents, while the complex molecules could assist a stabilization of the synthesized AgNPs.[15]

One interesting group of plants that can be an excellent source of active reducing and stabilizing agents for green synthesis of AgNPs is phytoestrogenic plants, which their active biomolecules are phytoestrogens, the phenolic containing phytochemicals with estrogenic agonists and/or antagonists in animals and human that have been used in many food supplements and cosmetic products.[16] Phytoestrogens are divided into three major classes; coumestans, prenylflavonoids and isoflavones. They have the chemical structure, especially phenolic ring and hydroxylation pattern, similar to estrogen, thus providing high affinity for binding to estrogen receptors.[17] Due to the high contents of phenolic compounds in phytoestrogenic plants, whether they exhibit greater activity to facilitate synthesis of AgNPs has been not investigated and compared with the non-phytoestrogenic plants. In Thailand, the dried root of *Dendrolobium lanceolatum*, the flowering plant in the legume family, is commonly used for the folkloric treatment of diuretic and urinary diseases. Our preliminary result revealed that its ethanolic extract exhibited a high estrogenic activity as determined by a yeast two-hybrid system. Thus, in this work, we studied the use of the root extract of *D. lanceolatum* to mediate a green synthesis of AgNPs. In addition, the white radish (*Raphanus sativus*) that contains no phytoestrogenic activity was also used as the comparison.

2 Materials and methods

2.1 Chemicals

Folin-Ciocalteu reagent and gallic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). Silver nitrate was purchased from QRC chemical (Auckland, New Zealand). All chemicals used were of analytical grade.

2.2 Preparation of plant extracts

Tuberous roots of *D. lanceolatum* (phytoestrogenic plant) and *R. sativus* (non-phytoestrogensic plant) were purchased from a local market in Nakhon Ratchasima, Thailand. Samples were sliced in small pieces and dried in a hot-air oven at 70°C. The dried samples were extracted with 80% ethanol at a ratio of 1:10 (w/v) in a shaker at 80 rpm at ambient temperature for 3 days. After passing through a Whatman No. 1 filter paper, the evaporation of the extract was at 60 °C in a rotary evaporator. Crude plant extracts were kept in a tightly-capped tube and stored at -70°C.

2.3 Total phenolic assay

The total phenolic content (TPC) was determined by the Folin-Ciocalteu assay with some modifications.[18] Briefly, 50 μL of each plant extract (31.25-500 mg/L) or the standard solution of gallic acid (1-500 μg/mL) were mixed with 750 μL of 20% sodium carbonate, 250 μL of 1 M Folin-Ciocalteu reagent and 3.95 mL of distilled water. A reagent blank was used the distilled water instead of the plant extract. The mixture was incubated at 50 °C for 2 h with light protection. The absorbance against the reagent blank was measured at 765 nm using an UV-Visible Specord® 250 Plus spectrophotometer (Analytik-Jena, Jena, Germany). The TPC was expressed as milligram gallic acid equivalent per gram of dried plant extract (mg GAE/g dry weight).

2.4 Reducing activity assay

The reducing activity of the plant extracts was determined using the slightly modified method from the previous publication[19] . The *D. lanceolatum* extract (2.5 mL) of various concentrations (0-1 mg/mL) was mixed with 2.5 mL of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. After adding 2.5 mL of 10% trichloroacetic acid (w/v), the mixture was centrifuged at 3000 ×g for 10 min. The upper layer of the solution (1.25 mL) was removed to a new tube before mixing with distilled water (1.25 mL) and a freshly prepared 0.1% ferric chloride (0.25 mL). The reducing power of the tested samples was evaluated by the color changes and the measured absorbance at 700 nm. All determinations were from five replications and the results were expressed as mean±standard deviation.

2.5 Synthesis and characterization of AgNPs

The reaction (10 mL) to synthesize AgNPs contained 8.6 mL of 200 mg/mL plant extract (D. lanceolatum or R. sativus) and 1.4 mL of 300 mM silver nitrate. The reaction was carried out at 60 °C with the light protection for 24 h. The formation of AgNPs was monitored from the reaction color change to dark brown and the presence of the characteristic surface plasmon resonance (SPR) peak of silver by measuring the absorbance at the wavelengths of 300-900 nm. The reactions containing silver nitrate and various concentrations (20-200 mg/mL) of D. lanceolatum extract were carried out at 60 °C at 12

h to study the effect of the extract concentrations. In addition, to study the effect of reaction times, the formation of AgNPs was monitored in a time course of 48 h, which the reactions contained silver nitrate and the plant extract (200 mg/mL) and incubated at 60°C.

To determine their crystalline structure, the synthesized AgNPs were subjected to X-ray diffraction (XRD; Bruker, Bremen, Germany) analysis using D8 Advance diffractometer with Cu Kα radiation, λ=1.54 $\mathring{A}$ in the 2θ range of 30°-80°. The instrument was calibrated by using the lanthanum hexaboride (LaB_6) before analysis.

The morphology and size of AgNPs were determined from the taken scanning electron microscope (SEM) images using a JSM 7800F SEM (JEOL, Tokyo, Japan) provided with a Schottky type field emission and lower electron detector at an accelerating voltage of 15 kV. The suspension of AgNPs was dropped on a carbon tape, allowed to completely dry at room temperature and sputter-coated with gold immediately before observing. An average diameter of AgNPs was determined from the SEM images at random locations (n=300) using the ImageJ open-access software.[20]

2.6 Antibacterial Assay

The antibacterial activity of the produced AgNPs against bacteria was evaluated by the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC). The representative Gram-negative and Gram-positive bacteria in this work were Escherichia coli (ATCC 25922) and Staphylococcus aureus (ATCC 25923), respectively. The MIC referred to the minimum concentration of AgNPs that inhibited bacterial growth. The MBC referred to the minimum concentration of AgNPs that completely killed the bacteria. The stock suspension of AgNPs was serially two-fold diluted by Mueller-Hinton (MH) broth (0.81-26.0 μg/mL) and incubated with the tested bacteria at a concentration of 5×10^5 colony-forming units/mL (CFU/mL) at 37 °C for 24 h. The bacterial growth was measured by the optical density at 600 nm to determine the MIC. To determine the MBC, the cultures (100 μL) at MIC and two above concentrations were cultured on MH-agar plates at 37°C for 24 h. The MBC was determined by the concentration of AgNPs showing no bacterial growth on the culture plate.[21]

2.7 Statistical analysis

The statistical analysis of two data groups was performed using the independent-samples t-test. The analysis of more than two data groups was performed using the one-way analysis of variance (ANOVA) with SPSS 18.0 for Windows software (SPSS Inc., Chicago, Illinois, USA). The multiple comparisons among data groups were analyzed by Tukey's honest significant test. The significant difference among groups was considered at a level of $P<0.05$.

3 Results and Discussion

3.1 Total phenolic content and reducing activity of the plant extracts

The tuberous roots of *D. lanceolatum* (phytoestrogenic plant) and *R. sativus* (non-phytoestrogenic plant) were extracted in 80% ethanol, which their extraction yields were 1.00±0.01 and 5.10±0.01 g/100g dried weight of the plants, respectively. The total phenolic contents (TPC) of the plant extracts are shown in Figure 1A. The TPC of the *D. lanceolatum* extract was 423.3±19.3 mg GAE/g dry weight, which was approximately 14.7 folds higher than that of the *R. sativus* extract (28.8±3.6 mg GAE/g dry weight). The major phenolic compounds of the *D. lanceolatum* extract, especially ones possessing phytoestrogenic activity, are likely in the groups of prenylflavonoids and dibenzocycloheptene derivatives.[22]

The reducing activities of *D. lanceolatum* and *R. sativus* extracts are shown in Figure 1B, which both extracts exhibited the reducing activity in a dose-dependent manner. However, the reducing activity of the *D. lanceolatum* extract was significantly higher than that of the *R. sativus* extract. At the concentration of 1 mg/mL, the reducing activity of the *D. lanceolatum* extract was approximately 3.4 folds higher than that of the *R. sativus* extract, well corresponding to the different TPCs of both plant extracts. It is likely that the hydroxyl groups of the phenolic compounds of these plant extracts may play a crucial role as the radical scavengers.[23] Thus, they exhibited the reducing activity but at different levels according to their phenolic compound contents.

3.2 Synthesis and characterization of AgNPs

The *D. lanceolatum* and *R. sativus* extracts were used to synthesize AgNPs by incubating with silver nitrate at 60°C for 24 h without the addition of other chemical reducing and stabilizing agents. The UV-Vis spectra of the reactions are shown in Figure 2. The color of the reaction changing from orange to dark brown color suggested the formation of AgNPs. In addition, the presence of synthesized AgNPs was determined by the characteristic surface plasmon resonance (SPR) peak of AgNPs at 416 nm.[24] In this reaction, it is likely that the phenolic compounds of the *D. lanceolatum* extract serving as the reducing agents to reduce Ag^+ into Ag^0 and eventually

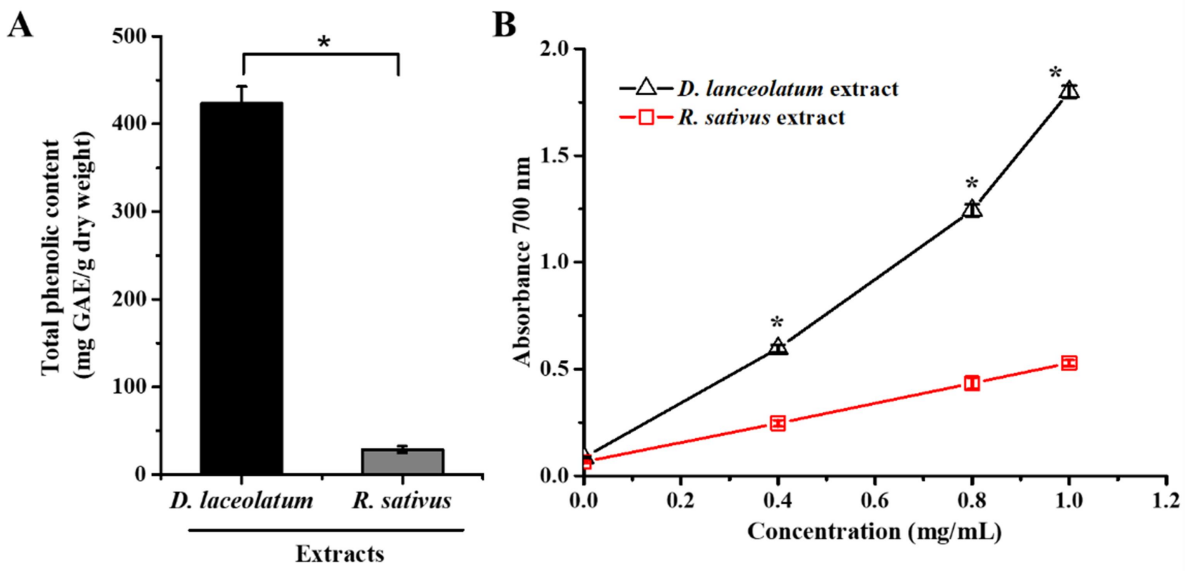

Figure 1. The total phenolic contents (A) and the reducing activities (B) of the *D. lanceolatum* and *R. sativus* extracts

form AgNPs, while the complex structure of proteins and carbohydrates of the extract assists the stabilization of colloidal AgNPs in an aqueous environment.[25] In contrast to, this characteristic SPR peak was not detected in the reaction containing the *R. sativus* extract, suggesting that the *R. sativus* extract was unable to promote the synthesis of AgNPs at this condition. It was noted that the presence of the absorption peak at 350 nm of both reactions was probably due to the absorption of phenolic compounds of the extracts.[26]

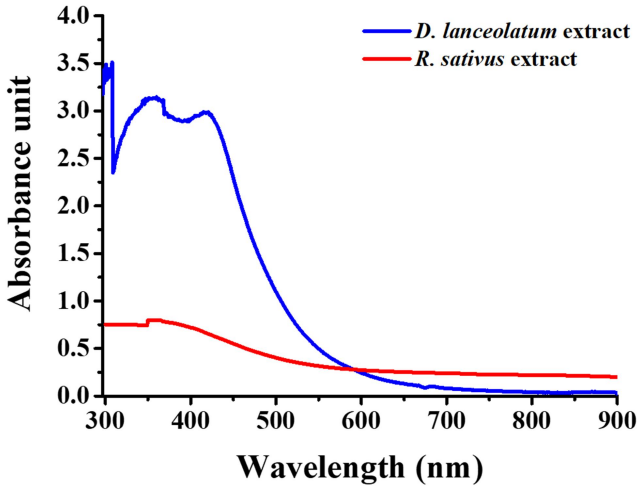

Figure 2. Different UV-Vis spectra of the reactions to synthesize AgNPs that were mediated by *D. lanceolatum* and *R. sativus* extracts

The effects of the concentration of *D. lanceolatum* extract and the reaction time on the synthesis of AgNPs were also studied. The UV-Vis spectra of the reactions containing various concentrations (20-200 mg/mL) of the *D. lanceolatum* extract at 12 h of incubation are shown in Figure 3A. The formation of AgNPs as indicated by the characteristic SPR peak was observed only in the reactions containing 100 and 200 mg/mL of *D. lanceolatum* extract. The SPR peak intensity was increased according to the increased concentrations of *D. lanceolatum* extract, suggesting that the formation of AgNPs depended on the concentration of the extract. The phenolic compounds of the *D. lanceolatum* extract might play the significant role to reduce Ag^{+} to Ag^{0} for a formation of silver nuclei via the protein and electron transfer mechanisms as well as to stabilize the antioxidant molecules in the reaction. In addition, the phenolic compounds could facilitate the growth of AgNPs via the binding to the silver clusters and assist the reduction of silver ions at the surface of the clusters to form AgNPs.[27]

In addition, the effect of the reaction time on the synthesis of AgNPs was studied. The formation of AgNPs in the reaction containing the *D. lanceolatum* extract (200 mg/mL) was monitored in a time course of 48 h (Figure 3B). The formation of AgNPs was detected in the reactions at 12-48 h as determined by the characteristic SPR peak of AgNPs and the changes of reaction color (light yellow to dark brown). The production yield of AgNPs in a time course of 48 h was increased in a dose-dependence as indicated by the SPR peak intensity.

The shape and size of the synthesized AgNPs were determined by the taken SEM images. Figure 4A shows the representative SEM image revealing the spherical morphology of the synthesized AgNPs. The diameters

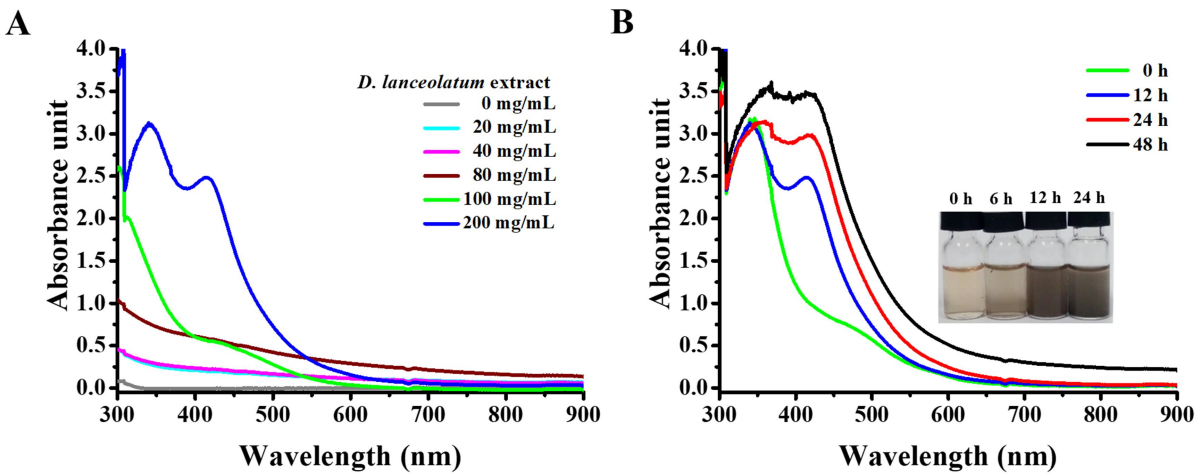

Figure 3. The UV-Vis spectra of the formation of AgNPs using different concentrations of *D. lanceolatum* extract (A) and different reaction times (B)

of 300 particles, randomly picked, were in a range of 40.8-134.0 nm (Figure 4B), which their average size was 74.60±17.11 nm. Although the formation of spherical AgNPs has not fully understood, it speculates that the binding between Ag^+ and biomolecules derived from the *D. lanceolatum* extract leads to isotropic growth of the silver clusters and formation of spherical nanoparticles.[28]

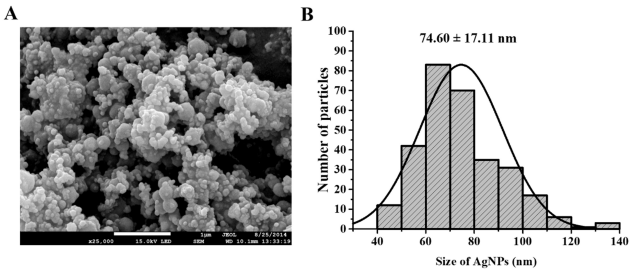

Figure 4. The representative SEM image (A) and the histogram of size distribution (B) of the synthesized AgNPs

In Figure 5, the XRD pattern shows the numbers of Bragg reflections with 2 theta values of 38.11°, 44.30°, 64.44° and 77.39° corresponding to the (111), (200), (220) and (311) lattice planes, indicating the face-centered cubic structure (fcc) of silver according to the JCPDS file No. 03-065-287.[29] Two unassigned peaks observed in the XRD pattern were likely the crystallization of bioorganic phases derived from the plant extract that occurred on the surface of the nanoparticles.[30]

3.3 Antibacterial Activity

The antibacterial activity of the synthesized AgNPs mediated by the *D. lanceolatum* extract was determined by a microbroth dilution method against the representative Gram-negative *E. coli* and Gram-positive *S. aureus*. The growths of both bacterial strains in response to various concentrations of AgNPs was monitored in a time course of 24 h. As seen in Figure 6, AgNPs exhibited the antibacterial activity against both bacterial strains in a dose-dependent response, which the increased concentrations of AgNPs resulted in more growth reduction of both bacterial strains. The minimal concentrations of AgNPs causing the inhibition of the *E. coli* and *S. aureus* growths (MICs) were equally at 6.5 μg/mL. The minimal concentrations of AgNPs that completely killed the bacteria (MBCs) were higher than the MICs, which the MBCs of AgNPs against *E. coli* and *S. aureus* were 13.0 and 26.0 μg/mL, respectively. The less susceptibility of *S. aureus* to AgNPs as compared with *E. coli* was reported to relate to the thicker peptidoglycan layer of

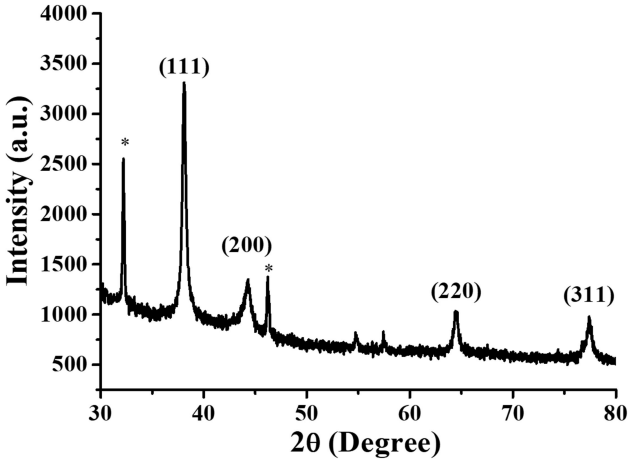

Figure 5. XRD analysis of the synthesized AgNPs. Unassigned peaks indicated as *

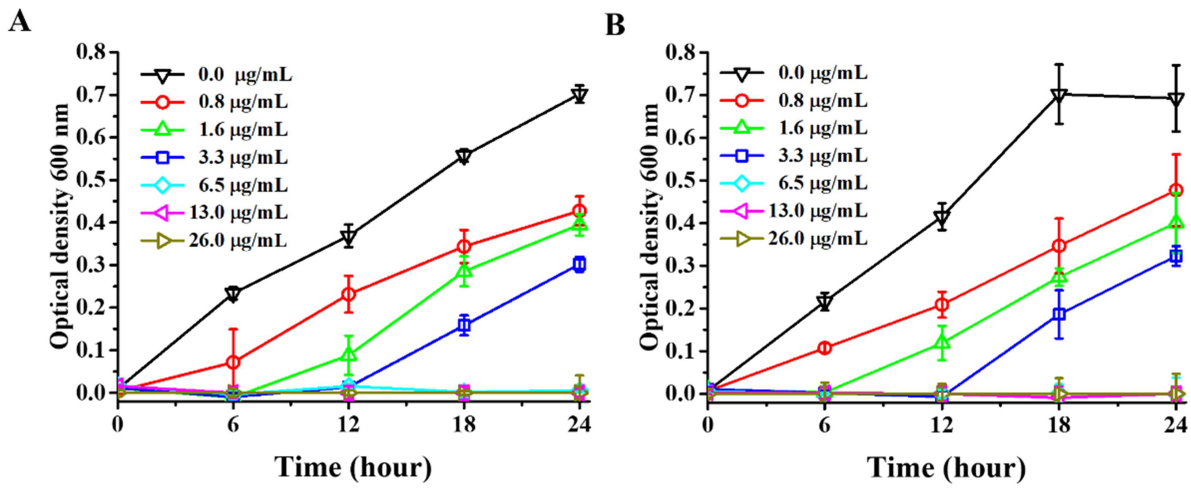

Figure 6. The growth curves of *E. coli* (A) and *S. aureus* (B) in response to different concentrations of AgNPs in a time course of 24 h

the Gram-positive bacteria.[31] The penetration of AgNPs inside the bacterial cells is proposed via direct diffusion and endocytosis, depending on their sizes.[32] AgNPs with the diameters in a range of 10-100 nm can enter the cells via the endocytosis mechanism, while AgNPs of less than 10 nm prefer to penetrate the cell wall via direct diffusion since their lower adhesion and stretching energy are not sufficient for endocytosis.[33] The penetrated AgNPs can disrupt bacterial enzyme function, interfere DNA transcription, interrupt DNA replication and eventually cause cell death.[34] In addition, some AgNPs attached on the cell surface can damage and disrupt the cell permeability and respiration as well as cause a formation of reactive oxygen species (ROS) in bacterial cells.[35]

4 Conclusion

This work demonstrated that the extract derived from phytoestrogenic *D. lanceolatum* actively induced the formation of AgNPs as its biomolecules, especially phenolic compounds, served as the reducing agents and the complex structural compounds stabilized the formed AgNPs. In comparison with the extract derived from the non-phytoestrogenic *R. sativus*, at the same condition, no formation of AgNPs was detected as determined by the presence of the characteristic SPR peak of AgNPs, which was likely due to the less TPC and reducing activity of the *R. sativus* extract. The synthesized AgNPs were spherical with an average diameter of 74.60±17.11 nm. The identity of the synthesized particles was confirmed by XRD analysis, which the crystalline structure of the synthesized particles was the face-centered cubic geometry of silver. The produced AgNPs exhibited the antibacterial activity against both *E. coli* and *S. aureus*. However, *E. coli* was more susceptible to AgNPs than *S. aureus* as indicated by the MBCs, well corresponding to the different thickness of their cell walls.

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