Antibacterial activity and catalytic activity of biosynthesised silver nanoparticles by flavonoids from petals of *Lilium casa blanca*

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Biosynthesis of silver nanoparticles (AgNPs) by flavonoids extracts from petals of *Lilium casa blanca* was reported. Reaction conditions such as pH, concentration of flavonoids or silver nitrite, reaction temperature and reaction time were optimised to control the formation of the AgNPs. Synthesised AgNPs were characterised by UV–Vis spectroscopy, transmission electron microscopy (TEM), dynamic light scattering (DLS), zeta potential measurement and Fourier transform infrared (FT-IR) spectroscopy. Stability, antibacterial and catalytic activities of the synthesised AgNPs were also discussed. The optimum synthetic conditions were 10 mL 20 mM $AgNO_3$ added to 1 mL flavonoids extracts, adjusting pH to 10, and reacted at 70°C for 1 h. The formation of AgNPs was confirmed by UV–Vis spectroscopic analysis, which showed maximum absorption peak at 404 nm. TEM analysis revealed spherical particles with size about 12.7 nm. FT-IR spectra revealed flavonoids were present on the surface of the AgNPs. AgNPs showed highest antibacterial activity against *Escherichia coli* and *Salmonella*, and the lesser antibacterial activity of AgNPs was found against *Bacillus subtilis* and *Staphylococcus aureus*. The biosynthesised AgNPs showed good catalytic activity as well by hydroboration of *p*-nitrophenol. Catalytic reduction followed pseudo-first-order kinetic.

1. Introduction: Recently, nanoparticle research has drawn intense attention in many scientific and technological fields. In comparison with bulk materials, nanomaterials show many interesting properties, such as quantum size effect, small size effect, surface effect and macroscopic quantum tunnelling effect. Among all the nanoparticles, silver nanoparticles (AgNPs) have drawn the attention of researchers because of their suitable applications in the fields of electronic, material science and medicine [1]. Property of AgNPs varies due to the size, the shape and the distribution of the nanoparticles [2]. Therefore, lots of researchers focus on the controllable synthesis of AgNPs with different sizes and shapes. Chemical reduction method is the most widely used way. However, some chemical regents used in this method to reduce or stabilise AgNPs are considered hazardous, which affected the biological applications of AgNPs [3, 4]. So biological processes are preferred for environmental and economic concerns.

Gardea-Torresdey and co-authors firstly reported the synthesis of gold nanoparticles (AuNPs) by alfalfa biomass in 1999 [5], which inferring the potential of biosynthesis of nanoparticles by plant extracts. AuNPs were simply formed by adding alfalfa biomass to hydrochloroauric acid solution, while different shapes or sizes of AuNPs were obtained by controlling the pH of the reaction. This method was considered as a green method to synthesise AuNPs, and then in 2002, AgNPs synthesised by live alfalfa plants were reported [6]. Recently, many plant extracts or biomass had been reported to synthesise AgNPs with different shapes and sizes, such as citrus fruit [2], *Caulerpa racemosa* [7], *Erythrina indica lam* [8], *Vitex negundo* [9], *Butea monosperma* [10] and so on. Many biochemical compounds in plant extracts or biomass were found to act as reducing or stabilising regents during the synthesis course, such as flavonoids, terpenoids, polysaccharides, proteins and alkaloids [11]. Among all these active compounds, flavonoids and reducing sugars were proved to be the most important reductants during the synthetic process [12]. Synthesised AgNPs showed some good biomedical activities, for instance antimicrobial activity [13–15], catalytic activity [4] and antitumor activity [16].

Herein we reported the biosynthesis of AgNPs by flavonoids extracts from petals of *Lilium casa blanca* for the first time. *Lilium casa blanca*, which has pure white colour and strong smell, is one of the most popular flowers in China, and often regarded as 'Queen of Lilies' [17]. In our previous study, flavonoids of the dried powder of *Lilium casa blanca* were extracted and showed quite high, then the flavonoids extracts were successfully used in the synthesis of AuNPs [18, 19]. To control the shape and the size of AgNPs, concentrations of flavonoids extracts, pH, temperature and reaction time have been optimised. UV–Vis spectroscopy, transmission electron microscopy (TEM), dynamic light scattering (DLS), zeta potential measurement and Fourier transform infrared (FT-IR) spectroscopy were used to characterise the synthesised AgNPs. Stability, antibacterial and catalytic activities of the synthesised AgNPs were also discussed.

2. Material and methods

2.1. Chemicals and materials: *Lilium casa blanca* were bought in a local market of Nanchang, China. Silver Nitrate ($AgNO_3$, Mr = 169.87) was purchased from Shanghai No. 1 Reagent Factory (China). *p*-Nitrophenol and sodium borohydride were all purchased from Aladdin (China). All other regents used in this experiment were of analytical grade, tested bacterial strains such as *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Salmonella* were all purchased from ATCC or CMCC. Ultrapure water (Milli-Q 18.2 MΩ) was used all through the experiments.

2.2. Preparation of flavonoids extracts: Petals of *Lilium casa blanca* were collected and washed, then dried overnight at 60°C and powdered. Flavonoids from petals of *Lilium casa blanca* were extracted by 70% (v/v) ethanol-water solution. 0.5 g dried powder was mixed with 15 mL 70% ethanol-water solution, and extracted at 60°C for 1.5 h subsequently. Then the extracts were filtered, and diluted to 100 mL. Contents of the flavonoids were measured by aluminium chloride–sodium nitrite spectrophotometric method, while rutin was used as the reference substance [18].

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2.3. Biosynthesis of AgNPs: Different volume of $AgNO_3$ (20 mM) was added to 1 mL flavonoids extracts, and then diluted to 100 mL. After adjusting the solution to appropriate pH, the mixture was reacted under water bath heating at 70°C by reflux for 1 h. Several reaction parameters for AgNPs synthesis [pH (7–12), temperature (40–90°C), reaction time (0.5–6 h), the ratio of flavonoids to $AgNO_3$ (1:1–1:15)] were optimised. pH of the reaction mixture was adjusted by 4% (m/v) sodium hydroxide solutions.

2.4. Characterisation of the synthesised AgNPs: UV–Vis spectroscopy of the synthesised AgNPs was recorded by a Specord 200 double beam spectrophotometer (Analytikjena, Germany) with the scanning wavelength from 250 to 600 nm, using water as the baseline. To get the hydrodynamic diameter and zeta potential of the synthesised AgNPs, a colloidal nanoparticle solution was used in a quartz cuvette and measured by a zetasizer nano S90 (Malvern, UK). A droplet of the aqueous AgNPs solution was spread onto a coated copper grid (300 meshes) and dried under IR lamp. The TEM images were recorded in a JEOL 2100F (JEOL, Japan). The synthesised AgNPs were dialysed, and dried by lyophilisation. FT-IR spectra of the AgNPs were obtained using a NICOLET IS5 FT-IR spectrometer (Thermo Scientific, USA).

2.5. Study on the stability of the synthesised AgNPs: Stabilities of the synthesised AgNPs under different pH or different concentration of NaCl were studied. Two hundred microliters of the synthesised AgNPs solutions were diluted to 1 mL by solutions of different pH. The pH of the solutions was adjusted by 0.1 M HCl or NaOH. Two hundred microliters of the synthesised AgNPs solutions were added with different concentration of NaCl, and diluted to 1 mL. The UV–Vis spectra of all the solutions were measured.

2.6. Study on the antibacterial activity of the synthesised AgNPs: Antibacterial activities of the synthesised AgNPs were tested by a double layer plate-Oxford Cup method. Both Gram-positive (*S. aureus* CMCC 26003 and *B. subtilis* ATCC 6633) and Gram-negative bacteria (*E. coli* ATCC 14028 and *Salmonella* ATCC 25922) were used as the tested strains. Typically, the bacteria were cultured in a 37°C nutrient broth for 16–20 h, and then diluted to 1×10^6–1×10^7 CFU/mL by aseptic water for further use. Two per cent agar solution were firstly added to the Petri dish to keep the bottom of the plate flat, then the Oxford cups were placed on the surfaces agar-layer. Fifteen millilitres of molten nutrient agar with 200 µL bacterial suspension were added into the Petri dishes and cooled. After solidification, the Oxford cups were removed, and the AgNPs solutions were added into the hole by dripping. The plates were incubated at 37°C for 24 h and the inhibition zone (diameter in mm) of each hole was measured by vernier calliper. All the experiments were repeated thrice.

2.7. Study on the catalytic activity of the synthesised AgNPs: To evaluate the catalytic activity of the synthesised AgNPs, the hydroboration reaction of *p*-nitrophenol was chosen as the model reaction. Typically, 30 µL of 0.01 M *p*-nitrophenol solutions and appropriate volume of ultrapure water was firstly taken into a 1 cm quartz colourimetric utensil, and then AgNPs solution was added and mixed. In the end, 200 µL of 0.1 M sodium borohydride was dripped and the total volume of the mixture was kept at 3 mL. Then the utensil was quickly placed in a UV–Vis spectrophotometer, and the UV–Vis spectra of the reaction mixture at different times were recorded [20].

3. Results and discussion

3.1. UV–Vis spectroscopic analysis: Chemical reduction method is the most widely used method for the synthesis of AgNPs. Some regents acted as reductants or stabilisers were added to silver nitrate solutions to form AgNPs in colloidal form. During the synthesis process of AgNPs using plant extracts, plant extracts always acted as both reductants and stabilisers, colours of the AgNPs solutions varies from yellow to deep brown due to different reaction conditions. UV–Vis spectra of AgNPs differ substantially due to the size or the shape of the nanoparticles [21], so the synthesised AgNPs were firstly characterised by UV–Vis spectroscopy [22], and the results were shown in Fig. 1. Fig. 1 shows the UV–Vis spectra of the silver nitrate solution (a), flavonoids extracts solution (b) and the mixture of the two solutions (c) under same conditions. From line c, a maximum absorption peak at 404 nm was obtained, which was the surface plasmon resonance (SPR) band of AgNPs. No peaks were shown in the range of 250–600 nm for the reaction solution without silver nitrate or flavonoid extracts. As can be seen from visual observation, colour changed from colourless to yellow after reaction, while solutions without silver nitrate or flavonoid extracts did not show any changes in colour. These results showed that mixing silver nitrate and flavonoids extracts from petals of *Lilium casa blanca* could form AgNPs, while flavonoids extracts acted as both reductants and stabilisers, and silver nitrate acted as precursors.

Optical properties of nanoparticles were affected by the shape or size of the nanoparticles. So several parameters that could influence the synthetic procedure were optimised, for example pH, concentration of flavonoids extracts or $AgNO_3$, temperature and reaction times [23]. The effects of different parameters on the synthesis of AgNPs were explored, which were shown in Fig. 2.

pH always plays a crucial factor in the formation of nanoparticles [24]. Fig. 2*a* shows the UV–Vis spectra of AgNPs synthesised at different pH. When the pH of the reaction system was 7, no obvious absorption peak at 400–450 nm was found, but a big absorption peak at 319 nm, which may due to the absorption of flavonoids, and no colour changes were observed. While the pH reached to 8, the absorbance from 350 to 600 nm increased greatly, with the maximum absorption peak at 426 nm, indicating the formation of AgNPs. When the pH reached to 10, SPR wavelength had a small shift to shorter wavelength (404 nm), and consequently full width at half maximum decreased, indicating the decrease in particle size [25]. However, further increase the pH of the reaction system (pH = 12), two shoulder peaks were observed at 349 and 421 nm, respectively. This may suggest that AgNPs with various sizes or shapes were formed [26]. Many researchers reported that the alkaline environment was the necessary condition for the reduction reaction of silver ions [27]. In our experiment, AgNPs with

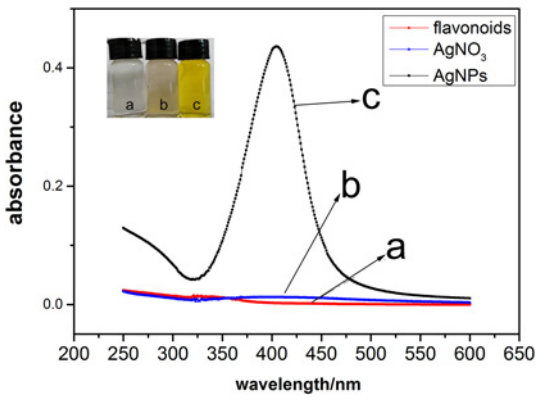

Fig. 1 *Synthesis of AgNPs. UV–Vis spectra. Inset: visual images of corresponding solutions*
a Flavonoids extracts
b $AgNO_3$
c AgNPs

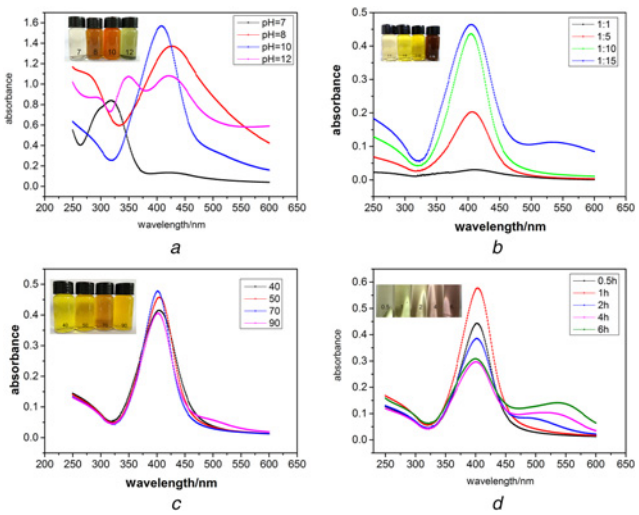

Fig. 2 *Optimum of the reaction conditions. Inset: visual images of corresponding solutions*
a UV–Vis spectra AgNPs synthesised at different pH
b UV–Vis spectra AgNPs synthesised at different volume of silver nitrate
c UV–Vis spectra AgNPs synthesised at different temperature
d UV–Vis spectra AgNPs synthesised at different reaction time

uniform sizes and shapes were willing to obtained, so pH 10 was chosen as the optimal pH conditions for the synthesis of AgNPs.

Concentration of silver nitrite or flavonoids extracts could also affect the formation of AgNPs. Fig. 2*b* shows the UV–Vis spectra of the synthesised AgNPs at different volume of silver nitrite with 1 mL of flavonoids extracts. When the volume of the silver nitrite was very low (1 mL), synthesis of AgNPs hardly happened, showing low absorption and colourless solution. With the increase of the volume of silver nitrite, the production of the AgNPs solutions gradually increased, with the enhancement of the maximum absorption intensity, and the characteristic yellowish solution. Furthermore, when the volume of the silver nitrite reached to 15 mL, absorption intensity at 404 nm increased, while a new absorption peak at 550 nm was formed, showing other sizes of AgNPs were formed. Colours of the solutions turned to dark brown as well. So 10 mL 20 mM $AgNO_3$ solution was used for further use.

Temperature always reported to be one of the most crucial factors on the synthesis of nanoparticles [28]. Effects of temperatures on the UV–Vis spectra of the synthesised AgNPs were studied, which were shown in Fig. 2*c*. Interesting, compared to varying the pH or the concentration of $AgNO_3$ of the reaction system, changes of temperatures showed no big influence on the synthesis of AgNPs. Either the maximum absorption peak or the maximum absorption intensity changed slightly when the reaction temperature varied from 40 to 70°C. When the temperature of the reaction system reached at 90°C, a new absorption peak at about 500 nm was found, indicating the formation of AgNPs with different sizes. In our experiment, 70°C was chosen as the optimal reaction temperature.

Reaction time is also one of the main factors that could affect the synthesis of AgNPs [29]. Fig. 2*d* shows the UV–Vis spectra of synthesised AgNPs after different reaction times. When the mixture reacted at 70°C for 0.5 h, AgNPs begun to form, which could be convinced from the colour change of the solution and the absorption intensity at 404 nm. When the reaction time reached to 1 h, the highest intensity at 404 nm was observed. However, with the increase of the reaction time, the intensity at 404 nm decreased, while a new peak at 500–550 nm was formed and the colour changes from yellow to pale red, corresponding to the formation of larger size of AgNPs. So we chose 1 h as the best reaction time.

Above all, the optimum conditions for the synthesis of AgNPs were as follows: 10 mL 20 mM $AgNO_3$ added to 1 mL flavonoids extracts, adjusting pH to 10, and reacted at 70°C for 1 h.

3.2. Characterisation of AgNPs: AgNPs synthesised under optimum conditions were characterised, which were shown in Fig. 3. Shape and size of the synthesised AgNPs were obtained with the help of TEM, which were shown in Figs. 3*a* and *b*. TEM results showed that the synthesised AgNPs were spherical in shape with an average size of 12.7 ± 2.3 nm. Compared to other AgNPs synthesised by plant extracts, our AgNPs showed narrower size distributions. Size distribution of the AgNPs colloid solution was measured by DLS principle, and the result was shown in Fig. 3*c*. The result showed that the average diameter of the AgNPs colloid solution was 50.88 nm, which was much larger than the result obtained from the TEM analysis. This may be due to that DLS measurement always gives the hydrodynamic radius of the particles, which indicating that the chemical compounds were enveloped to the core of the AgNPs [30]. Zeta potential of the synthesised AgNPs was also measured, which was shown in Fig. 3*d*. The value of the average zeta potential of the synthesised AgNPs was −33.9 mV. Large value of zeta potential obtained by the AgNPs revealed the good stability in the long run [31]. FT-IR spectra were always used to verify the chemical groups on the surface of synthesised AgNPs after reduction process, which was shown in Fig. 3*e*. FT-IR spectra showed the absorption peaks at 3432, 1481, 1384 and 881 cm^{-1} which represent various functional groups of flavonoids.

3.3. Evaluation of the stability of the synthesised AgNPs: For practical applications, AgNPs always dissolve in solutions with different pH or ionic strength. So the stability of the synthesised AgNPs was studied, which was shown in Fig. 4. Fig. 4*a* shows the UV–Vis spectra of the synthesised AgNPs solutions with different pH values. Generally speaking, synthesised AgNPs

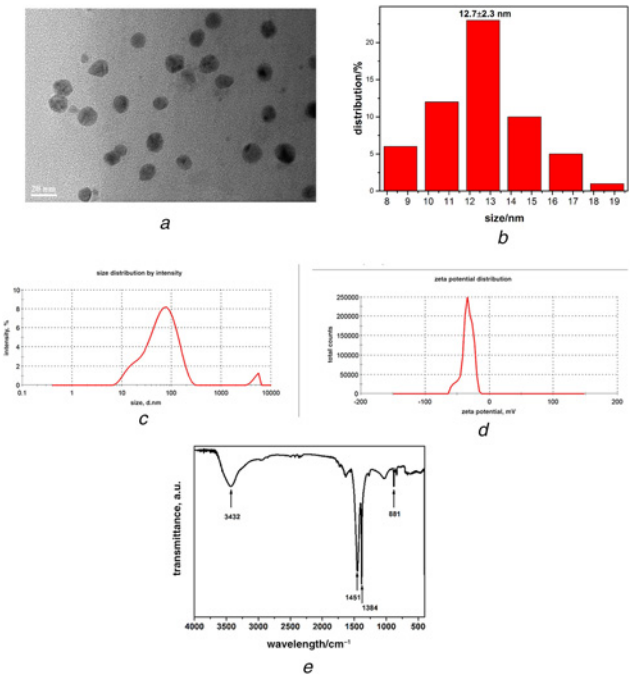

Fig. 3 *Characterisation of the synthesised AgNPs under optimum conditions*
a TEM image
b Particle size distribution histogram
c Dynamic light scattering (DLS) pattern
d Zeta potential measurement
e FT-IR spectra

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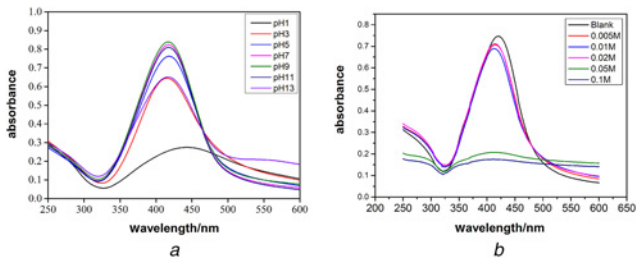

Fig. 4 *Stability of the synthesised AgNPs*
a UV–Vis spectra of synthesised AgNPs solutions dissolve at different pH
b UV–Vis spectra of synthesised AgNPs solutions dissolve at different concentration of NaCl

showed more stability in alkalis conditions than in acidic environment. Initial pH of the AgNPs solutions was 10, so the synthesised AgNPs could keep stable in neutral and alkalis conditions (pH = 7, 9 and 11), but aggregated in strong alkalis condition (pH = 13). Compared to the good stability in alkalis conditions, synthesised AgNPs showed less stability in acidic environments (pH = 3 and 5). When AgNPs dissolved in pH = 1 solution, AgNPs totally aggregated and the UV–Vis spectra changed a lot. Fig. 4*b* shows the stability of AgNPs at different concentration of NaCl. Synthesised AgNPs remained stable with the concentration of NaCl lower than 0.02 M. By contrast, when the concentration of NaCl exceeded 0.05 M, the UV–Vis spectra of the synthesised AgNPs changed tremendously, suggesting the aggregation of AgNPs. In biomedical use, AgNPs always dissolve in 0.9% NaCl, which are equal to 0.15 M. As can be inferred from the result of our experiment, synthesised AgNPs can keep stable when dissolves in 0.9% NaCl for biomedical application.

3.4. Evaluation of antibacterial activity of the synthesised AgNPs: Lots of researchers reported the antibacterial activity of AgNPs, so the antibacterial activity of the synthesised AgNPs was evaluated by Oxford Cup method, using *S. aureus*, *B. subtilis*, *E. coli* and *Salmonella* as the tested bacteria. Results were shown in Table 1. Synthesised AgNPs revealed good antibacterial activity against all four tested bacteria. The AgNPs showed highest antibacterial activity against *E. coli* (13.64 mm) and *Salmonella* (13.39 mm), lesser antibacterial activity of AgNPs was found against *S. aureus* (12.47 mm) and *B. subtilis* (10.77 mm). Minimum inhibitory concentrations (MICs) of the synthesised AgNPs for the four tested bacteria were measured as well, which were shown in Table 2. MIC of the AgNPs was 0.03125 mg/mL for *E. coli*, 0.0625 mg/mL for *Salmonella*, and 0.25 mg/mL for *S. aureus* and *B. subtilis*, respectively. In all, synthesised AgNPs showed higher antibacterial activity against Gram-negative bacteria than Gram-positive bacteria, which was in accordance with former reporters. The mechanism of the inhibitory effects may be attributed to the free radicals generated by AgNPs [13].

Table 1 Antibacterial activity of the AgNPs against selected pathogenic strains

Pathogenic microorganism	Zone of inhibition, mm
E. coli	13.64 ± 0.28
S. aureus	12.47 ± 0.07
B. subtilis	10.77 + 0.48
Salmonella	13.39 ± 0.07

Table 2 MICs for four tested bacteria (mg/mL)

Pathogenic microorganism	0.25	0.125	0.0625	0.03125
E. coli	+	+	+	+
S. aureus	+	+	+	–
B. subtilis	+	–	–	–
Salmonella	+	–	–	–

'+' means had inhibition zones; '–' means no inhibition zones.

3.5. Evaluation of catalytic activity of the synthesised AgNPs: To evaluate the catalytic activity of the synthesised AgNPs, we chose the transformation from *p*-nitrophenol (*p*-NP) to *p*-aminphenol (*p*-AP) reduced by sodium borohydride as the model reaction [30]. The maximum absorption peak of *p*-NP was at 317 nm initially. With the addition of sodium borohydride, the peak shifted to 400 nm, which was the characteristic peak of *p*-nitrophenolate ions [32]. Without any catalysis, the peak at 400 nm could stay stable for several days; however, with the addition of small volume of the synthesised AgNPs solutions, the yellowish colour of the reaction mixture faded quickly, and the absorption at 400 nm decreased gradually. Meanwhile, a new absorption peak at 300 nm was founded, which indicated the formation of *p*-AP. Time-dependent UV–Vis spectra were recorded, which were shown in Fig. 5.

Since the concentration of sodium borohydride was in great excess to the concentration of *p*-NP, so the concentration of sodium borohydride could be considered constant during the reaction. The reaction rate could be affected by the concentration of *p*-NP, and the reaction could be treated as pseudo first-order reaction. The kinetic equation could be shown as follows [33] (1):

$$\ln\left(\frac{C_0}{C_t}\right) = \ln\left(\frac{A_0}{A_t}\right) = k_{\text{app}}t \tag{1}$$

In this equation, C_0 and C_t were the concentration of *p*-NP at time 0 and t, respectively, while A_0 and A_t were the corresponding absorption at 400 nm. k_{app} was the apparent rate constant.

The absorption at 400 nm in the absence or presence of the synthesised AgNPs at different times was shown in Fig. 6. In the absence of AgNPs, absorption at 400 nm showed no decrement in 10 min, indicating that no reduction reaction happened. In the presence of 6.7 μg mL^{-1} AgNPs, absorption at 400 nm decreased dramatically, and a good linear relationship between $\ln(A_0/A_t)$ and the reaction time t could be observed (Fig. 6, the inset). The k_{app} could be calculated from the slope of the straight line, which was 0.276 min^{-1}.

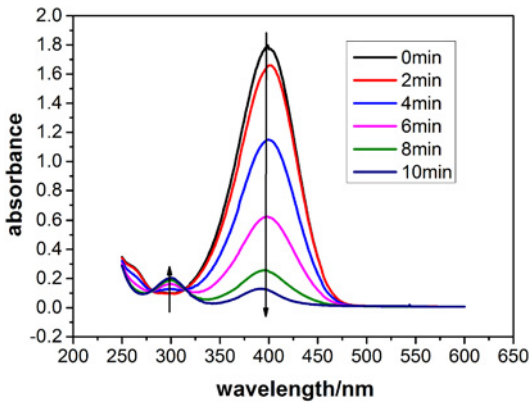

Fig. 5 *Successive UV–Vis spectra of the borohydride reduction of p-NP catalysed by AgNPs. Concentration of the AgNPs was 6.7 μg mL^{-1}*

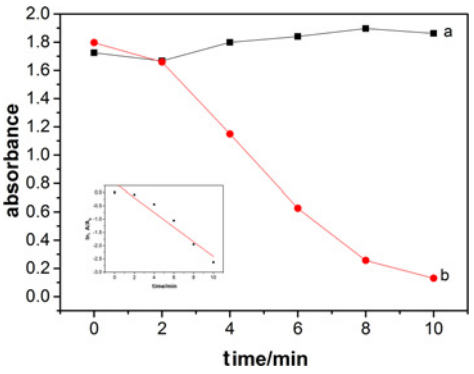

Fig. 6 *Absorbance at 400 nm. Inset: first-order linear plot of $\ln(A_t/A_0)$ versus time. Concentration of the AgNPs was 6.7 μg mL^{-1}*
a Absence of AgNPs
b Presence of AgNPs

4. Conclusion: A simple, economic and efficient method for the biosynthesis of AgNPs by flavonoids extracts from petals of *Lilium casa blanca* were established. By simply added flavonoids extracts to silver nitrate solution at appropriate pH, AgNPs formed with 1 h. Synthesised AgNPs showed good stability, antibacterial and catalytic activity. More applications of the synthesised AgNPs in biomedical fields were on the way in our lab.

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