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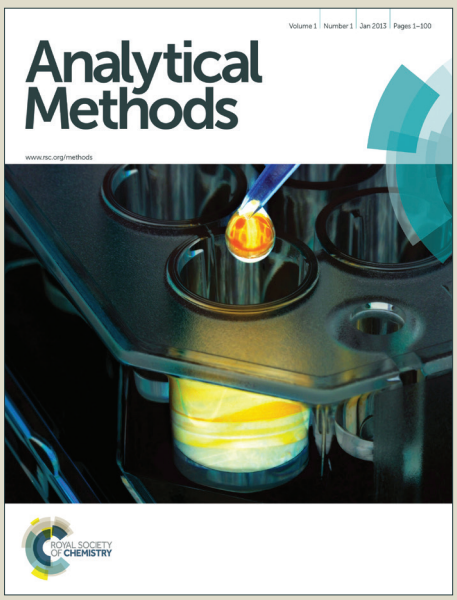

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ARTICLE TYPE

Green synthesis of gold nanoparticles with willow tree shells extract; sensitive colorimetric sensor for cysteine detection

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Abstract:

In this work, we report an easy and simple green synthesis method for gold nano particles, GNPs, using the willow tree shells extract. Willow tree shells include aspirin that acts as a reducing agent. In the present method there was no need to the addition of any stabilizing agent, such as a surfactant, to stabilize the synthesized GNPs. Therefore, green synthesis of gold nanoparticles with willow tree shell extract, as an alternative to chemical synthesis, is beneficial from its biological and medical application points of view. The optimum conditions for synthesis of the GNPs were obtained by studying the pH and amount of willow tree shell extract solution. Characteristics and morphology of GNPs were investigated by UV-Vis spectroscopy and transmission electron microscopy.

The interaction between the synthesized gold nanoparticles and cysteine was introduced as a new and high potential colorimetric sensor for the selective recognition and monitoring of cysteine among other amino acids. The sensitivity and selectivity of GNPs toward cysteine in comparison to other amino acids were studied.

Keywords: Gold nanoparticles, Willow tree shell, Colorimetric detection, Cysteine

1. Introduction

The characteristic of nanoparticles that makes them different from macroscopic solids is the surface atoms that have a very high percentage [1, 2]. Metal nanoparticles have numerous applications branch out in various fields of science and technology such as catalysis [3], optoelectronics [4], and biomedical applications [5].

There are a number of synthetic procedures for obtaining gold and other metal nanoparticles (NPs) over a wide range of sizes [6-14]. Gold nano particles (GNPs) often exhibit an

absorption band in the visible spectrum, which is usually termed as surface plasmon resonance (SPR) [15]. The peak position of SPR can be manipulated by varying the size, shape, surface properties, as well as the surrounding medium of the nanoparticles, which are the fundament for their applications in sensing [16-19].

Although there are lots of techniques available for the synthesis of GNPs and other metal nanoparticles [20-25], presenting new routes for the nano-particles synthesis without the need of adding any reducing agent is an interesting issue. Metallic NPs have often been produced by chemical reduction of the corresponding metal salts in solution in the presence of suitable reducing agents and organic stabilizing molecules. Although chemical methods are capable of producing a large number of nanometals using suitable reducing agents, the excess reducing agent ans also the oxidation product stay in the solution and it is very difficult to remove them. On the other hand, in many cases the reducing agent might have toxic characteristics; this further complicates the application of NPs to biomedicine [26,27].

Recently Vargas-Hernandez et al [28] showed that NPs synthesis could be performed by microwave-assisted technique without the need for the addition of any reducing agent.

Recently presenting new methods involving green materials which act both as reducing and stabilizing agents have been attracted great interests. Ciprian Tomuleasa et al [28] have used aspartate for the synthesis of GNPs. Aspartate in that work acts both as the reducing and capping agent.

Yong Shao et al [29] applied aspartate for synthesis of GNPs. They showed that no additional reducing agent and surfactant are needed for the synthesis of GNPs.

Recently we applied a new synthetic hydrogel to synthesis the GNPs. Poly (styrene-alt-maleic acid), a pH-sensitive hydrogel bearing negatively charged $–COO^-$ groups, was used for this purpose. There was no need to the additional reducing agent and/or any surfactant for the stabilizing of synthesized nanoparticles [30].

In this paper, a new green method is put forward for the production of GNPs using the Willow tree shells extract as the reduing agent and stabilizer. Willow tree shells include the aspirin that acts as reducing agent. In the proposed method no stabilizing agent is required. The results showed that pH 5 is suitable for the synthesis of GNPs using the Willow tree shells extract. The synthesized Au-nanoparticles were applied for the spectrophotometric measurements of cysteine.

Identification of light amino thiols as biological molecules, such as cysteine in human plasma, according to the following items are important; cysteine is an amino acid, a building block of proteins that are used throughout the body. When taken as a supplement, it is usually in the form of N-acetyl-L-cysteine (NAC). The body makes this into cysteine and then into glutathione, a powerful antioxidant. Antioxidants fight free radicals, harmful compounds in the body that damage cell membranes and DNA. Researchers think free radicals play a role in aging as well as the development of a number of health problems, including heart disease and cancer [31-32]. Also, cysteine is a sulfur containing amino acid, which is critical to the metabolism of a number of essential biochemicals, including coenzyme A, heparin, biotin [33]. Therefore, it is very important to find a sensitive, accurate and simple method for the measurement of cysteine.

The currently available methods relating to the detection of cysteine are based on the electrochemical, immunoassay and chromatography techniques that benefit from derivatization with chromophores or fluorophores, or often carried out in conjunction with High Performance Liquid Chromatography, Gas Chromatography, Mass Spectroscopy and capillary electrophoresis [34–45]. Although, these methods can well sense aminothiols their practical applications are limited because of suffering from some inherent drawbacks, such as requiring complicated instrumentation, expensive biological reagents or cumbersome sample preparation [33].

Recently Hormozinezhad et al [33] reported a highly sensitive colorimetric method for the determination of cysteine and glutathione, based on aggregation of the citrate capped GNPs which displays a linear relation in the range 1×10^{-6}–100×10^{-6} mol L^{-1} cysteine.

As mentioned, in this paper a new and green method is presented for the production of GNPs using the Willow tree shells extract. The interaction between cysteine and the synthesized nanoparticles was studied and used for the colorimetric identification and spectrophotometric determination of cysteine. Presence of cysteine in the solution caused a change in the SPR band of GNPs that was proportional to the cysteine in a certain concentration range. This interaction was a selective interaction for cysteine among other amino acids. Finally the blood compatibility and complement activation of the synthesized GNPs using willow tree shell extract were also evaluated to confirm its potential towards blood contacting applications. The stable gold nanoparticles synthesized with biological extract could be highly beneficial for drug delivery, gene delivery and biosensor applications where there is a direct contact of these nanoparticles with blood [47].

Also dynamic light scattering (DLS) analysis of synthesized GNPS were used to show and admit the changes in the particle size of aggregates caused by cysteine.

2. Experimental

2.1. Material and Instruments

Hydrogen tetrachloroaurate (III) tetrahydrate solution (prepared using pure Au 99.9%), sodium hydroxide (Merck), hydrochloric acid (Merck) and nitric acid (Merck) were used during the experiments. Cysteine and other amino acids were all from Merck.

Spectrophotometric measurements were done with a PG mode T80 UV-Vis double-beam spectrophotometer (Japan) utilizing a 1-cm quartz cell (volume 3 mL)**.** Samples for transmission electron micrograph (TEM) were prepared by placing a drop of the aqueous solution of gold NPs on to a copper micro grid and drying at room temperature. TEM photograph were taken on (Zice-50 kv) transmission electron microscopes at acceleration voltage of 50 kv. The dynamic ligh scatter analyzing were done using a DLS analyzer, Zetasizer Ver. 6.00 (Malvern Instruments Ltd, Worcestershire, UK),

2.2. Synthesis of gold nanoparticles

A 10 grams of Willow tree shells was added to the 250 ml of distilled water and was boiled about 30 minutes to extract squeezed juice. The solution was filtered using Whatman paper and made to final volume of 250 mL using distilled water.A 2 ml of this solution was added to a beaker containing 1.5 ml of the

1mmol L^{-1} $HAuCl_4$ solution. The solution was mixed gently and heated for about 2 minutes on the heater**.** The color of the resulting solution was wine-red.

3. Results and discussion

3.1. Effect of pH on the synthesis of Au nanoparticles

Uv–Vis spectroscopy is an important and simple technique to obtain the formation and stabilization of aqueous metal nanoparticles. The addition of willow tree shell extract to 1 mmol L^{-1} aqueous $HAuCl_4$ solution resulted in the color change from yellow to wine red due the formation of GNPs. GNPs SPR band centered at about 525 nm was observed as shown in Fig. 1 in the visible spectrum of the nanoparticle suspension. These particles were suspended in phosphate buffer and this showed an SPR band centered at 525 nm indicating that these particles are stable at physiological conditions.

Uv-Vis absorption spectra of the nanoparticle solutions at different pHs were recorded. Fig. 2 represents the spectra and also the ratio of maximum absorbance (Abs. in 525 nm) to bandwidth of spectra (A_{max} / $W_{0.5}$) versus pH. This plot can be a good criterion which helps to find out the optimum pH. Narrowing the spectrum of synthesized nanoparticles shows uniform GNPs have been synthesized. The results showed that the SPR of synthesized Au nanoparticle increased by increasing pH of the solution up to 5 and decreased at higher pHs. Therefore, pH 5 was selected as the optimum pH for further studies. In this pH the peak height was higher and the peak width was lower than those in other pHs. At pHs higher than 7 broadening of the band and a corresponding change in color of the suspension were observed.

From these plots it can be concluded that synthesis of gold nanoparticles (GNPs) is accomplished at mild acidic media (4<pH<7) well. The results showed that at pH = 5 the synthesized GNPs are the most uniform particles. Therefore, the pH 5 was chosen for further studies .At lower pHs (lower than 4) the synthesis of Au nanoparticles was not done. This is may be because of protonation of aspirin regards to its pKa value. On the other hand at higher pHs, OH^- might hydrolysis the Au(VI) to $Au(OH)_n$ in which it might prevent GNPs synthesis.

3.2. Effect of squeezed juice amount

In order to study the effect of willow tree shells extract amount on the synthesis of GNPs, different amounts of the stock solution was added to the solutions containing constant amount of $HAuCl_4$. For this purpose 1.5 mL of 1×10^{-3} mol L^{-1} $HAuCl_4$ was placed in a beaker and different amounts of extracted solution of willow tree shells were added and the final pH was adjusted at 5 by dilute HCl or NaOH. The solutions were made up to 10 mL by distilled water. Then the solution was heated for 2 min on the heater. It must be mentioned that the 2 min incubation time was selected based on the color monitoring of the solutions in primary experiments. By more incubation time the color of the solutions were changed to black. The solutions were cold and their absorption spectra were recorded. The results showed that the SPR of synthesized Au nanoparticle increased by increasing the willow tree shells extract solution up to 2 mL of the stock solution and decreased at higher concentrations. Therefore 2 mL of stock solution was selected as optimum amount for further studies

As mentioned, in the presented GNPs synthesis rout, no additional reducing agent and surfactant were needed. It seems

the willow tree shells extract acted both as reducing and stabilizing agents and therefore there was no need to use any further reducing or stabilizing agent. It has been mentioned that the willow tree shell contains aspirin [Ref]. The history of aspirin (also known as *acetylsalicylic acid*), its medical uses and related substances stretches back to antiquity, though pure ASA has only been manufactured and marketed since 1899. Medicines made from willow and other salicylate-rich plants appear in Egyptian pharonic pharmacology papyri [47]. This drug is a reducing agent. It means that probably the aspirin reduced the Au(III) to Au(0). The infra red spectrum of the willow tree shell extract was recorded and is shown in Fig. 3. As can be concluded from this spectrum, the extracts contains compound with –OH/ –N–H , –C=O, C–C–, C==C–, etc. functional groups. Therefore, it can be assumed that the water soluble compounds like tanens, alkanoids, flavoinds and alkaloids which have been extracted to water act as the capping agent in the synthesis of gold nanoparticles which also help in the stabilization of nanoparticles at physiological conditions. Such statements has been recently reported elsewhere for stabilization mechanism of GNps using Zingbar officinal extract [47, 49].

3.3. TEM characterization

Transmission electron microscopy image (Fig. 4) displays and admits that the gold nanoparticles have been obtained under the optimum conditions. From TEM images, it has been clarified that GNPs were successfully fabricated of uniformly distributed spherical structure with below ~15 nm in size.

3.4. Stability of the synthesized gold nanoparticles

The gold nanoparticles synthesized with willow tree shell extract were checked for their stability in synhesis media in pH 5 and also in pH 7. Stability was evaluated by analyzing its variation in the spectra of nanoparticles solutions with time for one month period at room temperature. The absorbances of the solutions at 525 nm versus time are shown in Fig. 5. The results indicated that the synthesized nanoparticles were very stable in the studied period. The same results were obtained at pH 7 (0.05 mol L^{-1} phosphate buffer solution).

3.5. Application of the synthesized gold nanoparticles for detection of cysteine

The interaction of cysteine with gold nanoparticles was investigated. The solution of the cysteine in microliter amounts was added into the gold nanoparticles solution and the absorption spectra were recorded at room temperature and spectrophotometric determination of cysteine was done. Also the colorimetric detection was applied for detection of cysteine among other amino acids. Capping of the GNPs with cysteine was accomplished in the presence of 1×10^{-3} mol L^{-1} NaCl by a thiolate bond in less than 15 min (Fig. 6).

3.5.1. The effect of pH on the detection of cysteine

The effect of pH on the colorimetric evolution was investigated. The results are shown in Fig. 6. The results showed that pH 5 is suitable for interaction of cysteine with these green synthesized GNPs. The results also showed that the difference between the absorbance at pH 5 with that at pH 3 was due to rate of color change from wine-red to violet.

These evidence versus pH changes can be attributed to the interaction between cysteine and gold nanoparticles. Through the covalent combination with the –SH group and the electrostatic binding with the $–NH_3^+$ group of cysteine, gold nanoparticles can self-assemble to form a network structure. The experimental results demonstrate that the the pH ~5 is suitable because of pKa values of –SH group and $–NH_3^+$ group of cysteine.

3.5.2. Effect of electrolyte concentration

The effects of solution ionic strength on the cysteine-induced aggregation of the synthesized GNPs were studied. The effect of NaCl concentration on the aggregation of GNPs was studied by spectrophotometric monitoring of the solutions. The absorbance of the solutions versus electrolyte concentration is shown in Fig. 6. The results showed that although addition of NaCl was necessary to accelerate the

GNPs color change reaction, but low amounts of NaCl was enough to accelerate the aggregation process of GNPs with cysteine (a similar effect was also found when other salts, such as $NaNO_3$ and KCl was used).

3.5.3. Analaytical charachteristic of the method

The interaction between cysteine and gold nanoparticles was used to construct the calibration curve. Through the covalent combination with the –SH group and the electrostatic binding with the $–NH_3^+$ group of cysteine, gold nanoparticles can self-assemble to form a network structure. This evidence can be resulted as changing the color of solution from red to purple by addition of cysteine (Fig 7). .

To evaluate the sensitivity of this method and to investigate the minimum detectable concentration of cysteine in aqueous solutions, different concentrations of aqueous solution of cysteine was added into the solutions of the synthesized GNPs (2 mL) at room temperature in optimum conditions and the absorption spectra were recorded. As Fig. 7 shows, the absorbance peak of GNPs decreased at 525 nm and increased at 650 nm by increasing the concentration of cysteine. There was a linear relationship between the absorbance intensity changes and the concentration of cysteine over the range 2×10^{-7} - 20×10^{-7} mol L^{-1} at 650 nm.

The limit of detection (LOD) for cysteine was determined using the standard deviation of 6 replicate monitoring of SPR peak of GNPs and the slope of the calibration curve. The results are shown in Table 1. The relative standard deviation for 6 replicate determinations of 1 µmol L^{-1} cysteine using the synthesized GNPs is represented in Table 1.

Table 1.

3.5.4. Selectivity study of the proposed method toward cysteine

After addition of a known amount of cysteine (e.g. 10^{-6} mol L^{-1}) to GNPs, the color of the solution changed to violet. But adding of other amino acids (such as arginine, glutamic acide, isoliucine, threonine, alanine, trypthopan, aspartic acid, asparagine, valine, histidine, lysine, methionine, glycine, serine), at the concentration at least 100 times over the cysteine, did not change the color of the solution related to SPR of GNPs, monitored visually. The results of color changes are shown in

Fig. 8. The possible interactions between the mentioned amino acids and synthesized nanoparticles were studied also by spectrophotometric monitoring of SPR band of GNPs solution. The results admitted that the changes of SPR of synthesized GNPs were very negligible except for cysteine. Therefore the synthesized nanoparticles showed selective interaction toward cysteine among other amino acids.

3.5.5. Dynamic light scattering analysis

By using Dynamic light scattering (DLS), we assessed the aggregation of synthesized Au nano particles in the presence f cysteine. The size distribution by volume, number and intensity of synthesized AuNPS without cysteine were shown in Fig. 9 (A1, A2 and A3). The DLs analysis were also done after addition of cystein (final concentration 10^{-6} mol L^{-1}) into the synthesized GNPs at the optimum conditions (NaCl 5 mM and pH 5). The sze distribution were shown in Fig. 9 (B1, B2 and B3). These Figs clearly admit the aggregation of synthesized Au nano particles in the presence of cysteine. The analysis were done at 25^{O}C.

The Z-average is the intensity, weighted mean hydrodynamic size of particles and a second parameter, the polydispersity index (PdI), indicates the width of the particle size distributions. Before addition of cystein The Z-average and the polydispersity index (PdI) were 106.1 nm and 0.751respectively. These parameters after addition of cystein were 260.0 nm and 0.616 respectively. The results show that there are polydisperse particles in the sample. These results were based on the intensity analysis while the %number and % volume admit that nealy monodisperse particles with nearly 10 nm diameter and 2.12 nm have been synthesized. After addition which after addition of cystein because of aggregation of Au Nanopriticles with cysteine the Z-average hase been increased while the dispersity of the particles have been not changed moderately.

The DLS analysis results for size distribution by volume and number were also presented in Fig. 9. All of results clearly admite the aggregation happened by cystein.

3.5.6. Interaction of the synthesized GNPs with blood plasma

It has been mentioned that stable gold nanoparticles synthesized with biological extract could be highly beneficial for drug delivery, gene delivery and biosensor applications where there is a direct contact of these nanoparticles with blood [47]. The blood compatibility and complement activation of the synthesized GNPs using willow tree shell extract were also evaluated to confirm its potential towards blood contacting applications. For this purpose, a 1.5 mL aliquot of human plasma sample was mixed with a 1.5 mL of synthesized GNPs. The pH was adjusted at 7 using phosphate buffer. The stability of GNPs in the presence of plasma was studied by recording the Uv-Vis spectrum during 1 month. This mixture was kept at room temperature. This experiment was done tree times. The mean absorbance was measured at 525 nm and plotted versus time. The result is shown in Fig. 10.a. Also the bandwidth of each spectrum versus time is shown in Fig. 10.b. The color of the mixture was tested every day and no change in wine-red color of the mixture was observed. All these results admited that there was not any possible interaction between synthesized GNPs and blood contents which makes it beneficial for drug delivery, gene delivery and biosensor applications. It can be concluded that the stable gold nanoparticles synthesized with willow tree shell extract can be beneficial for drug delivery, gene delivery and biosensor applications where there is a direct contact of these nanoparticles with blood.

4. Conclusion

The willow tree shell was used for the first time as a green procedure to synthesis the Au-nano particles. We think the included aspirin acts both as reducing and capping agent in the synthesis of GNPs. Characteristics and morphology of GNPs were investigated by UV-Vis spectra and transmission electron microscopy (TEM). The easy preparation and high stability (>30 days) of the synthesized GNPs allow this method to be a very simple and easy to implement method. The colorimetric and spectrophotometric method for the determination of cysteine shows synthesized GNPs selectivity toward cysteine over other amino acids. The blood compatibility and complement activation of the synthesized GNPs using willow tree shell extract were also evaluated.

Compared to our previous work (a new synthetic hydrogel Poly (styrene-alt-maleic acid) pH-sensitive hydrogel)to synthesize the gold nanoparticles (GNPs). [30], in this work, a new green method is put forward for the production of GNPs using the Willow tree shells extract as the reduing agent and stabilizer. The optimum pH was the same for both methods. Instead of applying chemical compounds and a difficult pre-synthesis and identification steps, a very simple extraction process was applied to prepare the willow tree shell extracts. The previously synthesized GNPs using the hydrogel containing –COO- groups, were also evaluated to test its potential towards blood contacting applications. The results showed the advantages of the Willow tree shells extract method regards to stability of the synthesized AuNPs.

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Notes

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Table 1. Analytical characteristics of the proposed method for detecting the cysteine

Regression equation	**y = 209561 C - 0.0042**
Linear range	**2×10^{-7} - 20×10^{-7} mol L^{-1}**
Regression coefficient	**0.994**
Relative standard deviation (n=6)	
(2×10^{-7} mol L^{-1})	**5.3 %**
(5×10^{-7} mol L^{-1})	**4 %**
(10×10^{-7} mol L^{-1})	**3.2 %**
(20×10^{-7} mol L^{-1})	**3.5 %**
Limit of detection (n=7)	**0.63×10^{-7} mol L^{-1}**

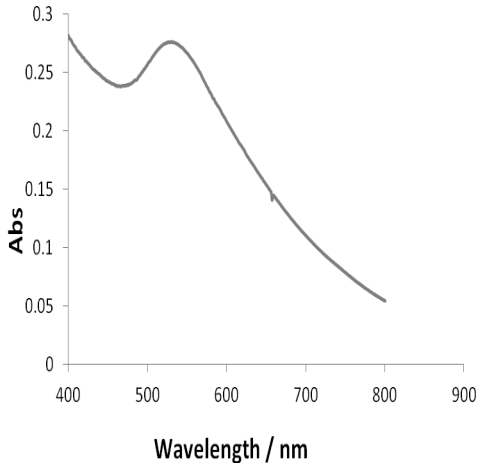


Fig. 1. The Uv-Vis spectrum of synthesized gold nanoparticles using willow tree shels extract in pH 5.

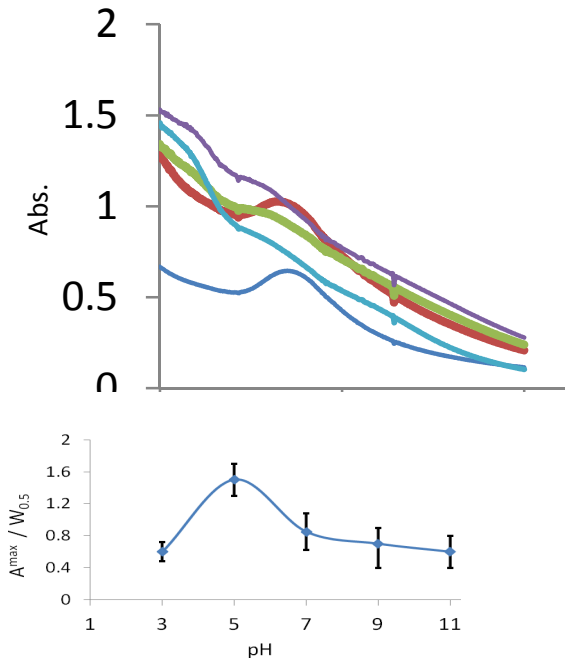


Fig 2. The absorption spectra of synthesized GNPs at different pHs and the absorbance of synthesized nanoparticles at 520 nm versus pH; the ratio of maximum absorbance to bandwidth of the spectra (A_{max} / $W_{0.5}$) of synthesized gold nanoparticles versus pH.

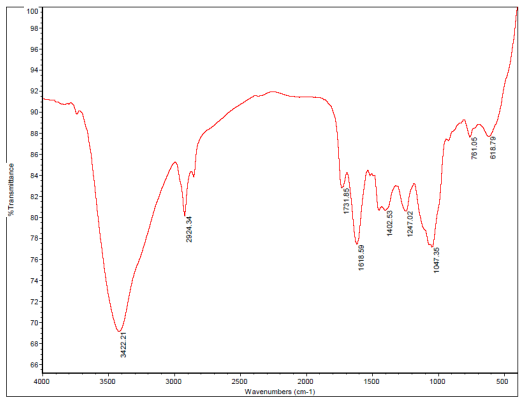


Fig3. The IR spectrum of willow tree shell extract

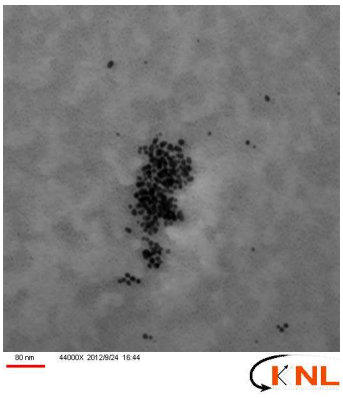


Fig 4.TEM image taken from the gold nanoparticles synthesized by Willow tree shells extract.

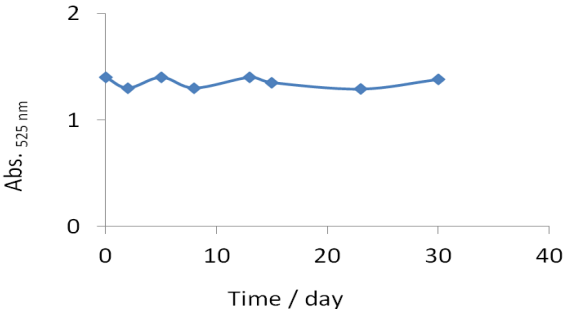
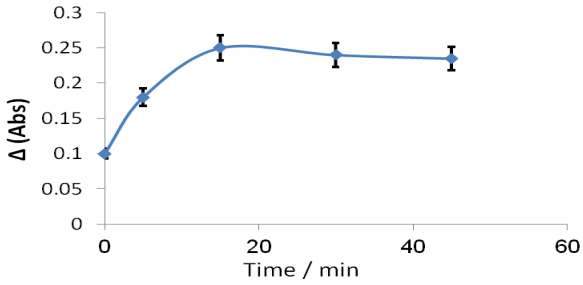
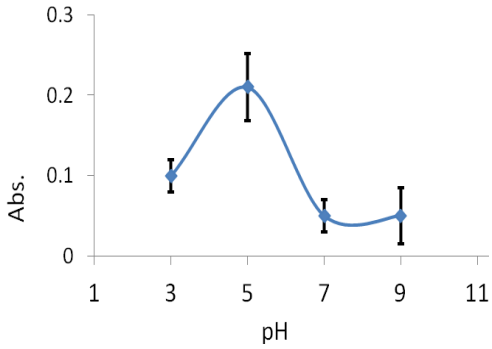


Fig. 5. The stability of synthesized gold nanoparticles studied by monitoring the absorbance of solutions versus time at 525 nm.





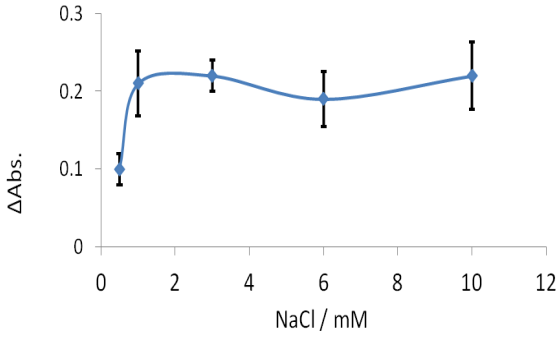


Fig.6. The compellation of GNPs – cysteine interaction, The effect of pH on the detection of cystein using synthesized GNPs and The effect of NaCl concentration on the aggregation of GNPs by cystein recorded after 15 min.

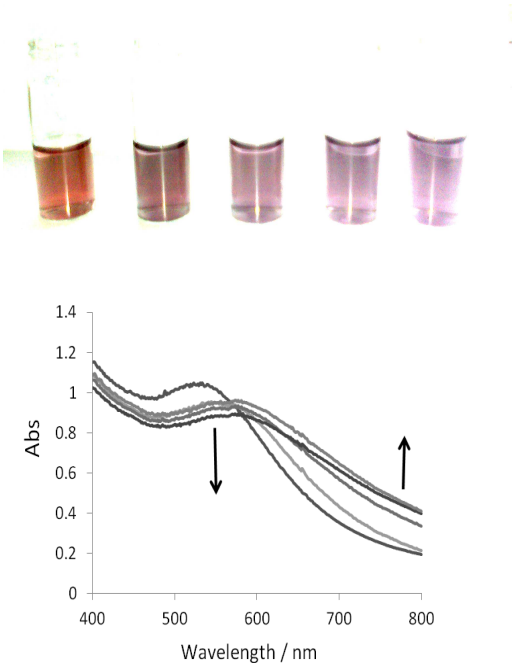


Fig. 7. The color changes and Uv-Vis spectra of 2 mL GNPs solution upon addition of cysteine (final concentration 0.2 – 2 µmol L^{-1}).

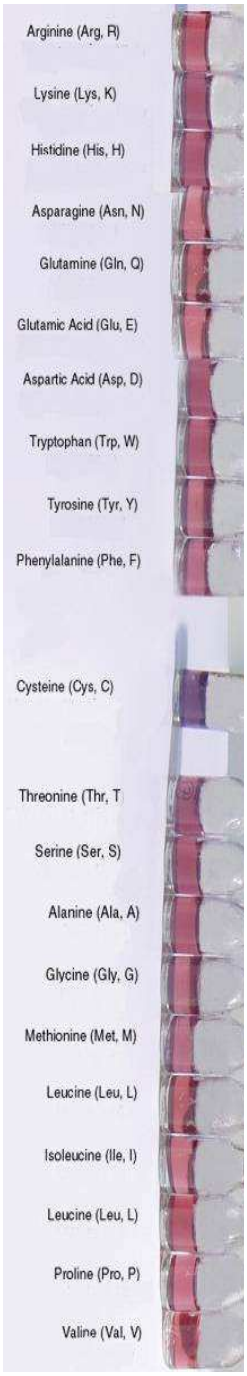
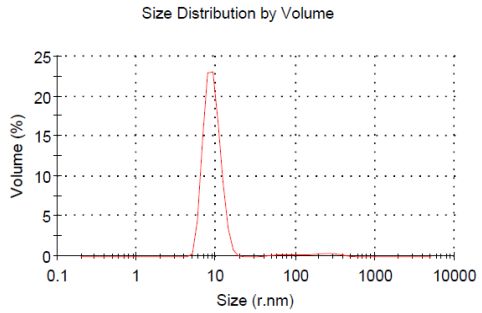
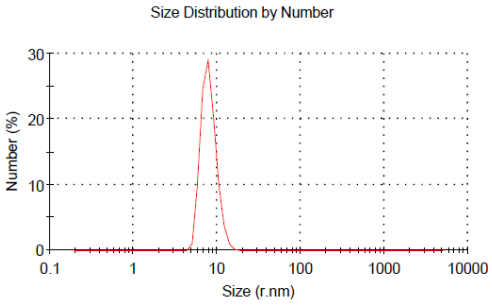


Fig. 8. Colorimetric responses of the synthesized GNPs toward cystein. cystine and 20 standard amino acids other than Cys (each 20 µmol L^{-1}); othe amino acids, 1200 µmol L^{-1}. The solutions contained 2 mL synthesized GNPs at pH ~5

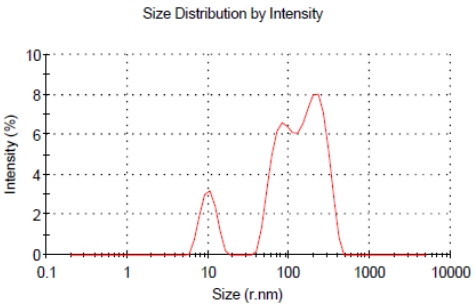
A1



A2

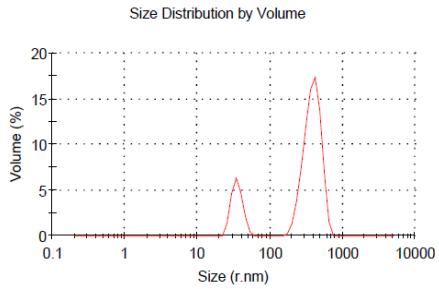


A3

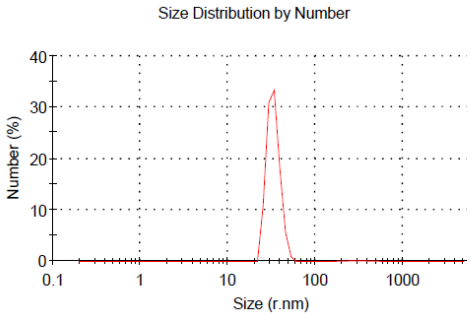


	Diameter / nm	% Number	% Volume	% Intensty	Width / nm
Peak 1	9.12-10.2	100	96	12.2	2.12
Peak 2	82.21-87.10	~0	1.7	38.7	27.5
Peak 3	217-244	~0	2.6	49.0	69-83

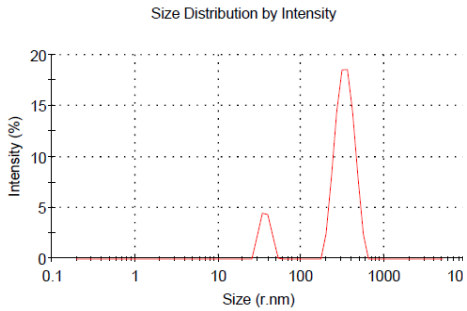
B1



B2



B3



	Diameter / nm	% Number	% Volume	%Intensity	Width / nm
Peak 1	35	99.6	80.4	86.8	5.53
Peak 2	350	0.4	19.6	13.2	82-89

Fig. 9. DLS analysis for synthesized Au nanoparticles (Size distribution by Volume, Number and Intensity) without cysteine (A1 and A2) and in the presence of cysteine (B1 and B2) to show the aggregation.

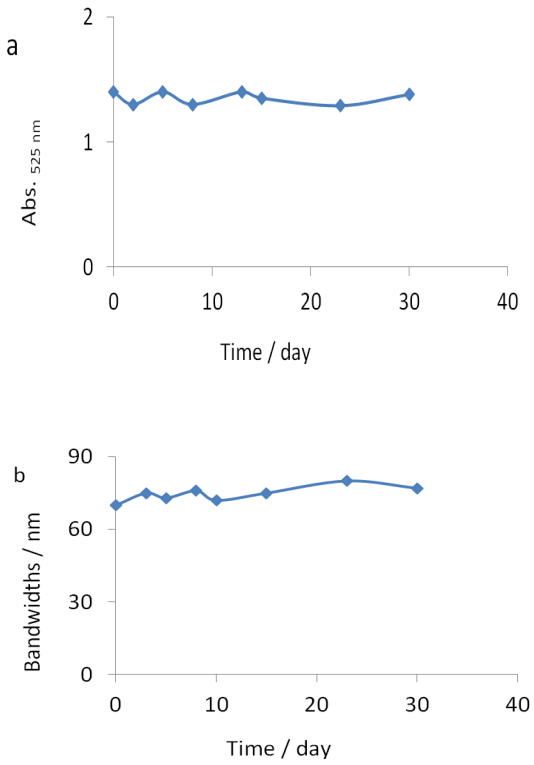


Fig. 10. Variation in maximum absorbance at 525 nm (a) and bandwidth of spectra of gold nanoparticles (b) prepared with willow tree shell extract with time at pH 7.