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Hepatoprotective and Urease Inhibitory Activities of Garlic Conjugated Gold Nanoparticles

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Abstract

A robust synthetic method is reported for the synthesis of highly stable, poly disperse and spherical gold nanoparticles conjugated to aqueous garlic extract (G-AuNPs). Ionic solution of gold was mixed and stirred with dilute aqueous garlic extract at room temperature for 4 hours. The G-AuNPs were characterized by UV–visible spectroscopy. The morphology and size of the G-AuNPs were determined by atomic force microscopy. FTIR spectroscopy revealed that secondary metabolites present in the garlic extract worked as capping agents around the gold nanoparticles. The G-AuNPs were quite stable even at higher temperature or with salt concentrations up to 10 mM or with various pH ranging from 4 to 11. Two different model systems were tested for evaluating the biological roles of G-AuNPs. They showed excellent hepatoprotective activity in a CCl_4-induced acute hepatic injury model which was better than the positive control, silymarin. They also showed urease inhibitory activity with an IC_{50} values of 180.61 ± 1.06 μg/mL. Therefore, the G-AuNPs can be useful in the treatment of diseases caused by injury or inflammation.

Keywords

Allium sativum, garlic, gold nanoparticles, AFM, hepato-protection, urease.

Introduction

Liver is a vital organ primarily responsible for the metabolism of drugs, alcohol and many other chemicals. It detoxifies chemicals (i.e. CCl_4, or Paracetamol), pollutants or even viral infections. During this detoxification, if overwhelmed, the liver experiences cellular damages which can lead to liver diseases.[1] Acute injury to the liver known as acute hepatitis is a major cause of morbidity and mortality around the world. Carbon tetrachloride (CCl_4)-induced liver injury in rats is a well characterized and widely used animal model for acute and chronic hepatitis and shows hepatic lesions similar to human liver disorders.[2] The CCl_4-induced acute liver injury involve the death of hepatocytes and other nonparenchymal cells.[3]

Garlic (*Allium sativum*) is one of the oldest of medicinal plants which is known for its antimicrobial,[4] antithrombotic,[5] hypolipidemic,[6] antiarthritic,[7] hypoglycemic,[8] antitumor,[9] and antioxidant[10] activities. The aqueous garlic extract has been explored for its biological activities both in *in vivo* and *in vitro* studies.[11] Garlic extracts showed hepatoprotective effect in mice/rats against acute hepatitis induced by hepatotoxins such as paracetamol, CCl_4, or galactosamine/lipopolysaccharide.[12] *In vitro* studies with silver nanoparticles (25 μg/mL) performed for cytotoxicity assays indicated no decrease in cellular proliferation for vascular smooth muscle cells and 3T3 fibroblasts.[13] G-AuNPs remained non-toxic against *S. cerevisiae* up to 100 μM concentration as assessed by well diffusion and inhibition of colony forming efficiency assay.[14] Surprisingly, *in vivo* studies of G-AuNPs have been ignored in the past. Currently, gold nanoparticles and biomolecules are considered for a wide array of medical applications, including sensing,[15-17] photodynamic therapy,[18] drug delivery,[19] imaging,[20] and hyperthermia.[21]

Here we are reporting a robust method for the synthesis of G-AuNPs. Both the aqueous garlic extract (G-H_2O) and their conjugated gold nanoparticles (G-AuNPs) were tested for hepatoprotective activity in a CCl_4-induced acute liver injury model. They were also subjected to urease, xanthine oxidase, and carbonic anhydrase-11 inhibitory activities.

Results and Discussion

Characterization of Garlic Conjugated Gold Nanoparticles

Gold nanoparticles usually exhibit a pink color in water due to surface plasmon excitations.[22] During the nanoparticles synthesis, a pink color was observed that showed a strong absorption band at 574 nm (Figure. 1). Different synthetic batches of G-AuNPs showed similar peak location and but different surface plasmon resonance (SPR) intensities. Furthermore, when the gold concentration was doubled while keeping the concentration of extract constant SPR did not change but the peak intensity increased proportionally. This behavior can be attributed to the quantitative increase of gold nanoparticles. The quantitative effects associated with the concentration of ligands and gold salts in the reaction media was evaluated by varying the concentration of gold salt while keeping the concentration of extract constant. A proportional increase in the intensity of the absorption band was observed with the increment in the gold concentration (Figure 1). These behaviors can be attributed to the quantitative increase of gold nanoparticles.[21, 23] The G-AuNPs were found to be stable up to 80 ^0C[24] (Figure S2), 3-11 pH range (Figure S3) and up to 10 mM NaCl concentration (Figure S4).

AFM analysis revealed polydispersed, spherical G-AuNPs with varying sizes. A typical size distribution of G-AuNPs is shown in Figure 2. The particles were found in the 16-60 nm diameter range with a maximum particle size distribution of about 30-60 nm.

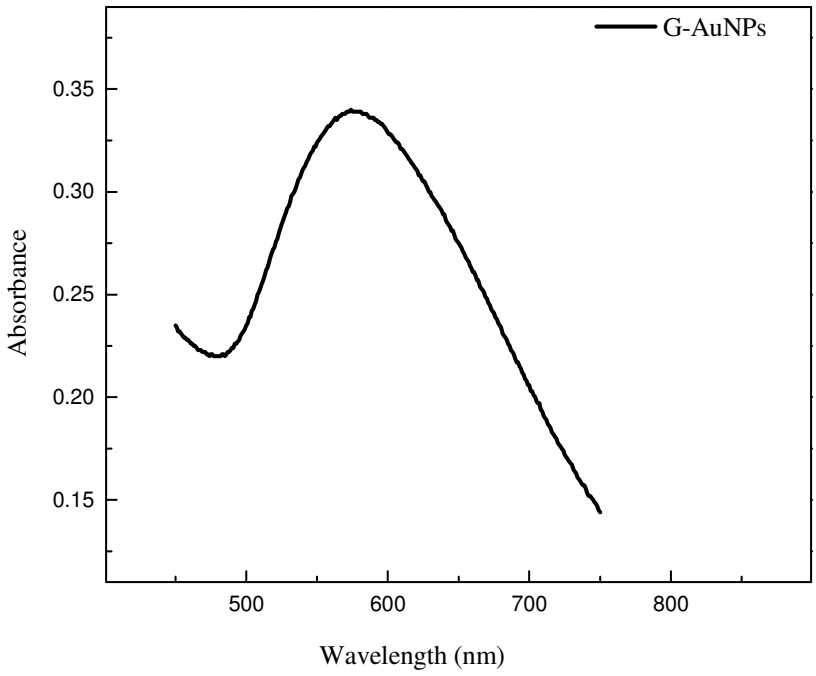
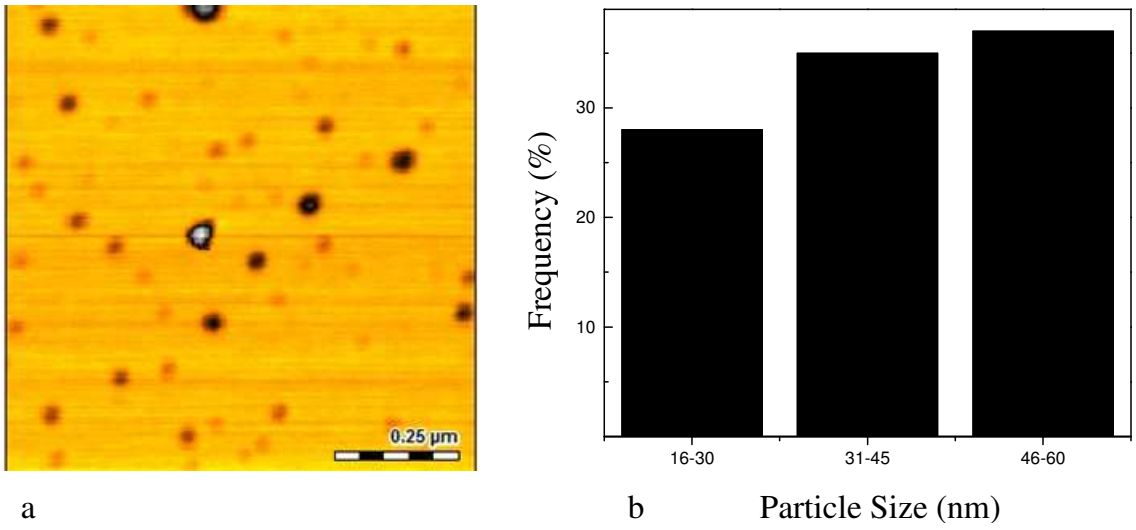


Figure 1 UV-Visible spectrum of G-AuNPs



a b

Figure 2 AFM analysis of G-AuNPs. (a) Topography (b) Particles size distribution

Fourier transform infrared spectroscopy (FTIR)

The G-AuNPs were also characterized by FT-IR analysis.[25-27] In order to identify the functional groups that stabilize G-AuNPs, FT-IR analysis was carried out and compared with the IR spectrum of G-H_2O as reference. The FT-IR spectrum of G-H_2O showed stretchings at 3232, 2926, 1633, 1427, 1191, 1026, 943, 819, and 603 cm^{-1}, respectively, whereas G-AuNPs showed stretchings at 3324, 2931, 1631, 1408, 1262, 1060, 927, 819, and 602 cm^{-1}, respectively. The absorption bands assignment was as follows: 3324 cm^{-1} to hydroxyl O–H stretching, 2931 cm^{-1} to asymmetric C–H stretching, 1631 cm^{-1} to carbonyl C=O of amides, 1408 cm^{-1} to carboxylic acids O–H bending, 1262 cm^{-1}, and 1060 cm^{-1} to primary amines C–N stretching, 927cm^{-1} to CH_2, C-H deformation, and 819 cm^{-1} to primary amines N–H bending. After reduction and stabilization of gold with aqueous garlic extract and the subsequent formation of G-AuNPs, a number of changes in the IR spectrum was observed (Figure S1). Enhancement in the band intensity at 3324 cm^{-1} was observed which suggests electronic interactions of OH with gold. Similar increment in the band intensity at 1631 cm^{-1} of amide was also evident suggesting electronic interactions of NH and gold. The IR spectrum of G-AuNPs showed the changes in the bands (3441, 1633, 1262, 1060, and 780 cm^{-1}) that belong to proteins suggesting their role in the stabilization of the nanoparticles.[25] It is known that gold nanoparticles can bind to proteins via their cysteine residues, or free amine groups[26] or through electrostatic attraction of the negatively charged carboxylate groups.[27] Keeping the changes in the IR stretching's in mind, it can be assumed that the gold nanoparticles were stabilized by proteins present in the garlic extract.

Hepatoprotective activity of G-H_2O and G-AuNPs

Histological analysis of the liver of normal control group showed well characterized central veins surrounded by hepatic cord of cells; with clear sinusoidal spaces lined by endothelial cells (Figure 3 A, B, green arrow). After 48 h of CCl_4 treatment, the liver showed necrosis around the central veins as clear pale areas (Figure 3 A, B, red arrow). Infiltration of inflammatory cells in the necrotic region was also observed. Interestingly, damage in the necrotic region was limited to hepatocytes only supporting the notion that free radicals are generated only in the hepatocytes where they cause membrane damage. In some cases, hydropic degeneration of hepatocytes was also evident in the pale necrotic areas around the central vein. Healthy hepatocytes (Figure 3 A, B, green arrow) were also observed at the vicinity of the necrotic area around the central vein and could be easily distinguished from the necrotic cells due to their darker staining characteristics with the H/E staining. Histopathological analysis (Figure 4 B) revealed ~49% damage in the CCl_4 treated group. In contrast, the CCl_4+silymarin treated group showed large decrease ($p<0.001$) in the necrotic area around the central vein (Figure 3 C, D) compared to the CCl_4 treated group. However, ~3% damage was detected by histopathological analysis (Figure 4 B). Hepatoprotective screening of G-H_2O and G-AuNPs was carried out on rats treated with CCl_4. In both the cases of G-H_2O and G-AuNPs, no sign of necrosis was observed in the whole tissue including the central vein region (Figure 3 E, F). The CCl_4+G-H_2O group at a dose of 100 mg/kg showed 0% damage (Figure 4B) and excellent hepatoprotective activity ($P<0.001$) compared to the positive control silymarin at a dose of 200 mg/kg. Similarly, the CCl_4+G-AuNPs group showed excellent protective effect with no sign of inflammation or necrosis (Figure 3, G, H) and 0% damage (Figure 4, B) surrounding the central vein region even at a dose of 10 mg/kg G-AuNPs and therefore, was more potent than the G-H_2O extract. G-AuNPs

treatment completely resolved the CCl_4 induced hydrophic degeneration and necrosis. From histological observation it was clear that the hepatocytes have distinct morphology with densely stained nucleus and clear sinusoidal spaces. A negative control group with non-conjugated gold nanoparticles were also performed which showed no effect against hepatotoxicity. These results indicate that the hepatoprotective activity of G-AuNPs is better than the aqueous extract of *Allium sativum* or the positive control silymarin.

Biochemical results revealed that the CCl_4 treatment significantly elevated (Figure 4, A) the level of serum ALT (1245 ± 111), AST (387 ± 19) and ALP (490 ± 7) compared with the normal control group's ALT (104 ± 6), AST (125 ± 8) and ALP (136 ± 11). The positive control silymarin prevented ($P<0.001$) this increase in the serum level of hepatic enzymes (ALT (254 ± 19), AST (233 ± 14) and ALP (206 ± 11)) to a great extent. However, it did not bring the level of the enzymes down to the normal control group indicating a small amount of damage still remaining in the silymarin group. Interestingly, G-H_2O also prevented ($P<0.001$) the increase in the serum level of hepatic enzymes (ALT (189 ± 22), AST (142 ± 7) and ALP (141 ± 21)). Similar to the histopathology results, the G-AuNPs were more effective ($P<0.001$) in this activity compared to the silymarin group, or the G-H_2O group (Figure 4 B). The G-AuNPs brought the level of the enzymes down to normal control levels indicating no damage to the liver parenchyma despite the CCl_4 treatment. Taken together, the histopathological and biochemical results indicate that the hepatoprotective activity of G-AuNPs is better than the G-H_2O or silymarin.

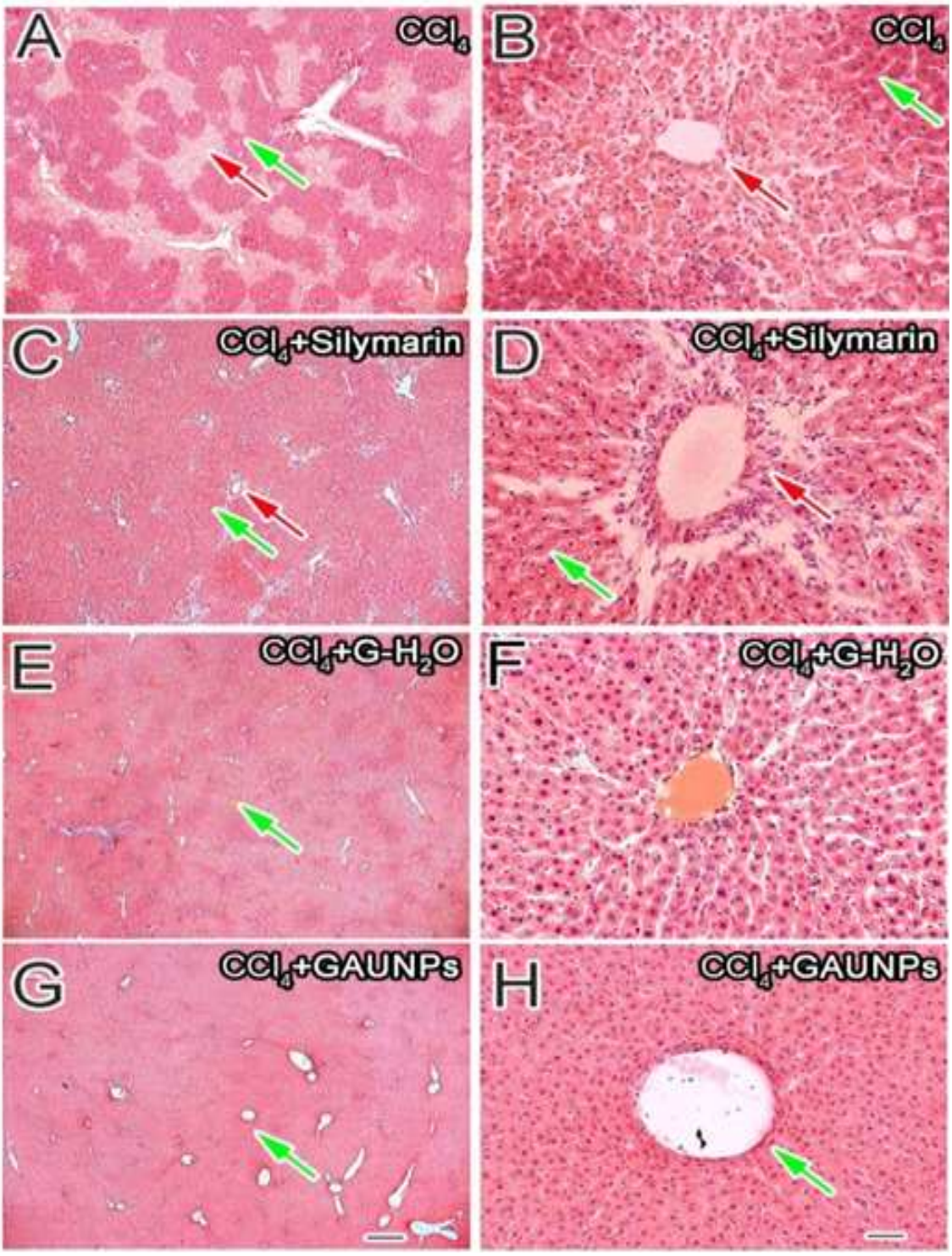


Figure 3 Histological-analysis of acute-liver-injury induced by CCl_4. Control-group (A, B), CCl_4 treated group (C, D), positive-control-group treated with CCl_4+silymarin (E, F), CCl_4+GAuNPs treated Group (G, H). Red arrows indicate injured or necrotic areas which appear as pale-regions while green arrows indicate healthy areas with normal hepatic cords, sinosoids and central vein. Scale-bar of first column is 250 µm (A, C, E, G) while scale-bar of second column is 25 µm (B, D, F, H).

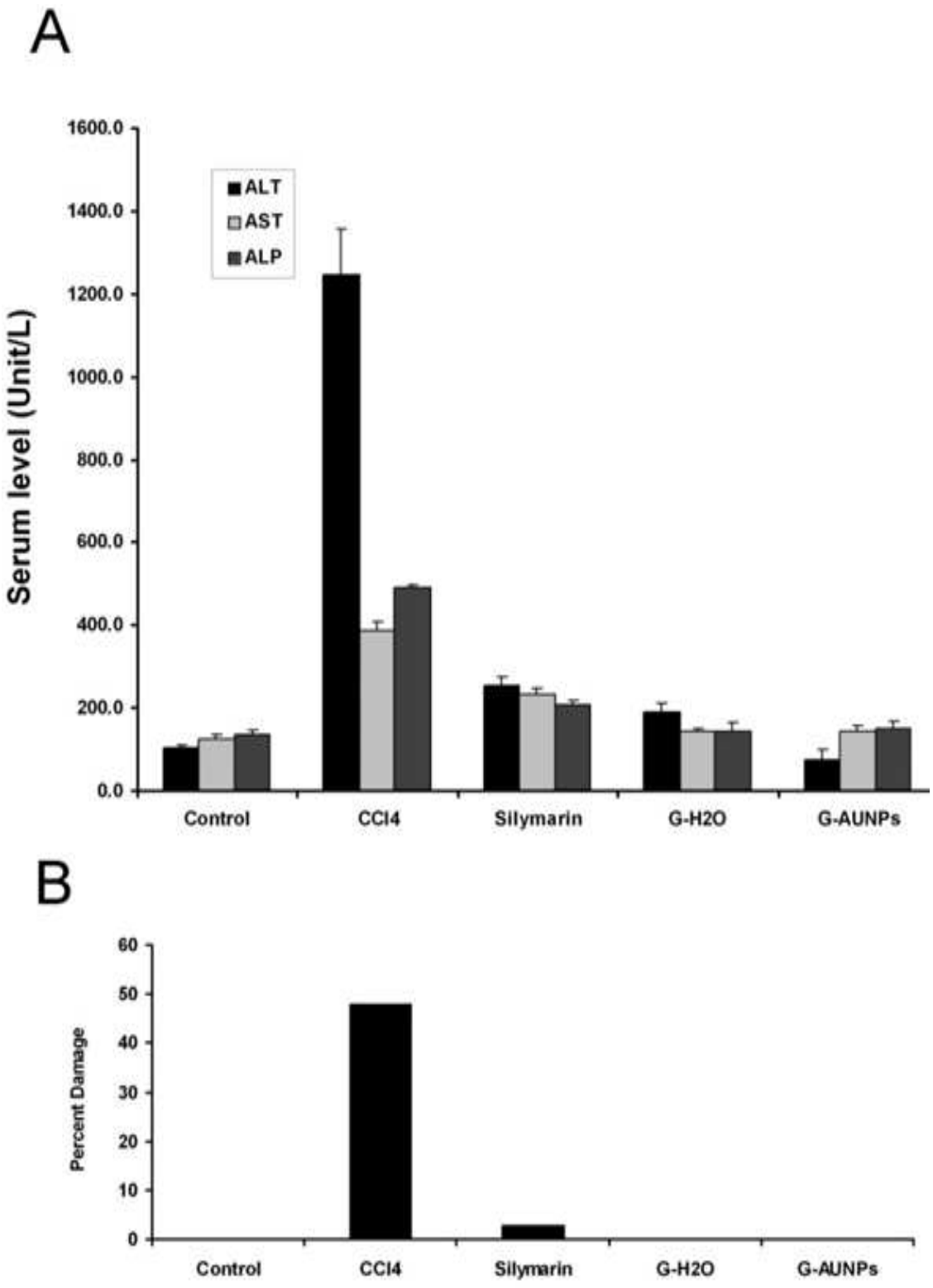


Figure 4 Acute-liver-injury markers, serum ALT, AST and ALP. Acute-liver-injury induced by CCl_4 was completely protected by GAuNPs (**, P<0.001) in comparison with CCl_4+silymarin treated group (*, P<0.001). B. Nikon NIS-elements-software was used for histopathological-analysis of stained-sections.

Enzyme Inhibitory Activity of G-AuNPs

Both G-H_2O and G-AuNPs were screened for their inhibition potential against jack bean urease,[28] xanthine oxidase,[29] and carbonic anhydrase-II[30] enzymes. Garlic extract is known to have significant inhibition against *helicobacter pylori* bacterium and jack bean urease.[30, 31] The G-H_2O and G-AuNPs showed selective urease inhibitory activities and remained inactive against xanthine oxidase, and carbonic anhydrase-II enzymes (Table-1). G-H_2O showed urease inhibitory activity with IC_{50} value of 192.28 ± 2.04 μg/mL. The activity of G-H_2O increased after stabilization with gold nanoparticles with IC_{50} value 180.61 ± 1.06 μg/mL.

Table-1: The enzyme activities of G-H_2O and G-AuNPs

Compounds	IC50±S.E.M(µg/ML)		
	Urease	Xanthine oxidase	Carbonic anhydrase-II
G-H_2O	192.28 ± 2.04	--	--
G-AuNPs	180.61 ± 1.06	--	--

Conclusion

We have successfully synthesized gold nanoparticles (G-AuNPs) using aqueous garlic (*Allium sativum*) extract (G-H_2O) — via a robust procedure which is fairly quick. The synthesized G-AuNPs remained stable at 80°C or higher concentrations of NaCl up to 10 mM, or between pH 4-11. In this study we found better hepatoprotective and urease inhibitory activities by the G-AuNPs. The G-H_2O with IC_{50} value of 192.28 ± 2.04 μg/mL and G-AuNPs with IC_{50} value of 180.61 ± 1.06 μg/mL were found selective against the urease enzyme. The G-H_2O and G-AuNPs remained inactive against xanthine oxidase, and carbonic anhydrase-II enzymes. It appears that

the G-AuNPs may exert this increased biological activity because of increased bioavailability, better stabilization of the active ingredients of garlic, decreased biotransformation or accessibility to active sites inside the hepatocytes. We found no signs of any toxicity of these G-AuNPs *in vivo* during the whole period of the experiment.

Experimental

Materials and Methods

The garlic cloves (1 kg) were purchased from the local market and identified by Prof. Dr. Surayia Khatoon, a plant taxonomist at the Department of Botany University of Karachi. A voucher specimen GC-0045-DBKU was already present at the herbarium. These cloves were peeled, washed with distilled water and extracted with EtOH (3 x 10 L, 10 d each) at room temperature. The EtOH was concentrated under reduce pressure to yield a crude residue (10 g), which was suspended in distilled H_2O (500 mL), successively extracted with n-hexane (01 g), $CHCl_3$ (900 mg), EtOAc (02 g), and n-BuOH (01 g) and consequently, residual 3 gm aqueous extract was obtained. Dried aqueous extract (100 mg) was dissolved in 50 mL of deionized water. The resulted clear solution was filtered and analyzed through IR (Bruker Victor 22) and UV-Vis (Thermo Scientific Evolution 300) spectroscopy.

The formation of gold nanoparticles was monitored by UV-Visible spectrophotometer. The instrument was operated at a resolution of 2 nm and the spectra were obtained in the range of 400-700 nm by using water as a reference. Topographical images were obtained by an atomic force microscope (Agilent 5500) operated at tapping mode using a silicon nitride soft triangle-shaped cantilever (Veeco, model MLCT-AUHW); with 0.01 N/m and 0.1 N/m nominal spring constant. The samples were prepared as follows: a drop of freshly prepared suspension of G-

AuNPs in deionized water was deposited on freshly cleaved mica wafer surface; left for 30 min to allow the absorption of G-AuNPs. Then, the mica wafer was rinsed with deionized water (3 times) to remove the weakly absorbed/adsorbed moieties and dried under argon.

FTIR measurements (Bruker Victor 22, diffuse reflectance mode, resolution 2 cm^{-1}) were carried out to obtain information about the role of functional groups in the stabilization of GAuNPs. The IR spectra of G-H_2O and G-AuNPs were obtained using dried KBr discs.

Synthesis of gadeionized water rlic conjugated gold nanoparticles (G-AuNPs)

In a volumetric flask 250 mL deionized water was taken and 85 mg $HAuCl_4$ (1 mM) was added which was followed by adding freshly prepared aqueous garlic extract and 0.1 mL (10 mM) $NaBH_4$ solution under constant stirring. A gradual change in the color of the solution from yellow to pink was observed which indicated the formation of G-AuNPs. The reaction mixture was stirred for 4 hours to reduce gold completely. The reaction was optimized at 2:1 (v/v) gold solution to garlic extract.

Hepatoprotective activity *in vivo*

Male wistar rats weighing 200 to 220 g were housed in individual cages and kept at 22 to 26 ºC under 12-hour light/dark cycles, with free access to standard laboratory chow and tap water ad libitum. All procedures involving animals and their care were carried out according to the guidelines of the institutional ethical committee for care and use of laboratory animal (ICCBS, Karachi University).

The animals were randomly divided into 5 groups of six rats each in the following manner: Group 1 (normal control): injected with vehicle only (1 mL/Kg body weight olive oil); Group 2

(acute liver injury model): injected with CCl_4 intraperitoneally (1 mL/Kg with 1:1 olive oil); Group 3 (positive control): injected with CCl_4 intraperitoneally (1 mL/Kg with 1:1 olive oil) and also received silymarin 200 mg/kg (oral) daily, 3 days before treatment and one day after treatment; Groups 4 (G-H_2O): injected with CCl_4 intraperitoneally (1 mL/Kg with 1:1 olive oil); and also received G-H2O at a dose of 100 mg/kg body weight daily for 3 days before treatment and one day after treatment; Group 5 (G-AuNPs): injected with CCl_4 intraperitoneally (1 mL/Kg with 1:1 olive oil) and also received G-AuNPs at a dose of 10 mg/kg daily for 3 days; before experiment and one day after experiment.

After 48 hours of treatment with CCl_4, blood was collected by cardiac puncture. Serum was separated to determine alanine aminotransferase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) using a dry chemistry analyzer (Roche Diagnostics, Mannheim, Germany). Liver tissues were rapidly removed and fixed in 10 % neutral buffered formalin (24 hours), dehydrated with graded series of alcohol, embedded in paraffin, and cut into 6 µm thick sections. The tissue sections were stained with Hematoxylin–Eosin (H&E) and examined under a bright field microscope (Nikon 90*i*) at different magnifications. Histopathological analysis of the liver was carried out under different conditions. The necrotic area of the liver was measured in 30 different sections using the NIS-elements software (Nikon, Japan). Then, the damaged area was expressed as percentage of the whole area of the section.

Urease Inhibition Assay

Jack bean urease enzyme (25 μL solution) and test compound (5 μL, 0.5 mM) were incubated in buffers (55 μL) containing urea (100 mM) for 15 min at 30 °C in each well of 96-well plates. Ammonia evolution was measured by indophenol method as described by Weather burn.[26] Final

volumes were maintained as 200 μL by adding 45 μL phenol reagent (1% w/v phenol and 0.005% w/v sodium nitroprusside), and 70 μL of alkali reagent (0.5% w/v NaOH and 0.1% active chloride NaOCl) to each well. Using a microplate reader (Molecular Devices, CA, USA), increase in absorbance was measured at 630 nm after 50 minutes at pH 6.8. The results (change in absorbance per minute) were collected using softMax Pro software (Molecular Devices, CA, USA). Thiourea was used as the standard inhibitor and % inhibitions were calculated as follow:

% inhibition = 100-(OD test well/OD control) ×100.

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