

Facile method for the synthesis of silver nanoparticles using 3-hydrazino-isatin derivatives in aqueous methanol and their antibacterial activity

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Introduction: A new method for preparation of silver nanoparticles (AgNPs) based on using hydrazino-isatin derivatives in an aqueous methanol reaction medium is reported here. AgNPs were prepared using silver nitrate solubilized in a water core as the source of silver ions and 3-hydrazino-isatin derivatives (3-hydrazino-isatin [IsH] and 1-benzyl-3-hydrazino-isatin [BIsH]) solubilized in methanol core as a reducing agent. The proposed method is effective, rapid, and convenient. X-ray diffraction (XRD), energy dispersive X-ray analysis, scanning electron microscope (SEM) and transmission electron microscopy (TEM) were used for characterization of the AgNPs. The TEM micrographs confirmed that the nanopowders consist of well-dispersed agglomerates of grains with a narrow size distribution of 18–21 nm and 17–20 nm. The AgNPs, as well as BIsH, showed high antimicrobial and bactericidal activity against the Gram-positive *Bacillus subtilis* and Gram-negative *Micrococcus luteus* and *Proteus vulgaris*, as well as anti-fungal activities against *Saccharomyces cerevisiae*. On the other hand, they were not effective against the Gram-negative *Escherichia coli*.

Purpose: A simple, effective, rapid, and convenient chemical reduction method for the synthesis of AgNPs in an aqueous methanol reaction medium using hydrazino-isatin derivatives and studying their antibacterial effect.

Results: IsH and BIsH are remarkably powerful reductants for Ag⁺ ions in an aqueous methanol medium, which could be considered as a simple chemical reduction method for formation of AgNPs. The AgNP formation depends on the solubility of the hydrazino-isatin derivatives. BIsH gave more AgNPs than IsH, as observed from XRD. The formation of AgNPs is attributed to the adsorption of hydrazine derivatives and/or interparticle interaction on the surface of AgNP through electrostatic interactions between the lone pair electrons of the hydrazino group (C=N-NH₂) and the positive surface of AgNPs. AgNPs and BIsH showed high antimicrobial and bacterial activity.

Conclusion: In summary, it is shown that IsH and BIsH are remarkably powerful reductants for Ag⁺ ions in an aqueous methanol medium. BIsH gave more AgNPs than IsH, as observed from XRD due to better solubility of the BIsH than IsH in aqueous-methanol. The formation of AgNPs is attributed to the adsorption of hydrazine derivatives and/or interparticle interaction on the surface of AgNPs through electrostatic interactions between the lone pair electrons of the hydrazino group (C=N-NH₂) and the positive surface of AgNPs. The AgNPs as well as BIsH ligand showed high antimicrobial and bactericidal activity.

Keywords: silver nanoparticles, 1-benzyl-3-hydrazinoisatin, SEM, TEM, antimicrobial

Introduction

Nanoparticles (NPs) are emerging materials that have been the subject of focused research with a broad range of applications, due to their unique physical and chemical

properties that are significantly different from those of bulk materials.¹⁻⁵ Silver nanoparticles (AgNPs) are one of the most attractive inorganic materials not only because of their broad application in catalysis,⁶ photography,⁷ biosensors,⁸ biomolecular detection,⁹ diagnostics,¹⁰ and antimicrobial activities.¹¹⁻¹⁸ A number of methods are used for the synthesis of AgNPs, such as the electrochemical method,^{19,20} thermal decomposition,²¹ laser ablation,²² microwave irradiation,²³ and sonochemical synthesis.²⁴ The simplest and most commonly synthetic method for metal NPs is the chemical reduction of metal salts.²⁵⁻³⁰ The chemical reduction methods have been applied to synthesize stable and various shapes of AgNPs in water by the use of different reducing agents (eg, ascorbic acid,³¹ hydrazine,³² ammonium formate,³³ dimethylformamide [DMF],³⁴ and sodium borohydride).³⁵ The shape, size, and size distribution depend on the tendency of organic substrates to reduce the silver ions. Compared with physical and chemical methods, biologic methods using microbes and plants are regarded as an ecofriendly process.³⁶ Several biologic methods employing bacteria,³⁷ fungi,³⁸ yeast,³⁹ or plant extracts^{40,41} have been used as an alternative to chemical methods. The presence of various natural products such as carbohydrates, alkaloids, steroids, proteins, and/or peptides in plant extract, and the tendency of silver ions to form a variety of complexes with them, makes the systems of considerable complexity. Using a chemical reduction method for the synthesis of different morphology NPs can be advantageous over other biosynthetic methods because it involves reduction of an ionic salt in the presence of a reducing agent, is cost-effective, is easily scaled up, and there is no need to use high pressure, energy, and temperature.

Recently,⁴² aniline was used as a stabilizer for controlling the morphology and particle size of AgNPs using hydrazine and sodium citrate as the reducing agents. Later, Hussain et al⁴³ used aniline in the presence of cetyltrimethylammonium bromide as a simple chemical reduction aniline route for the synthesis of silver nanocrystals. The studies revealed that the reaction conditions (cetyltrimethylammonium bromide and aniline) have great influence on the morphology of AgNPs.

Because isatin derivatives have been the subject of extensive research, particularly in the pharmaceutical and agrochemical areas,⁴⁴⁻⁵² the present work describes a simple chemical reduction employing 3-hydrazino-isatin (IsH) derivatives as reducing agent for the synthesis of AgNPs in aqueous methanol. The antimicrobial activity is also described for both of the reducing agents and AgNPs.

Materials and methods

Silver nitrate (AgNO₃), hydrazine hydrate, and isatin were purchase from Sigma-Aldrich (St Louis, MO, USA). All chemicals were used as received. Double-distilled deionized water was used. The evaluation of crystal structure was achieved by X-ray diffractometer (XRD) (X'Pert PRO, PANalytical BV, Almelo, the Netherlands) using CuK α radiation. The studies of size, morphology, and composition of the NPs were performed by means of scanning electron microscope (SEM) and transmission electron microscopy (TEM), equipped with energy dispersive X-ray (EDX) analysis. Histograms of AgNPs' size distribution were calculated from the TEM images by measuring the diameters of at least 50 particles. Samples for TEM studies were prepared by placing drops of the AgNP solutions on carbon-coated TEM grids. ¹H- and ¹³C-NMR spectra of compounds were run on a JEOL-NMR spectrometer (400 MHz) (JEOL, Tokyo, Japan). The chemical shifts are expressed in ppm downfield from tetramethylsilane as the internal standard.

Synthesis of 1-benzylisatin (3)⁵²

A mixture of isatin (5 mmol) and potassium carbonate (8 mmol) in DMF (10 mL) was stirred for 10 minutes at room temperature. Benzyl bromide (6 mmol) was added dropwise to the reaction mixture and then the reaction was microwave irradiated using a multimode reactor (Synthos 3000, Anton Paar GmbH, Graz, Austria) (1,400 W maximum magnetron). The vessel was placed in the corresponding rotor, fixed by screwing down the upper rotor place, and finally the rotor was closed with a protective hood. The vessel was heated for 5 minutes at 80°C and held at the same temperature for a further 5 minutes (~2 bar pressure, 400 W). Cooling was accomplished by a fan (5 minutes). The final product was dried and recrystallized from ethanol to give orange red crystals in yield 88%, mp: 134°C–136°C; ¹H-NMR (CDCl₃) δ (ppm): 4.93 (s, 2H, C₆H₅CH₂), 6.77 (d, $J=7.7$ Hz, 1H), 7.08 (t, $J=7.35$ Hz, 1H), 7.33 (s, 5H, Ar), 7.47 (t, $J=8.07$, 1H), 7.61 (d, $J=8.07$, 1H). ¹³C-NMR (CDCl₃) δ (ppm): 42.94, 109.90, 122.74, 124.31, 126.31, 127.04, 127.93, 133.37, 137.18.

General method for preparation of the hydrazino-isatin derivatives 4 and 5

Hydrazine hydrate (3 mL) was added to a solution of isatin (1, 5 mmol) or 1-benzylisatin (3, 5 mmol) in ethanol (10 mL). The reaction mixture was microwave irradiated using a multimode reactor (Synthos 3000, Anton Paar GmbH, 1,400 W maximum magnetron). The vessels were placed in the corresponding rotor, fixed by screwing down the upper rotor

place, and finally the rotor was closed with a protective hood. The vessel was heated for 3 minutes at 80°C and held at the same temperature for a further 3 minutes (~2 bar pressure, 400 W). Cooling was accomplished by a fan (5 minutes). The final product was dried and recrystallized from ethanol to give a yellow solid.

1-Benzyl-3-hydrazino-isatin (4)

The product obtained was a yellow crystal from ethanol, yield 93%, mp: 114°C–115°C. ¹H-NMR (dimethyl sulfoxide [DMSO]-d₆) δ (ppm): 4.97 (s, 2H, C₆H₅CH₂), 6.96 (d, *J*=8.04 Hz, 1H), 7.02 (t, *J*=7.32 Hz, 1H), 7.17 (t, *J*=8.08 Hz, 1H), 7.32 (s, 5H, Ar), 7.43 (d, *J*=7.36 Hz, 1H), 9.77 (d, *J*=14.64 Hz, 1NH), 10.58 (d, *J*=13.96 Hz, NH); ¹³C-NMR (DMSO-d₆) δ (ppm): 42.8, 109.8, 117.9, 122.13, 122.6, 125.6, 127.5, 127.8, 127.9, 129.2, 137.3, 139.5, 161.3. Elemental analysis calculated. for C₁₅H₁₃N₃O: C, 71.70; H, 5.21; N, 16.72. Found: C, 71.91; H, 5.19; N, 16.98.

3-hydrazino-isatin (5)

The product obtained was a yellow crystal from ethanol, yield 90%, mp: 240°C–242°C. ¹H-NMR (DMSO-d₆) δ (ppm): 6.86 (d, *J*=8.08 Hz, 1H), 6.98 (t, *J*=7.32 Hz, 1H), 7.14 (t, *J*=7.32 Hz, 1H), 7.35 (d, *J*=8.08 Hz, 1H), 9.55 (d, *J*=13.96 Hz, 1NH), 10.54 (d, *J*=14.68 Hz, NH); ¹³C-NMR (DMSO-d₆) δ (ppm): 110.5, 118.01, 121.9, 122.8, 126.8, 127.6, 139.2, 163.3. Elemental analysis calculated for C₈H₇N₃O: C, 59.62; H, 4.38; N, 26.07. Found: C, 59.88; H, 4.52; N, 26.31.

Preparation of AgNPs

For the synthesis of AgNPs, silver nitrate solution (0.1 mmol in 50 mL H₂O) was used as a metal salt precursor and added slowly (5–10 minutes) to a solution of IsH derivatives (1 mmol in 50 mL methanol). The reaction mixture was stirred at 25°C–30°C for 1 hour and then at room temperature overnight. The transparent, colorless solution was converted to the characteristic pale yellow and then finally to black with formation of a silver mirror on the wall. The AgNPs were collected and purified by centrifugation and then washed three times with deionized water. A dried powder of the AgNPs was obtained by drying under vacuum. The dried particles were used to carry out all characterization methods and interaction of the AgNPs with bacteria.

Micro-organisms

The microbial organisms used in this study included the Gram-positive bacterium *Bacillus subtilis* NRRL B-543;

the Gram-negative bacteria *Escherichia coli* JM 105 DSM 3949, *Proteus Vulgaris*, and *Micrococcus luteus*; as well as the fungal yeast *Saccharomyces cerevisiae*.

Assay for the antimicrobial activity

The prepared silver complexes were evaluated for their antimicrobial activity by the modified agar diffusion technique of Perez et al.⁵³ The compounds were dissolved in DMSO (Panreac Sintesis, Barcelona, Spain) to a final concentration of 1 mg/mL working solution. The test bacterial and fungal strains were maintained on nutrient agar (Merck Millipore, Billerica, MA, USA) and ISP-2 mediums at 37°C and 26°C, respectively. Prior to the assay, the cultured strains were cultivated on their corresponding broth mediums for at least 12 hours on a reciprocal shaker at 150 rpm. Samples in the form of 1 mL were taken each hour and the growth was determined by measuring the optical density of the growing cells at 623 nm against control growth medium. When the growth entered the exponential phase, the cells were used to inoculate agar media to achieve a cell concentration of 0.015 mL⁻¹. After that, the medium was poured on to 9 mm agar plates to provide thin agar layers with a thickness of 3.5–4.5 mm. After solidification, wells of 10 mm diameter were cut using a sterile cork-borer, and 0.1 mL of the prepared stock solution was poured into the wells. For comparison, 1% DMSO was used as a negative control. Additionally, standard tetracycline and erythromycin sensitivity discs at 15 µg/disc were used as positive controls. The agar plates were then incubated at 4°C for 1 hour to ensure the diffusion of the tested compounds, and they were then incubated for 24 hours at 37°C and 26°C for bacterial and yeast strains, respectively. After incubation, the diameter of the resulted inhibition zone was measured.

Results and discussion

N-benzylisatin 3 was prepared by reaction of isatin 1 with benzyl bromide 2 in the presence of K₂CO₃ and DMF as solvent under microwave irradiation for 10 minutes (Figure 1). Reaction of 3 with hydrazine hydrate (80%) under microwave irradiation for 5 minutes afforded 1-benzyl-3-hydrazino-isatin (4, BIsH), yield 92% (Figure 1). Isatin (1) was reacted with hydrazine hydrate (80%) under the same conditions described to afford IsH (5), yield 90% (Figure 1). The structures of the synthesized compound were confirmed by spectral data.

The synthesized compounds 4 and 5 were used as a reducing agent for preparation of AgNPs in aqueous methanol at room temperature. A solution of AgNO₃ in water was added to a solution of hydrazino derivative 4 or 5 in methanol. The

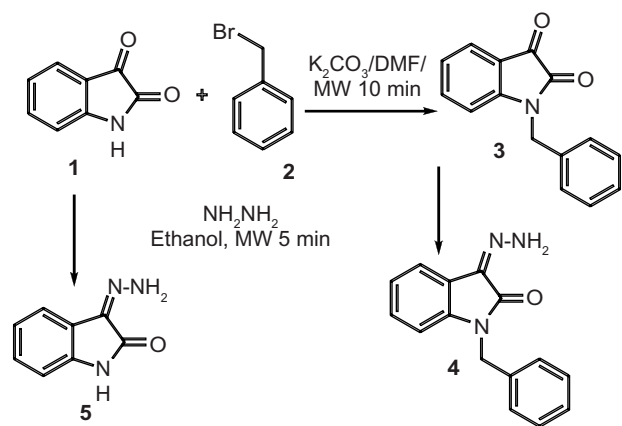


Figure 1 Synthesis of 1-benzyl-3-hydrazino-isatin **4** and 3-hydrazino-isatin **5**.
Abbreviations: MW, microwave irradiation; DMF, dimethylformamide.

reaction mixture was slowly stirred at room temperature for 24 hours. The color of the reaction mixture was changed from colorless to yellow and finally to black, indicating that AgNPs were formed. The precipitate was collected and dried under vacuum.

The elemental analysis of the AgNPs was performed using EDX on the TEM. EDX analysis confirmed the presence of the elementary silver signal of the prepared AgNPs, as shown in Figure 2A and B. Signal peaks in the range of 2.5–4 keV were observed, which correspond to the binding energies of crystalline silver.^{54–56} Also, a strong signal peak near 0.2 keV corresponded to carbon in the ligand connected to AgNPs. On the other hand, several peaks for CuK α and CuK β showed, which correspond to the TEM holding grid. These results were confirmed by previous reports that showed that AgNPs are crystalline in nature with the same EDX results.

The typical powder XRD patterns of the prepared AgNPs from IsH (IsHAg) and from BIsH (BIsHAg) are shown in Figure 3. The XRD patterns (Figure 3) showed peaks at about 38.1°C, 44.09°C, 64.36°C, 77.29°C, and 81.31°C for both the prepared materials, which corresponded to 111, 200, 220, 311, 222, 400, 331, and 420 planes, respectively, to indicate a typical face-centered cubic structure of silver as per the available literature (Joint Committee on Powder Diffraction Standards, JCPDS file No 04-0783).^{57,58} The high peaks intensity observed in BIsHAg indicates that a large amount of AgNPs were produced compared with IsHAg. This is attributed to the higher solubility of BIsH in an aqueous methanol medium.

A scanning electron microscope (JSM-5800 JEOL) was employed to study the structure of the prepared silver–ligand composite, as shown in Figures 4 and 5. The composite

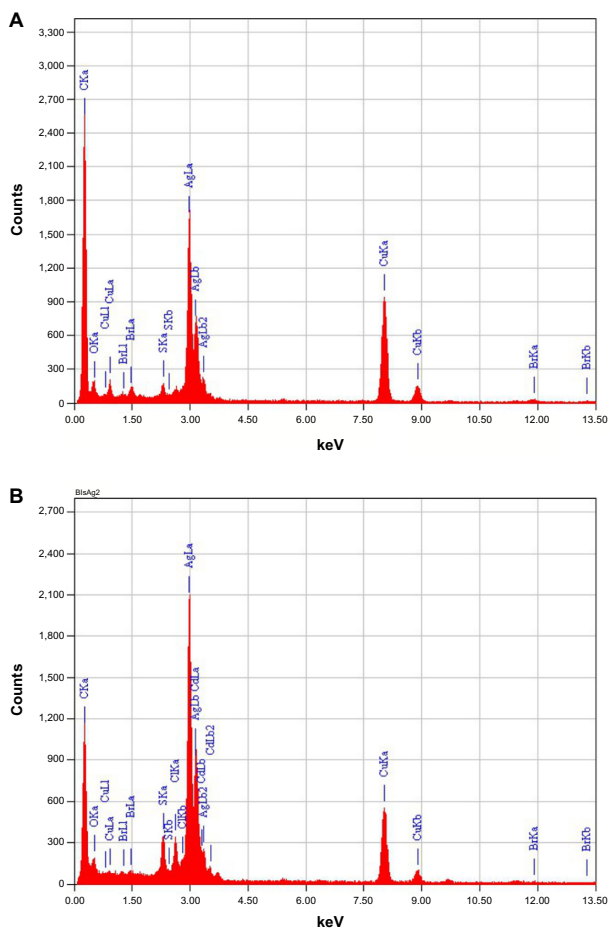


Figure 2 Energy dispersive X-ray spectra of the prepared silver nanoparticles from 3-hydrazino-isatin (**A**) and 1-benzyl-3-hydrazino-isatin (**B**) composite powders.

that formed was uniform in structure, and a homogenous distribution of AgNPs over ligand surface is observed as shown in Figure 4.

The TEM image of the synthesized silver is represented in Figure 6A and B. Analysis of TEM imaging shows that

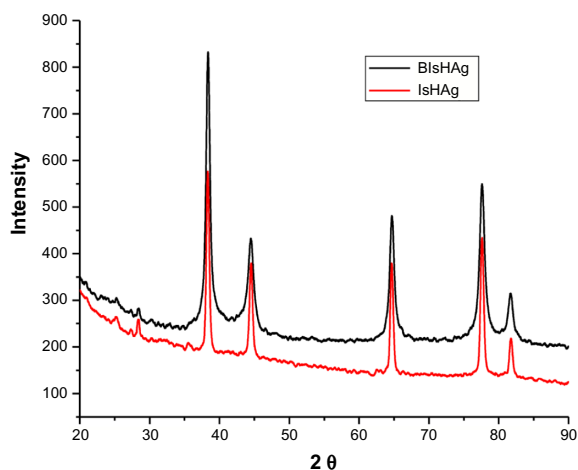


Figure 3 X-ray diffraction pattern of the silver nanoparticles produced from 3-hydrazino-isatin (IsHAg) and 1-benzyl-3-hydrazino-isatin (BIsHAg).

prepared AgNPs are monodispersed spheres in the range of 18–21 nm and 17–20 nm in size for IsHAg and BIsHAg, respectively. Nanosized silver particles were formed by nucleation and growth mechanism after reduction by the lone pair of electrons of the hydrazino group ($C=N-NH_2$) located at the ligand structure. On the other hand, some particles whose diameters were longer than 40 nm were formed because of aggregation during preparation of the TEM holding grid.

Finally, the antimicrobial susceptibility of synthesized AgNPs was investigated. The obtained results showed that prepared AgNPs from BIsH (BIsHAg) and from IsH (IsHAg) exhibited both antibacterial and antifungal activities (Figure 7). The nanosilver compounds were active against Gram-positive *B. subtilis* and Gram-negative *M. luteus* and *Pr. vulgaris*, though they were not effective against *E. coli*. The prepared compounds showed similar antifungal activities against *S. cerevisiae*, where the reported inhibition zone ranged from 1.5 cm to 1.6 cm. The inhibition zone reported for the Gram-positive *B. subtilis* was 5 cm, 3.6 cm, and 3.4 cm for BIsH, IsHAg, and BIsHAg, respectively. The Gram-negative *Pr. vulgaris* was similarly affected by IsHAg and BIsHAg, where both compounds gave an inhibition zone of 3 cm, representing 75% of the activity recorded for BIsH. On the contrary, the growth of all tested microorganisms was not inhibited upon the addition of 1% DMSO. The standard tetracycline antibiotic showed inhibition zones of 1.3 cm and 1.8 cm for *B. subtilis* and *M. luteus*, respectively, whereas the erythromycin showed inhibition of 2.8 cm and 3.2 cm for the same two microbes, respectively. Hence,

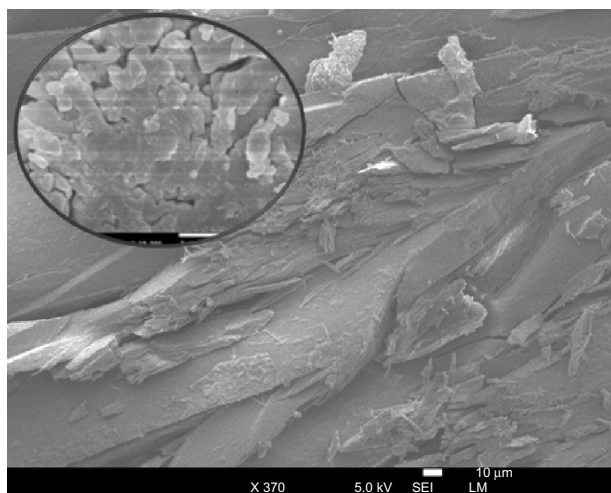


Figure 4 Low-resolution scanning electron microscope (SEM) micrograph of silver-ligand composite using secondary electron imaging mode (SEI).

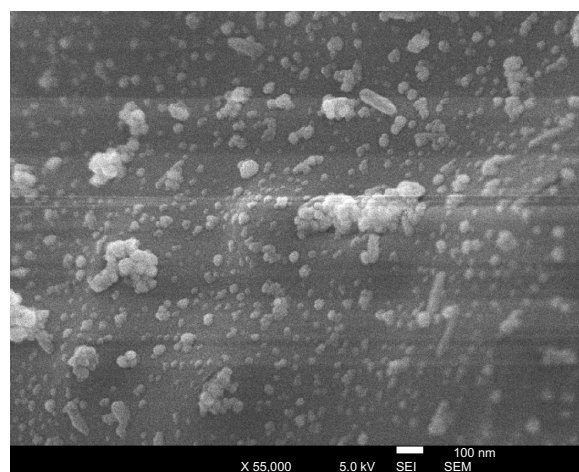


Figure 5 High-resolution electron microscope (SEM) micrograph of silver-ligand composite using secondary electron imaging mode (SEI).

the activity of the prepared NPs showed comparable activities with standard antibiotics.

The resulting antimicrobial effects of the prepared AgNPs can be attributed to the fact that AgNPs can interact with the microbial cell surface, which will lead to the rupture of the

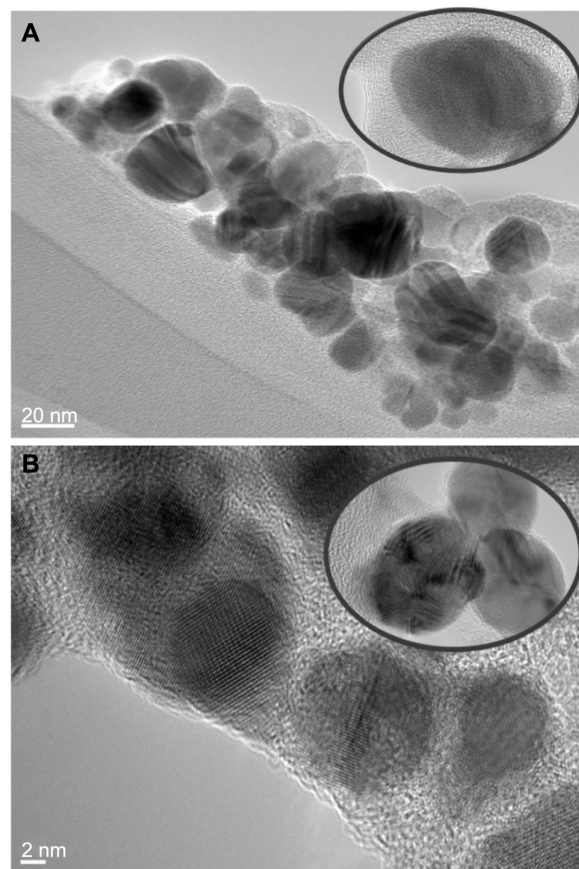


Figure 6 High-resolution transmission electron microscopy micrographs of prepared nanosilver from 3-hydrazino-isatin (IsHAg) (A) and 1-benzyl-3-hydrazino-isatin (BIsHAg) (B).

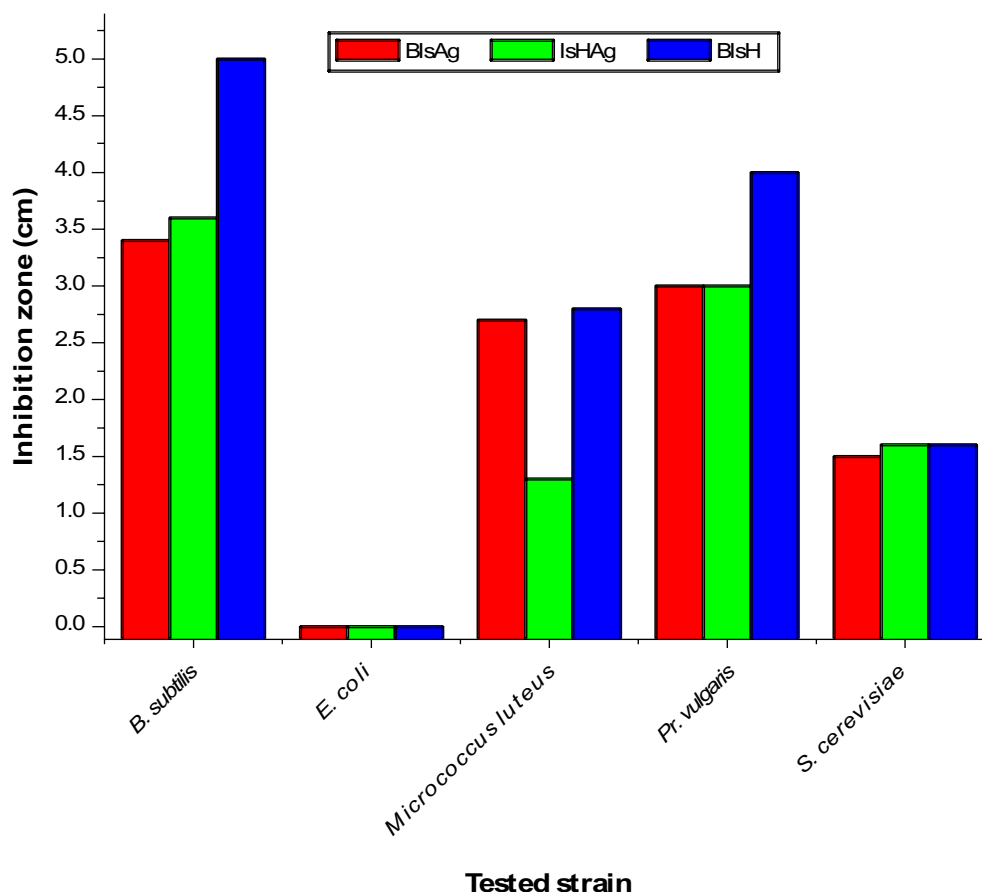


Figure 7 Antimicrobial activity of silver nanoparticles against Gram-positive, Gram-negative, and fungal micro-organisms.

Abbreviations: Ag, silver; BlsH, 1-benzyl-3-hydrazinoisatin; IsH, 3-hydrazinoisatin; *B. subtilis*, *Bacillus subtilis*; *E. coli*, *Escherichia coli*; *Pr. vulgaris*, *Proteus vulgaris*; *S. cerevisiae*, *Saccharomyces cerevisiae*.

cell membranes. Many mechanisms have been proposed to explain the antimicrobial effect of AgNPs. One mechanism suggests the incorporation of AgNPs in the cell membrane, leading to the leakage of intracellular components and eventually resulting in cell death.⁵⁹ Another hypothesis suggests that the NPs will be oxidized, dissolve, and generate reactive oxygen species, which will, in turn, enter the cytoplasm, causing bacterial death.^{60–62} Moreover, small-sized nanoparticles may accumulate intracellularly and lead to cell malfunction.⁶³

Our results showed varying responses of different microbial micro-organisms as affected by the prepared AgNPs. This can be explained based on the fact that the antimicrobial activity of AgNPs differs according to the microbial strain tested. Yoon et al⁶⁴ showed that *E. coli* and *B. subtilis* have different susceptibility to silver and copper NPs. This is due to the differences in the membrane structure of different microbial strains, mainly the thickness of the peptidoglycan layer of the cell membrane.⁶⁵ On the other hand, our results showed that *E. coli* was insensitive to the prepared AgNPs. Although these results contradict most of the work presented

in the literature showing positive effects against the same microbe, few reports reported the same finding. Rawani et al⁶⁶ found that their two investigated human pathogens, *E. coli* and *Pseudomonas aeruginosa*, were not affected by their prepared NPs. Recently, this was attributed to the finding that AgNPs stimulated the activation of anaerobic respiration-related regulators and enzymes, which play an important role in the resistance of *E. coli* to AgNPs.⁶⁷

Conclusion

In summary, it is shown that IsH and BlsH are remarkably powerful reductants for Ag⁺ ions in an aqueous methanol medium, which could be considered as a simple effective, rapid, and convenient chemical reduction method for formation of AgNPs. The NP formation depends on the solubility of the hydrazino-isatin derivatives. BlsH gave more AgNPs than IsH, as observed from XRD. The formation of AgNPs is attributed to the adsorption of hydrazine derivatives and/or interparticle interaction on the surface of AgNP particles through electrostatic interactions between the lone pair electrons of the hydrazino group (C=N-NH₂) and the positive

surface of AgNPs. The TEM micrographs indicate that the nanopowders consist of well-dispersed agglomerates of grains with a narrow size distribution of 18–21 nm and 17–20 nm. The AgNPs, as well as BIsH ligand, showed high antimicrobial and bactericidal activity against the Gram-positive *B. subtilis* and Gram-negative *M. luteus* and *Pr. Vulgaris* for both BIsH and BIsHAg, as well as antifungal activities against *S. cerevisiae*. On the other hand, they were not effective against the Gram-negative *E. coli*.

Author contributions

Ayman El-Faham and Ahmed A Elzatahry carried out the preparation, designed the proposed methods, and statistically analyzed the data together. Zeid A Al-Othman carried out the analysis and characterization for silver nanoparticles. Elsayed Ahmed Elsayed carried out the biologic studies. All authors took part in drafting the article or revising it critically for important intellectual content. All authors read and approved the final manuscript.

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Disclosure

The authors report no conflicts of interest in this work.

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