

Antimicrobial Activities of Gold Nanoparticles against Major Foodborne Pathogens

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Abstract: Spherical gold nanoparticles (Au) were chemically prepared and characterized by transmission electron microscope (TEM) and UV spectra. Their antimicrobial activities against major foodborne pathogens were studied. Antimicrobial activities of Au nanoparticles had been increased with their higher volume. Best antifungal activity was observed on using fluconazole coated with 40 μ l Au nanoparticles with zone of inhibition 14mm against *A. niger*, 13mm *C. albicans* and 12mm *A. flavus*. Minimum inhibitory concentration test (MIC) revealed synergistic effect of Au nanoparticles with ciprofloxacin when compared with ciprofloxacin alone. Best results were shown against *S. Typhimurium* (0.097, 0.19), *B. cereus* (0.19, 0.39), *E.coli* O157 (0.39, 0.39), *P. aeruginosa* and *L. monocytogenes* (0.39, 0.78) and finally *S. aureus* (0.78, 6.25) respectively. Gold nanoparticles and fluconazole coated with Au nanoparticles showed variable MIC against *C. albicans*, *A. niger* (6.25, 3.125) and *A. flavus* (12.5, 6.25), respectively. TEM revealed small size of gold nanoparticles (range 9-19 nm) trapped by the biofilm released by *S. Typhimurium* and easily attached to the surface of cell membrane which drastically disturbed its proper function like respiration and permeability. Interaction between *S. Typhimurium* and ciprofloxacin coated with gold nanoparticles revealed that the cell wall was loosened and separated from the membrane or disrupted with complete absence of flagella. TEM of *S. Typhimurium* using ciprofloxacin alone showed intact bacterial cell wall with the accumulation of antibiotic on the cell wall and partial destruction of flagella. Drugs capped gold particle act as a single group against the microorganism which was indicated by using disk diffusion method with increase zone of inhibition of Au alone, ciprofloxacin alone and Au coated ciprofloxacin from 12, 26 and 30 mm, respectively. Also, it was clarified by the decrease in MIC from 6.25, 0.19 to 0.097, respectively. Results indicated that drugs coated with nanoparticles were highly effective against tested isolates so that Au nanoparticles can minimize treatment durations and side effects of drugs.

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Introduction

Nanotechnology offers unique approaches to control a wide variety of biological and medical processes that occur at nanometer length and it is believed to have a successful impact on biology and medicine (West and Halas 2000; Zandonella, 2003). By controlling the structure precisely at nano scale dimensions, one can control and modify their surface layer for enhanced aqueous solubility, biocompatibility or bio-conjugation. Nanoparticles exhibit attractive properties like high stability and the ability to modify their surface characteristics easily. The basic necessities for drug targeting are that the carrier should be capable of extended circulation in the blood stream; it must be small enough to gain access to target tissues and target cells (Tom *et al.* 2004). Nowadays, research efforts are being concentrated on integrating nanoparticles with biology. It has been reported that antibiotics often disturb the bacterial flora of digestive tract which may develop multiple drug-resistant isolates, hence novel

ways of formulating biocide materials is an upcoming field of attraction (Jarvinen *et al.* 1993; Concannon *et al.* 2003; Altman *et al.* 2006; Daglia *et al.* 2007). For this reason, there is a need for the use of an agent which does not generate resistance and presents a good bactericidal property. Gold nanoparticles have a great bactericidal effect on a several range of microorganisms; its bactericidal effect depends on the size and the shape of the particle (Nirmala and Pandian, 2007). Nanoparticles can act as antibacterial and antifungal agents, due to their ability to interact with microorganisms (Hernandez *et al.* 2008; Dror-Ehre *et al.* 2009; Eby *et al.* 2009; Panacek *et al.* 2009). Exerting their antibacterial properties, nanoparticles attach to the surface of the cell. This interaction causes structural changes and damage, markedly disturbing vital cell functions, such as permeability, causing pits and gaps, depressing the activity of respiratory chain enzymes, and finally leading to cell death (Rai *et al.* 2009; Sharma *et al.* 2009; Li *et al.* 2010). *In vitro* antibacterial activities

of vancomycin protected gold nanoparticles have been reported by (Gu *et al.* 2003).

In the present study, we evaluated the antimicrobial activity of spherical gold nanoparticles against six food poisoning bacteria and three fungal isolates using agar disk diffusion method and MIC. Gold nanoparticles synergistic effect with reference drugs was studied. Also, transmission electron microscopic analysis was used for investigating the interaction of ciprofloxacin, gold nanoparticles and ciprofloxacin coated with nanoparticles with *S. Typhimurium*.

2. Materials and methods

2.1. Synthesis and Characterization of gold nanoparticles

Hydrogen tetrachloroaurate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$, 99.99%), Cetyltrimethylammonium bromide (CTAB, 99%) and 11-mercaptoundecanoic (MUA) were obtained from Sigma. The other reagents were obtained from Aldrich and were used as received. Ultrapure deionized water was used throughout the experiments. Gold colloids were prepared by citrate thermal reduction method (Yang *et al.* 2005). Typically in the process of thermal reduction, a gold sol was prepared by adding 1ml of 1 wt% HAuCl_4 aqueous solution and 1.5 ml of 38.8mM sodium citrate aqueous solution into 90 ml boiling water. The citrate ion acts as both a reductant and stabilizer. After the solution had turned purple red within 30s, the solution was cooled quickly in the ice bath. This resulted in a stable dispersion of gold particles with an average diameter of around 13.2 nm and 10% polydispersity (Yang *et al.* 2003 and Yang *et al.* 2005). 0.2 ml of 0.1M freshly prepared cetyltrimethyl ammonium bromide aqueous solution was added to 20 ml as prepared gold colloid at room temperature. Finally, 1 ml of 0.5mM MUA aqueous solution was added to the gold colloid modified by 0.1mM CTAB in order to restrain the overmuch aggregation process. The absorption optical spectra of these gold colloids were recorded using Jasco Ubest 570 UV-vis-NIR spectrophotometer. All the spectra were recorded in air at room temperature. The microstructure and morphology of gold nanoparticles in gold colloids was measured with a JEOL-JSGM T1230 transmission electron microscopy (TEM) operating at 200 kV. Those samples were prepared by dropping the colloid onto a carbon coated Cu grid underlying tissue paper, leaving behind a film.

2.2. Preparation of microbial suspensions.

Antimicrobial activities of gold nanoparticles were carried out against common food poisoning isolates. Three Gram positive bacteria (*L.monocytogenes*, *B.cereus* and *S. aureus*), three

Gram negative bacteria (*S. Typhimurium*, *E.coli* O157 and *P. aeruginosa*) and three fungal isolates (*C. albicans*, *A. flavus* and *A. niger*) isolated from food of animal origin were used. Agar disk diffusion method (qualitative) and minimum inhibitory concentration method (quantitative) were used in this study. Wherein a suspension of bacterial and fungal isolates were freshly prepared by inoculating fresh stock culture from each isolate into separate broth tubes, each containing 7ml of Mueller Hinton Broth (Difco) for bacterial isolates and Sabouraud Dextrose broth (Difco) for fungal isolate. The inoculated tubes were incubated at 37°C and 28 °C for 24 hr, respectively. Serial dilutions were carried out for each isolate, dilution matching with 0.5 McFarland was selected for screening of antimicrobial activities. Ciprofloxacin (5µg/ml) and fluconazole (100µg/ml) were used as reference drugs (Oxoid).

2.3. Preparation of drug coated gold nanoparticles (Nirmala and Pandian, 2007).

The drugs coated nanoparticles were prepared as follows; 0.1mM citrate stabilized gold nanoparticles [10 ml of 0.5mM Au diluted to 50 ml] was mixed with 5 ml of 3mM drugs in water and stirred effectively for 2 hrs. This was marked as the control sample. Similarly, antibiotic protected gold were prepared at concentration of gold particles 0.5mM, using different volumes (20 and 40 µl) to study the effect of nanoparticles on the microbial activities.

2.4. Determination of antimicrobial activity by Disk-diffusion method (Nirmala and Pandian 2007; Bansod and Rai 2008).

Mueller Hinton agar plates (Difco) and Mueller-Hinton agar supplemented with 2% glucose and Methylene Blue (0.5 mg/L) were prepared for testing antibacterial and antifungal activities, respectively. The colony forming units of suspension of the tested isolates were determined and tested inoculums were adjusted to 1×10^5 cells/ml, matching with 0.5 McFarland. Inoculums (100µl) were applied on the surface of the agar plates and spread by using sterile glass spreader. For evaluation of antibacterial activities, Whatman no.1 filter paper disks were sterilized and saturated with different volumes of Au nanoparticles; 20, 40 and 50 µl. On the other hand, others were saturated with 50 µl ciprofloxacin (5µg/ml) or with ciprofloxacin mixed with different volumes of gold nanoparticles (20 and 40 µl). The same method was used for evaluation of antifungal activities using fluconazole (100µg/ml) as reference drug using drug coated with Au at volume 40 µl. Disks were placed onto inoculated agar plates and left for 1 hr at 25 °C to allow a period of pre-incubation diffusion in order to minimize the effects of variation

in time between the applications of different solutions. The plates were re-incubated at 37°C and 28°C for 24 hrs for bacterial and fungal isolates respectively. After incubation, plates were observed for antimicrobial activities by determining the diameters of the zones of inhibition for every sample. For an accurate analysis, tests were run in triplicate for each isolate to avoid any error.

2.5. Determination of Minimum Inhibitory Concentration (MIC)

Microtiter dilution plate quantitative method (Andrews, 2001), i.e. the minimum inhibitory concentration (MIC) was used for evaluation of the antimicrobial activity of the gold nanoparticles against inhibited organisms. Determination of MIC of gold nanoparticles against tested isolates was achieved using 96-well sterile micro plates. Initial concentration 100%, then two fold serial dilutions of the nanoparticles and reference drugs (ciprofloxacin or fluconazole) and drugs coated with nanoparticles were inoculated with 100µl of tested isolates (0.5 McFarland, about 1×10^5 cells/ml) and incubated at 37°C-28°C for 24 h for bacterial and fungal isolates respectively. After incubation, plates were examined visually for bacterial or fungal growth precipitation. The experiment was repeated three times. The lowest concentration that showed complete growth inhibition of the microbe was taken as MIC.

2.6. Transmission Electron Microscopy (TEM) examination

S. Typhimurium was subjected to gold nanoparticles, ciprofloxacin and ciprofloxacin coated with gold nanoparticles. The samples were examined using transmission electron microscope (TEM, JOEL JSM-1230). Samples were inoculated in Brain Heart Infusion (BHI) broth then centrifuged at 2100 rpm. Most of the bacterial cells settled at the bottom of the sample vial and the nanoparticles remained suspended in the solution under these centrifugation conditions unless they were bound to the cell walls of the bacterial cells or became large particles as a result of aggregation. The supernatant was then discarded after centrifugation. The remaining bacterial cells conjugated with nanoparticles were washed twice with deionized water (0.5 ml) under gentle vortex mixing for 10 min. The remaining bacterial cells that might be conjugated with the nanoparticles were then resuspended in deionized water (0.5 ml). After gentle vortex mixing for another 10 min, 2µl of this suspension solution was deposited on the copper holder. After drying, the sample was ready for TEM analysis (Ho *et al.* 2004).

3. Results and Discussion

3.1. Characterization of gold nanoparticles

Figure 1 shows TEM image of the obtained gold nanoparticles. As seen in Figure (1), the prepared gold nanoparticles were almost spherical shape, and separated from each other. The particle size is mainly in the range of 11–22 nm. It is also noted that the nanoparticles in the gold nanoclusters were nearly individually isolated.

Figure 2 presents the UV–vis absorption spectra of the prepared gold sols, in which the 530 nm absorption bands were characteristic of the surface plasmon bands of gold nanoparticles of 11–22 nm.

3.2. Agar disks diffusion test

Antimicrobial activities of gold nanoparticles differ according to the volume used and the tested isolates. Antimicrobial activities increased with higher volume; 50 > 40 > 20µl. Au nanoparticles (vol. 50 µl) showed great antimicrobial activities with the best zone of inhibition against *P. aeruginosa* (17mm), *B. cereus*, *L. monocytogenes* (14mm), *S. aureus* (13 mm) and *S. Typhimurium* (12mm) as shown in Figures 3&4.

The best antifungal activity was against *A. niger* and *C. albicans* (12mm each) followed by *A. flavus* (11mm) as shown in Figures 5&6 The antimicrobial ability of Au nanoparticles might be referred to their small size (9-19) nm which is 250 times smaller than a bacterium. This makes them easier to adhere with the cell wall of the microorganisms causing its destruction and leads to the death of the cell. Also, Au nanoparticles are able to maintain their constant shape and size in solution as indicating from Figure (7a). Metal nanoparticles are harmful to bacteria and fungi (Chwalibog *et al.* 2010). Nano-Au stimulate biofilm production and aggregate within this biofilm. They bind closely to the surface of microorganisms causing visible damage to the cells, and demonstrating good self-assembling ability. Gold nanoparticles possess well-developed surface chemistry, chemical stability and appropriate smaller size, which make them easier to interact with the microorganisms (Nirmala and Pandian, 2007). Also, the particles interact with the building elements of the outer membrane and might cause structural changes, degradation and finally cell death. During the interaction between *S.aureus* and gold nanoparticles, they were trapped by the biofilm and the substance released by cells causing distortion of the cell wall (Chwalibog *et al.* 2010). The effect of the ciprofloxacin as antibiotic or ciprofloxacin in combination with 20 and 40 µl Au nanoparticles was studied. It is indicated that the best results were against *S. Typhimurium* with zone of inhibition equal 26, 27 and 30mm), respectively followed by *B. cereus* (23, 24 and 26mm), *P. aeruginosa* (20, 21 and 23mm),

L. monocytogenes (20, 21 and 23mm), *E.coli* O157 (20, 22 and 23mm) and *S. aureus* (11, 12 and 21mm), as shown in Table (1) and Figure (3, 4). By increasing the number of gold atoms, more amount of drug gets adsorbed on the nanoparticle surfaces when the number of drug molecules increase, they act more effectively against the microorganisms. This means that the gold nanoparticles can act as an effective carrier to these drugs (Nirmala and Pandian, 2007).

The antifungal drug coated with Au nanoparticles using different volumes; 40 and 20 μ l was compared with fluconazole. The best antifungal activity was observed on using fluconazole coated with 40 μ l Au nanoparticles with the best zone of inhibition against *A. niger* 14mm, then *C. albicans* 13mm, followed by *A. flavus* 12mm as illustrated in Figures 5&6. The interaction between nanoparticles and fungal cells caused their damage (Nirmala and Pandian, 2007).

3.3. Minimum Inhibitory concentration (MIC)

Au nanoparticles showed variable MIC of bacterial and fungal cultures which differ according to the tested isolates; *P. aeruginosa* (1.56) followed by *B. cereus*, *L. monocytogenes* and *S. aureus* equal (3.125) then *S. Typhimurium* and *E.coli* O157 (6.25). MIC test revealed synergistic effect of Au nanoparticles with ciprofloxacin when compared with ciprofloxacin alone; best results were shown against *S. Typhimurium* (0.097, 0.19), *B. cereus* (0.19, 0.39), *E.coli* O157 (0.39, 0.39), *P. aeruginosa* and *L. monocytogenes* (0.39, 0.78) then *S. aureus* (0.78, 6.25), respectively. Gold nanoparticles and fluconazole coated with Au nanoparticles showed variable MIC against *C. albicans*, *A. niger* (6.25, 3.125), *A. flavus* (12.5, 6.25), as shown in Table (2). Au nanoparticles disintegrated the cell wall and the cytoplasmic membrane of *C. albicans* releasing homogeneous matter then attach to a filamentous substance, excreted from the disrupted cells. Fungicidal activity of Au nanoparticles was due to destroying cell membrane integrity (Chwalibog *et al.* 2010).

3.4. Transmission Electron Microscopy (TEM) Study of Treated Microorganisms

TEM visualization provides an extraordinary opportunity for the morphologic evaluation of ongoing interactions between microorganisms and nanoparticles. Au nanoparticles are stucked randomly with the surface of bacteria. Interaction between *S. Typhimurium* and gold nanoparticles indicates that the nanoparticles were trapped by the biofilm and the substance released by cells with distorted cell wall which might be referred to the thin layer of peptidoglycans found in the cell wall of Gram

negative bacteria. This might be due to the small size of gold nanoparticles (range 9-19 nm) which can easily attach to the surface of cell membrane and drastically disturbed the bacterial proper function like respiration and permeability as shown in Figure (7a). Interaction between *S. Typhimurium* and ciprofloxacin coated with gold nanoparticles revealed that the cell wall is loosened and separated from the membrane or disrupted with complete absence of flagella. Accumulated gold nanoparticles with ciprofloxacin on bacterial cell wall distorted the cells and disintegrated cell wall and cytoplasmic membrane with complete absence of flagella as shown in Figure (7b). TEM of *S. Typhimurium* treated with ciprofloxacin alone exhibited interacted bacterial cell wall with the accumulation of antibiotic on its surface and partial destruction of flagella as shown in Figure (7c&d). The results proved that interaction between Au nanoparticles and *S. Typhimurium* cause damage of parts of the bacterial cell and the flagella which is the organ of motion. Drugs capped gold particle act as a single unit against the microorganism. This led to increase the zone of inhibition from 12 mm (Au), 26 mm (ciprofloxacin) to 30 mm (Au coated ciprofloxacin) using disk diffusion. Also, this was confirmed by the decrease in MIC from 6.25(Au), 0.19 (ciprofloxacin) to 0.097(Au coated ciprofloxacin) as shown in Table (2). Previous study indicated that Au nanoparticles are more effective against Gram negative organisms due to the nature of materials present in cell wall. Gram positive organisms generally have thick mesh like cell wall made of peptidoglycans layer whereas Gram negative organisms possess a thin cell wall with peptidoglycans. Also, TEM images proved easier permeability in Gram negative organisms, which confirms the approach of nanoparticles to the *E. coli* which in turn supports the enhancement of antibacterial activity. Gold nanoparticles act as a good anchor carrying more amounts of drugs on its surface via electrostatic attraction between the amine groups of drugs and nanoparticles which give a better activity of streptomycin protected gold nanoparticles at 0.5mM Au concentration with an increase in the level of zone of inhibition from 82 to 96. Increasing the number of gold atoms, surrounded by a number of drug molecules makes an effective approach of the drug molecules as a group rather than acting alone towards the bacterial organisms (Nirmala and Pandian, 2007). Another studies indicated that drugs capped gold nanoparticles are effective against various isolates of bacteria when compared with the pure drugs. The process of targeting by Au nanoparticles can minimize treatment durations and side effects of drugs (Thomas and Klivanov, 2003; Tkachenko *et al.* 2004; Connor *et al.* 2005 and Mukherjee *et al.* 2007).

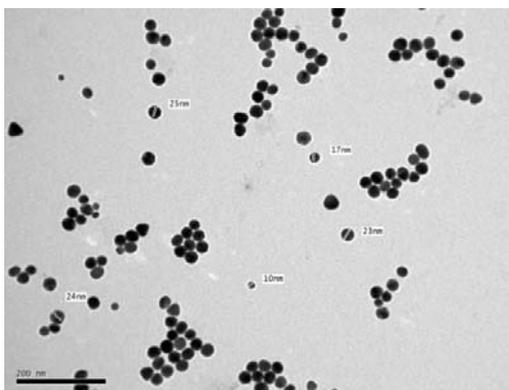


Figure 1: TEM image of gold nanoparticles

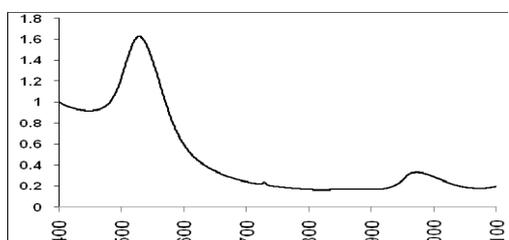


Figure 2: Absorption characteristic of gold nanoparticles

Table (1): Antibacterial activity of spherical gold nanoparticles as compared with ciprofloxacin (5µg/ml) and drugs coated with nanoparticles at different volumes.

Bacterial Isolates	Volume of Au nanoparticles (µl)					ciprofloxacin (5µg/ml)
	20µl	40 µl	50 µl	20 µl Au+ ciprofloxacin	40 µl Au+ ciprofloxacin	
<i>L.monocytogenes</i>	11	12	14	21	23	20
<i>S. aureus</i>	-ve	11	13	12	21	11
<i>B. cereus</i>	12	13	14	24	26	23
<i>S. Typhimurium</i>	10	12	12	27	30	26
<i>E. coli O157</i>	11	12	12	22	23	20
<i>P. aeruginosa</i>	10	16	17	21	23	20

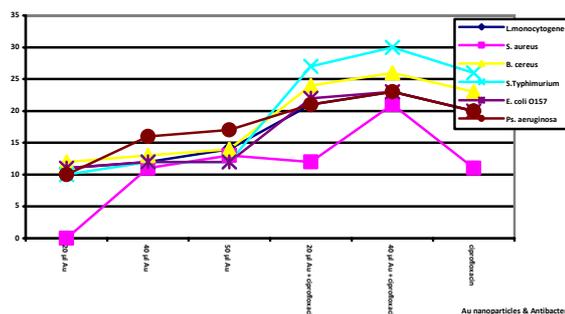


Figure 3: Antibacterial activity of spherical gold nanoparticles as compared with ciprofloxacin (5µg/ml) alone or coated with nanoparticles at different volumes.

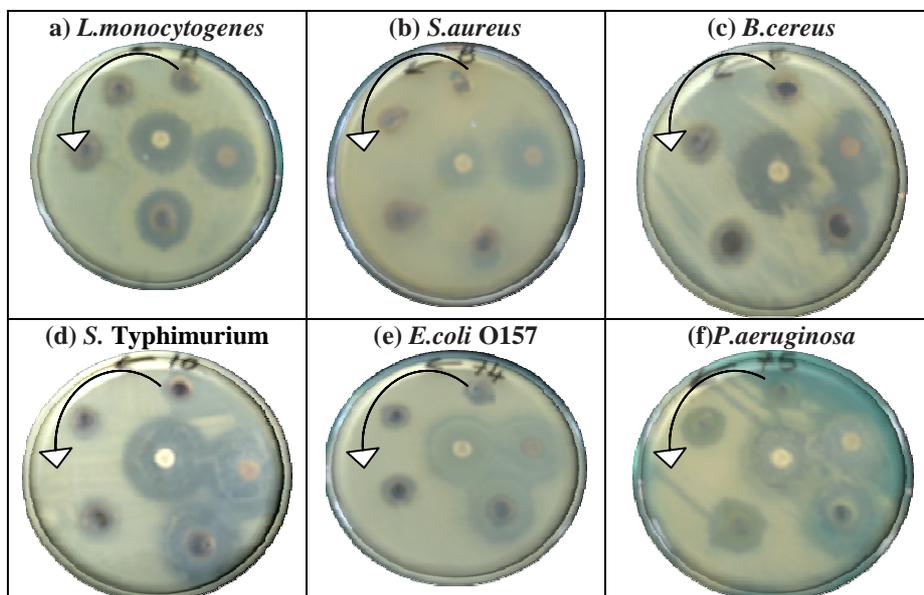


Figure 4: Zones of inhibition of different tested isolates using disk diffusion method showing the antibacterial activity of Au nanoparticle in different volumes; 20 µl, 40 µl, 50µl, (20 µl Au + ciprofloxacin), (40 µl Au + ciprofloxacin) then ciprofloxacin alone, in sequence with the arrow.

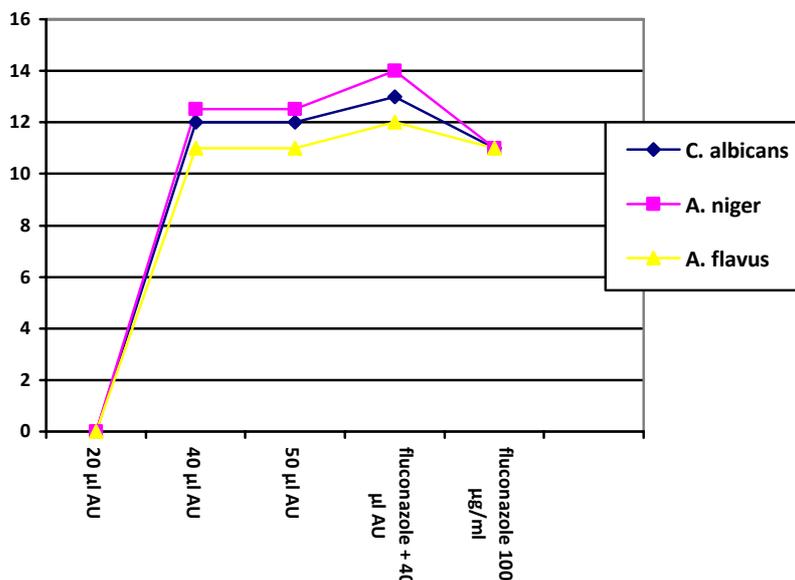


Figure 5: Antifungal activity of spherical gold nanoparticles as compared with fluconazole and drugs coated with nanoparticles at different volumes.

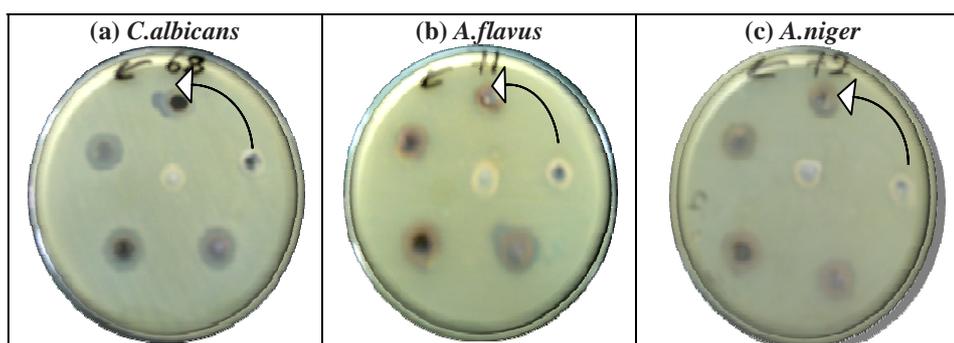


Figure 6: Zones of inhibition of different tested isolates using disk diffusion method showing the antifungal activity of Au nanoparticle in different volumes; 20 μ l, 40 μ l, 50 μ l, 50 μ l fluconazole only, then 40 μ l of Au nanoparticles + fluconazole, in sequence with the arrow. Control negative was at the center.

Table (2): Minimum Inhibitory concentration of gold spherical nanoparticles compared with reference drugs alone and coated with Au nanoparticles.

Isolates	MIC		
	AU nanogold	Reference drugs	Au & reference drugs
<i>L.monocytogenes</i>	3.125	0.78	0.39
<i>S. aureus</i>	3.125	6.25	0.78
<i>B. cereus</i>	3.125	0.39	0.19
<i>S. Typhimurium</i>	6.25	0.19	0.097
<i>E. coli O157</i>	6.25	0.39	0.39
<i>P. aeruginosa</i>	1.56	0.78	0.39
<i>C. albicans</i>	6.25	6.25	3.125
<i>A. niger</i>	6.25	6.25	3.125
<i>A. flavus</i>	12.5	6.25	6.25

Reference drugs: ciprofloxacin (5 μ g/ml), fluconazole (100 μ g/ml).

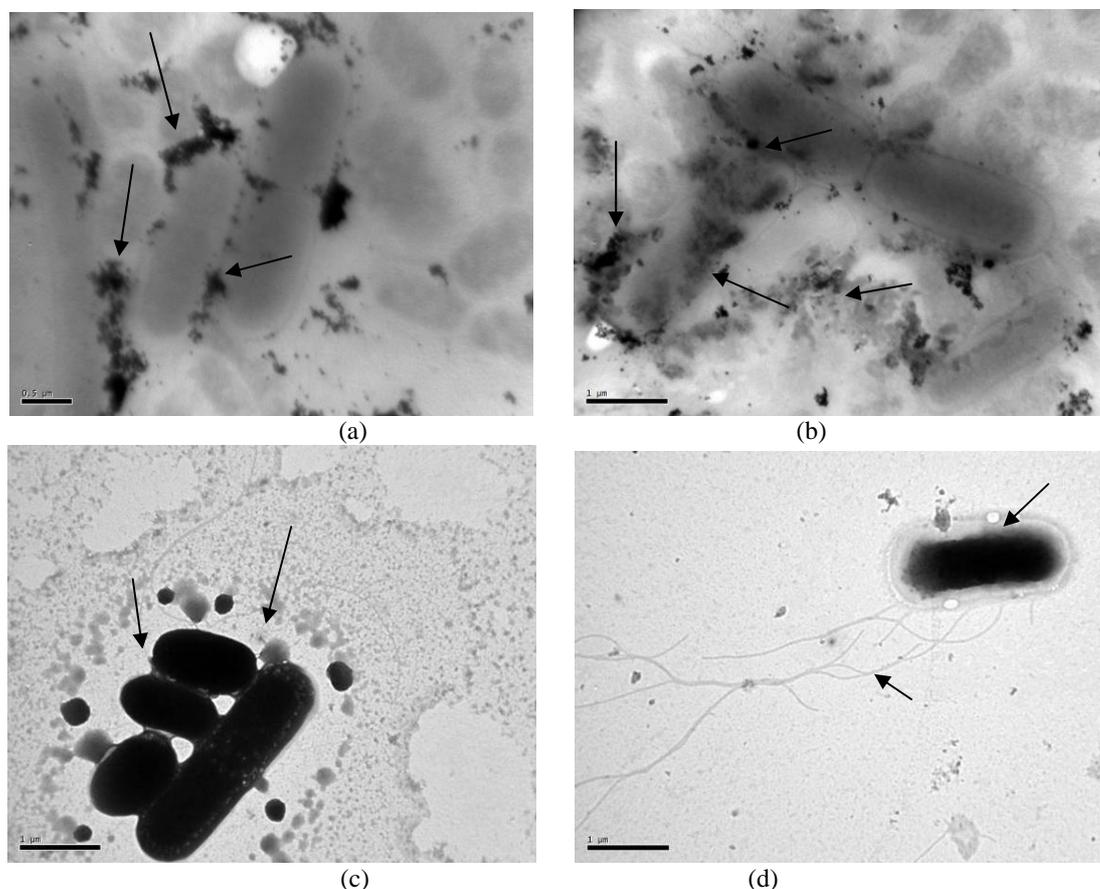


Figure 7: Transmission electron of *S. Typhimurium* treated with (a) Au nanoparticles alone. (b) Ciprofloxacin and Au nanoparticles. (c, d) Ciprofloxacin alone.

Conclusion

Gold nanoparticles are harmful to bacteria and fungi. They bind closely to the surface of the microorganisms causing visible damage to the cells with complete destruction of flagella, stimulate production of biofilm and aggregate within this biofilm. The results verified that drugs coated with gold nanoparticles had more hindrance activities than the pure drugs. They were more effective against Gram negative bacteria due to the thin peptidoglycan layer in the cell wall. Thus the use of Au nanoparticles coated drugs can minimize the treatment durations and side effects of drugs.

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