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Research Article

Antimicrobial Analysis of Biosynthesized Lectin-Conjugated Gold Nanoparticles

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To enhance the bioactivity of molecules through nanoparticles is being tested which has potential use in sustained-release drug delivery systems and to enhance the therapeutic effectiveness of drugs. Our current investigation **s** is to conjugate lectin to that of a gold nanoparticle (GNP) surface without disturbing the bioactive properties and enhances the antibacterial activity of lectin. Au-lectin nanoparticles were checked for their hemagglutination activity, characterized by transmission electron microscopy (TEM) and UV-visible spectrophotometer. The antibacterial effect of nanoparticle lectin, Au salt nanoparticle, and conjugated Au-lectin was estimated by Kirby-Bauer disc method; MICs were determined by microbroth dilution and compared with ciprofloxacin. These tests were done using known species of bacterial strain of multidrug resistant. The hemagglutination activity of lectin was improved to fourfold after purification. Lectin and Au nanoparticles combined had a significant effect on the inhibition of bacterial growth. No significant differences were observed in the inhibition zone diameters from killed bacteria and its supernatant towards any of the tested organisms. Lectin-conjugated gold particles showed good efficacy as antimicrobial agents and the nanoparticle-killed bacteria to work against the viable population of the same bacterium and/or other bacterial species too.

1. Introduction

Recent past is witnessing the increasing bacterial resistance due to inappropriate use of available antibiotics [1]; status of multidrug-resistant microorganism has become a severe problem to health of public [2]. Such scenario raised the need to develop new bioactive molecule with potency to overcome antibiotic resistance and thereby able to control infectious diseases [3]. Lectin is group of proteins or glycoproteins, heterogeneous in chemical nature having non-immune origin mostly occurring in animals, bacteria, viruses, fungi, and plants [4]. It has a wide application in many fields as antitumor, antifungal drug, antiviral, anti-insect, and antibiofilm [5]. Though they are used in wide applications, the outsourcing of lectins is very expensive. Several investigations have demonstrated the antimicrobial effects of lectins [6], which is originated of carbohydrate-recognition domain [7]. The properties of lectins to bind with carbohydrate provided with its ability to interact with eukaryotic cells and pathogens, defending pathogens, suppressing of viral infections, and inhibiting microbial propagation by reducing cell adhesion and migration [6].

To enhance the bioactivity of a molecule nanoparticles are being tested which has potential use in sustained-release drug delivery systems to enhance the therapeutic effectiveness of drugs. These approaches maximize the effect of drug, decrease the potential side effects, and increase their bioavailability in certain sites. Among the various drug

delivery systems, silver nanoparticles have been widely studied [8]. Next to silver nanoparticles, gold nanoparticles (AuNPs) are important and widely used in diagnostics, as well as in therapeutics. AuNPs have more surface area which allows dense arrangement of drugs and targeting agents thereby bioconjugates of AuNPs have important application in the design of innovative bioactive nanoparticles [9, 10]. In this paper, we report a comprehensive approach to conjugate lectin to that of a gold nanoparticle (GNP) surface without upsetting the lectin bioactive properties. We experimentally demonstrate that lectin bound gold particles have good efficacy as antimicrobial as well the nanoparticle-killed bacteria work against the viable population of different bacterial species.

2. Methods

2.1. Chemical Reagents. Potassium gold (III) chloride ($KAuCl_4$) was precure-**procured** from Sigma-Aldrich (St Louis, USA). Unless and until mentioned, the rest of all reagents in this study were obtained from Merck (Germany), and Sephadex G-150 column and DEAE Sephadex A-50 column were purchased from GE Healthcare (USA).

2.1.1. Isolation and Purification of Lectin. Lectin was extracted using red kidney beans (55 g). In brief, beans were softened in 1000 mL of buffered saline (0.85% NaCl; 1 : 8, w/v) overnight in room temperature and homogenization using blender. The diffused homogenate was filtered using an eighty-mesh grid after 24 hours. Filtrate is further set for centrifuging for 30 minutes at 6000 rpm, and the resulting supernatant fractionated by ammonium sulfate at concentrations 20-80% saturation. The obtained precipitate was centrifuged at 8000 rpm for 30 min and transferred in 0.02 M PBS, pH 7.2 and the protein concentration and hemagglutination activity were measured. DEAE -cellulose column (2.5 × 20 cm (was initially equilibrated with buffer and dialyzed protein was incorporated in the column. Before elution with salt gradient (0.1–0.5 M NaCl), the column was washed with buffer as used before. The fractions showing activity those peaks were collected mixed and run on to Sephadex G-150 column (2 × 80 cm) which was already equilibrated and later eluted in same the buffer; the fractions that revealed the protein and hemagglutination activity in the same peak were mixed and transferred to a new sterile tube for further study. The active peak represented the purified lectin [11].

2.1.2. HPLC Analysis of Purified Compound. We estimate and confirm the purity of the lectin extract by Luna C_{18} reverse phase column [CA, USA] was used to purify collected fractions using HPLC technique [Shimadzu, Kyoto, Japan].

2.2. Hemagglutination Assay. To check the lectin presence, we utilized the hemagglutination activity properties of lectin. In a round-bottomed 96-well micro titer, plate was placed at flat surface. To that, each of well 50 μl PBS and 50 μl of sample was mixed and suspended to wells. From last well, 50 μl was discarded. 50 μl of working solution of red cell blood cells was placed in well, mixing gently and left at 30°C for 30 min to develop agglutination if positive.

2.3. Preparation, Characterization of Au Nanoparticles by Laser Ablation and Conjugation with Lectin by Pulses Method. Laser ablation method was utilized for the preparation of Au nanoparticles that was prepared by laser ablation from Au metal salt pellets (purity 99.99%; 10 mm × 2 mm). The operation of Nd:Yag laser oblation was performed at 1 Hz repetition rate with 40 pulse, fluence (14.45 J/cm^2), and 1064 nm of wavelength. Au nanoparticles were characterized by UV-visible spectrophotometer and transmission electron microscopy (TEM) for determination of their distribution and particle size. The Au pellets were dispensed in 5 milliliter of purified lectin at 4°C and pulsed by using laser ablation technique at 1 Hz repetition rate with 40 pulses, fluence (14.44 J/cm^2), and wavelength of 1064 nm. The obtained particles were then confirmed lectin enzyme by estimating the hemagglutination activity. Au-conjugated lectin nanoparticles were characterized by UV-visible spectrophotometer and transmission electron microscopy (TEM) using drop casting method at 32°C for 30 minutes.

2.4. Bacterial Strains and Antimicrobial Activity. All the bacterial cultures like *Escherichia coli* O157:H10 and O157:H7, methicillin-resistant *Staphylococcus aureus*, and multidrug-resistant *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, and *Acinetobacte baumannii* were isolated at a microbiology department. All cultures were stocked and preserved at 4°C in their appropriate agar slants until their use.

2.4.1. Detection of Antibacterial Activity of Nanoparticles. The antibacterial effect of nanoparticle lectin, Au salt nanoparticle, and conjugated Au-lectin was estimated by Kirby-Bauer disc diffusion method and compared with ciprofloxacin. In brief, overnight grown pure culture of bacterial isolates was spread on to the Mueller-Hinton agar plates and sterile paper discs (6 mm) soaked separately with nanoparticles and antibiotics were placed on the surface of the media and incubated as prescribed. Zone of inhibition was calculated in millimeters by observing transparent ring around the disc [12].

2.4.2. Determination of the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Nanoparticles. MICs of all tested nanoparticle were determined by microbroth dilution method using bacterium inoculum size of 1×10^6 cfu ml-cfu/ml. Aliquots with no visible turbidity were plated and examined on next day for the presence or absence of bacterial growth. MBCs were estimated by drop plate technique from the tubes, where there is no visible growth observed [13].

2.4.3. Antimicrobial Efficacy and Durability of Nanoparticle-Killed Bacteria and Supernatant against the Tested Strains. Au-killed *E. coli* O157:H10 bacteria and the supernatant prepared using citrate Au at a concentration of 6 ppm were tested for their efficacy and durability against *E. coli* O104:H4, E. coli O157:H7, MDR *P. aeruginosa*, and MRSA. All the agents were prepared and stored in dark at 4°C and

the baseline line zone was determined for each of the tested agents. The tested agents were stored in dark for antimicrobial activity testing after 1, 2, 6, 16, 22, 34, and 40 days using agar diffusion protocol and the zone of inhibition was recorded as mentioned [14].

The overnight culture of *E. coli* O157:H10 isolate was centrifuged for 8 minutes at 4000 rpm and the pellet was washed in pyrogen-free water for three times. Pellet was resuspended in 5 ml pyrogen-free water and was adjusted to 0.5 McFarland standard. 1 ml bacterial suspension was added to 3 ml of citrate Au at increasing concentrations of Au nano and Au-lectin nano (1.5, 3, 6, 12, and 18 ppm) and the mixture was incubated at 37°C under dark conditions for 6 hrs. Au nanotreated bacteria were centrifuged for 8 minutes at 4000 rpm after proper neutralization and the pellet was resuspended in pyrogen-free water. Supernatant was filtered and two ml of this was dispensed in 1 ml of fresh viable *E. coli* O157:H10 culture and 2 ml of pellet suspension was dispensed in 1 ml of the fresh viable *E. coli* O157:H10 culture. Both supernatant and pellet suspension containing cultures were incubated at 37°C for 6 h. All the culture tube aliquots were serially diluted (10-fold dilution) in saline and then pour-plated. The plates were incubated for 24 h at 37°C; the bacterial colonies were counted and compared to the total no. of colonies of fresh viable *E. coli* O157:H10 culture (not treated) [15].

2.5. Statistical Analysis. All the experiments were performed in triplicate and data was represented as mean ± SD. By using SPSS, 17 statistical software (SPSS Inc., Chicago, IL), one-way ANOVA is performed to estimate any significant variations between control and Au-killed bacterial supernatant.

3. Results

3.1. Extraction and Purification of Lectin

3.1.1. Lectin Production. The activity of lectin production was checked at every step of purification and the result indicated that even crude extract had a significant hemagglutination activity (1 : 64 dilutions) and after purification the activity was increased fourfold (1 : 256). The purified content was enhanced purification steps.

3.1.2. Purification of Lectin. Crude extract solution which showed hemagglutination activity was disrupted using glass beads and fractionation in steps using solid ammonium sulfate with 20-80% saturation. The glass beads were very effective in disrupting the cells and also lead to the extraction of periplasmic proteins. Saturation led to increase the specific activity up to 56 U/mg and precipitate sample showed four protein peaks in HPLC run in which the hemagglutination activity was observed in the 2nd peak (Figure 1). The active fractions were then run through DEAE cellulose followed by Sephadex G-150 followed by elution revealed a single peak showing hemagglutination activity. The purified extract with hemagglutination was run in HPLC and exhibited a single peak and it has definite activity of 555 U/mg. The lectin was purified with 40.8 fold of purification and a definite activity of 555.12 U/mg protein.

3.2. Characterization of Au nanoparticles Conjugated with Purified Lectin. The absorption spectrum of the Au nanoparticles was estimated by UV-visible spectrophotometer wavelength range between 300 and 700 nm as displayed in Figure 2. Results showed that Au nanoparticles had a wavelength about 372 nm. The Au nanoparticle-lectin conjugation was shown and maximum absorption spectra with shift in wave length from 372 nm to 435 nm (Figure 2), indicating that Au nanoparticle produced by pulses and the shell thickness of the lectin around the Au nanoparticle led to a shift in the peak wave length, thus reflecting a conjugation of purified lectin with the Au nanoparticle. TEM images of these nanoparticles have a spherical shape ranging between 15 and 60 nm (Figure 3). The hemagglutination activity of Au nanoparticles conjugated to lectin was measured and found that the hemagglutination activity was increased from 256 to 1024 U/ml.

3.3. Detection of Antibacterial Activity of Nanoparticles. Ciprofloxacin was effective with high inhibition zone diameter than lectin and Au nanoparticle against all pathogens though it all were in resistant zone. The combined effect of lectin and Au nanoparticles had significant effect on inhibition with quite uniform range of zone ranging between 18 and 21 mm indicating it has broad spectrum activity with higher diameter of inhibition zone above sensitive cut-off (Table 1).

3.3.1. MIC and MBC Analysis of Conjugated Nanoparticle. The MIC and MBC of conjugated nanoparticles was checked against *E. coli* O157:H10 (8 ppm each) and MRSA *S. aureus* (5 ppm each). Both the pathogens were exposed to MBC value of nanoparticle which was 6 ppm and 4 ppm, respectively; the killed bacteria were pellet from media by centrifugation and washed with phosphate buffer saline. A suspension of killed bacteria was separated from supernatant and washed. Both pellet of the killed bacteria and filtered supernatant (0.2 μm bacterial filter) were impregnated in well agar and examined for antimicrobial action against fresh viable *E. coli* O157:H10 and MRSA *S. aureus*. This showed good size of inhibition zones (23 ± 0.22 mm) for pellet suspension and 24 ± 0.41 for supernatant against MRSA *S. aureus* (Figure 4).

3.3.2. Antimicrobial Efficiency and Stability of Au-Killed Bacteria and Supernatant against the Tested Strains. The supernatant and bacterial pellet also contain the toxin and enzyme of pathogens which may or may not be active by the time that were tested *in-vitro*. Further simple experiment was done to rule our possibility of antibacterial activity was due to nanoparticle-killed bacteria and not because of their excreted toxin or byproducts. Earlier experiment was again repeated using killed bacteria by autoclaved and results were not surprising but was as expected that on viable bacteria was examined. This time we did not see the zone of inhibition against tested pathogen which suggests that nanoparticle-treated-killed bacteria are reservoirs of these

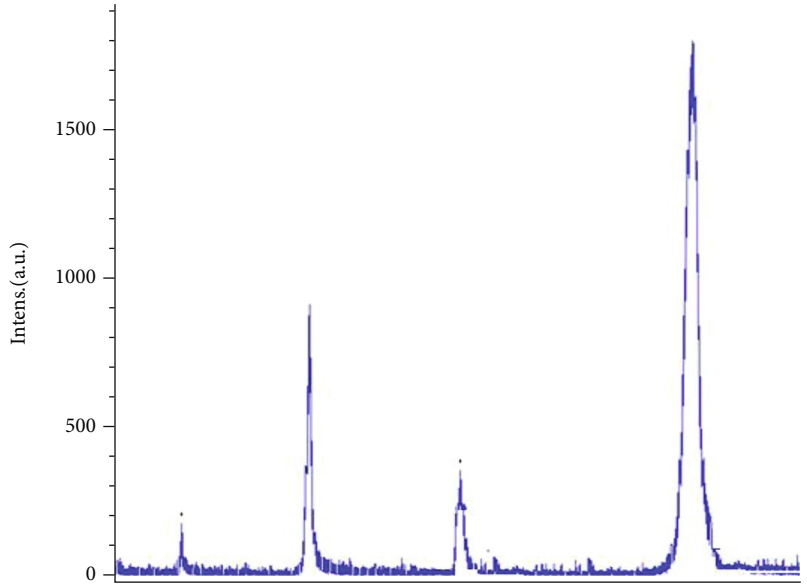


Figure 1: HPLC showing peaks of crude extract. The sample showed four protein peaks in HPLC run and second peak was positive for hemagglutination test.

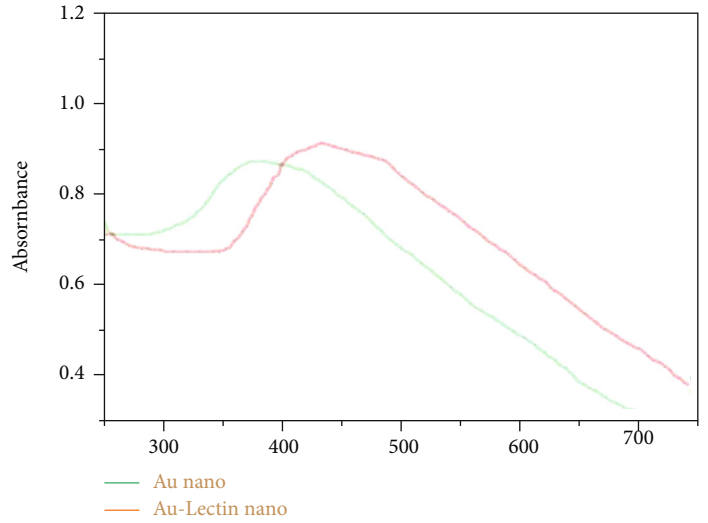


Figure 2: UV spectroscopy of nanoparticle showing the observance of particle.

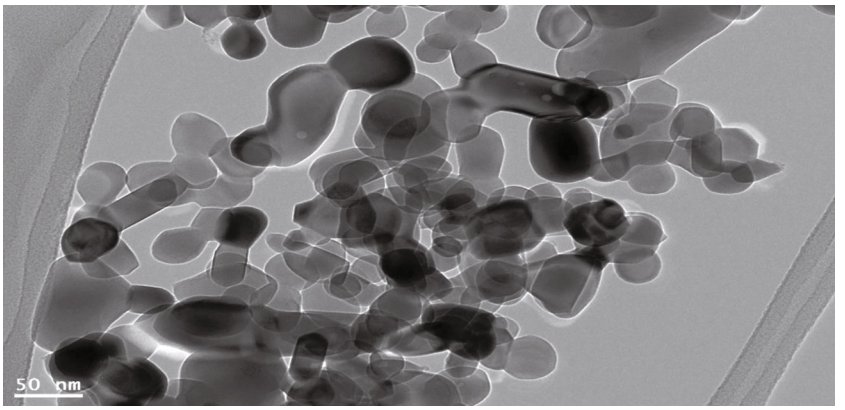
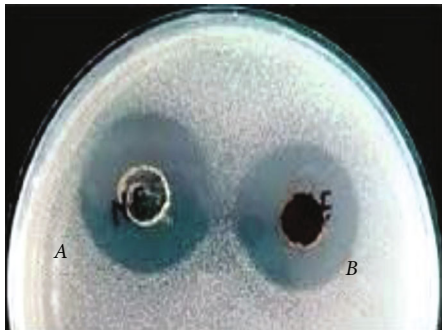


Figure 3: TEM images of Au-lectin conjugate nanoparticle prepared by laser ablation in deionized water.

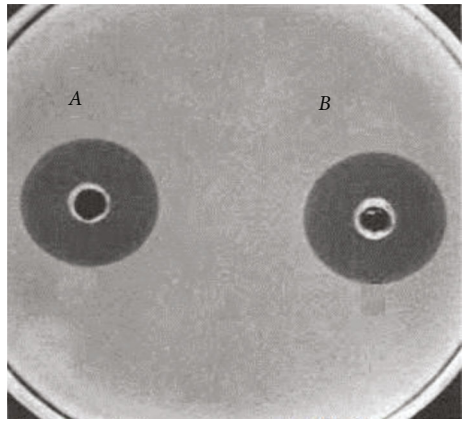
Table 1: Zone of inhibition of pathogens against lectin and Au nanoparticle.

ZIO (zone of inhibition in mm)	Ciprofloxin (30 μg/ml)	Au nano (10 ppM)	Lectin (10 μg/ml)	Au-lectin nano (10 ppM)
E. coli 0157:H10	18 mm	15 mm	14 mm	20 mm
E. coli 0157:H7	18 mm	15 mm	13 mm	21 mm
MDR *S. aureus*	19 mm	16 mm	15 mm	21 mm
MDR *P. aeruginosa*	19 mm	15 mm	12 mm	20 mm
K. penumoniae	18 mm	16 mm	14 mm	19 mm
A. baumannii	18 mm	16 mm	14 mm	18



E. coli O157:H10 A. Heat killed and B. Supernatant

Figure 4: The diameter of inhibition zone for *E. Coli* O157:H10. Zone of inhibition against tested pathogen suggests that nanoparticle-treated-killed bacteria are reservoirs of these particles.



S aureus A. Supernatant; B. Killed bacteria impregnation

Figure 5: The diameter of inhibition zone for *S. aureus*. Zone of inhibition against tested pathogen suggests that nanoparticle-treated-killed bacteria are reservoirs of these particles.

particles (Figure 5). Though there was light difference in zone of inhibition of supernatant and nanoparticle-killed bacteria, their killing activities were approximately equal and no significant differences were observed in inhibition zone diameters from killed bacteria and its supernatant towards any of the tested organisms (data not shown).

4. Discussion

The nanoparticles are tested for their better diffusion capability through the cell wall and they seem to be more effective then—than antibiotics in cases where antibiotics are though capable of bacterial killing but are failure against those inside the host cells or causing infection in deep tissue otherwise hiding in thick biofilms. The nanoparticles have the ability to enter into the bacterial cell and if they are loaded with known potent antibacterial agents, they boost their capability leading to a high inhibition zone [16]. Earlier studies reported that silica-made nanoparticles have broad spectrum of activity against bacterial strains [17, 18], due to their phosphate groups and their electrostatic interactions with cell wall as they were used as a carrier of gentamicin thereby entrapped the antibiotics in the sol-gel matrix and the biologically active gentamicin is released at site [19]. Apart from silica, silver was another most tested antimicrobial agent which has shown different effects against bacteria at high concentrations in form of silver nitrate. The various mechanism of its bioactivity against bacteria has been described which includes its binding to the thiol groups of protein and denaturing them, causing DNA to be in the condensed form which inhibits cell replication and leading to programmed cell death (apoptosis) [20].

The purified lectin was combined with Au nanoparticles by pulses and hemagglutination assay as well as antibacterial activity was increased for all tested Gram-positive and -negative bacteria when compared to ciprofloxacin. This may be of large surface area of each nanoparticle that had area volume then the ratio of area to volume would be higher; therefore, the presence of nanoparticles would be a good surface and medium to binding of the lectin with the receptors on the surface of erythrocytes then increasing its biological activity.

Compared to antibiotics, nanoparticles have high penetration through the cell wall [21], which lead to high inhibition zone [16]. The antimicrobial activity was enhanced when both the nanoparticle was conjugated and that indicates the delivery of the potent antimicrobial was enhanced. It was reported that nanoparticles once are closed to a bacterium cell; it releases thousands of atoms in the neighboring area. At this juncture, there is high concentration of particles around the bacteria and as the Au is positively charged get attracted towards negative charged bacterial cells through electrostatic forces [22]. Through diffusion, these nanoparticles get through cell wall inside the bacteria and deliver the bioactive molecule already conjugated with it. The bioactivity of both killed bacteria and supernatant towards other pathogens may be credited to the fact that nanoparticles

produce reactive oxygen species (ROS) which have shown cytotoxic effect on the treated bacteria [23].

The release of nanoparticles varies with depending factors on size of the nanoparticles, their surface area, the temperature, and neighboring environment [24]. Thus, small dose of nanoparticle was sufficient to kill high number of bacteria and it was observed that this element is released in a sustained and controlled manner providing adequate amount of the antimicrobial activity for an extended period of time [24].

5. Conclusion

Nanoparticle-killed bacteria can act as effective weapons against other viable bacteria which are present in their environment or media. This is by acting as a reservoir for adsorbed gold nanoparticles. This phenomenon helps in management of wound infections using wound dressing containing gold and for the disinfection of water using gold.

Data Availability

The data used to support to the findings of this study are included with in the article.

Conflicts of Interest

The authors declare there is no conflict of interest among authors.

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