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Extracellular Biosynthesis of AgNPs by the Bacterium *Proteus mirabilis* and Its Toxic Effect on Some Aspects of Animal Physiology

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Abstract

The development of a reliable green chemistry process for the biogenic synthesis of nanomaterials is an important aspect of current nanotechnology research. Silver nanoparticles (AgNPs) have been used as antimicrobial and disinfectant agents. However, there is limited information about its toxicity. Therefore, this study focused on the biosynthesis of AgNPs by the bacterium *Proteus mirabilis* and on determining its preliminary toxic effect on some aspects of animal physiology. A green method for the synthesis of AgNPs using culture supernatant of *Proteus mirabilis* has been developed in this study and the synthesized AgNPs were characterized by several techniques. The AgNPs showed a maximum absorbance at 445 nm on ultraviolet-visible spectra. The presence of proteins was identified by Fourier transform-infrared spectroscopy. The reduction of Ag^+ to elemental silver was characterized by X-ray spectroscopy analysis. The transmission electron micrograph revealed the formation of polydispersed nanoparticles of 5 - 45 nm. The AgNPs were evaluated for their toxic effect on pregnant female albino rat. The result showed that liver enzymes (AST and ALP) were decreased significantly in the group treated with AgNPs. Mean corpuscular hemoglobin concentration also showed significant increase.

Keywords

AgNPs, *P. mirabilis*, TEM, X-Ray, Liver Enzymes, Blood Parameters

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1. Introduction

The synthesis of metallic nanoparticles is an active area of applied research of nanotechnology. A variety of chemical and physical procedures could be used for the synthesis of metallic nanoparticles. However, these methods are fraught with many problems including use of toxic solvents, generation of hazardous by-products [1], and high energy consumption [2]. Accordingly, there is an essential need to develop environmental procedures for synthesis of metallic nanoparticles. A promising approach to achieve this objective is to use the array of biological resources in nature. Indeed, over the past several years, plants, algae, fungi, bacteria and viruses have been used for production of low-cost, energy-efficient and nontoxic metallic nanoparticles [3]. In recent years, interest in AgNPs and their applications has increased because of the important antimicrobial activities of these nanomaterials allowing their use in several industrial activities [4] [5]. However, together with these applications, there is increasing concern related to the biological impacts of the use of AgNPs on a large scale and the possible risks to the environment and health [6]. Similarly, the novel properties of nano-size materials may also reflect their potent toxic effects, since the same properties that make these nanoparticles very interesting for a wide range of applications might affect their toxicity [7]. In addition, different size and shape AgNPs have diverse toxicities [8]. Health effects of nanoparticles are also attracting considerable and increasing worldwide recognition. AgNPs can be ingested directly via water, food, cosmetics, drugs, drug delivery devices, etc. [9]. Uptake of particles of different size via the gastrointestinal tract can also lead to different toxicological effects [10]. However, reports about the toxicological research of AgNPs by the gastrointestinal tract are few. Moreover, silver and its nanoparticles are widely being applied to consumer products and medical uses. Some medical and biological reports have proved that, many medical devices release silver ions into the blood which finally accumulate in liver and kidney inducing toxic injuries of these organs [11]. According to the experiments done with a variety of metal nanoparticles, AgNPs have shown more toxicity than other metals such as iron, nickel, manganese and aluminum [12].

The production and usage of these materials in different industries lead to the necessity of this research, thus investigation of harmful effects of these substances is an essential and urgent matter [13]. In this study, a single AgNPs dose was prepared and its toxicity was evaluated after oral administration in pregnant female albino rat.

2. Materials and Methods

2.1. Chemicals

$AgNO_3$ was obtained from Sigma-Aldrich, USA. All other chemicals were purchased from Merck, Germany. Freshly prepared doubly distilled water was used throughout the experimental work.

2.2. Isolation and Characterization of Bacterial Strains

The soil samples were collected from the waste dump sites at Taif, KSA. The collected sample was serially diluted and plated on Luria-Bertani (LB) agar medium and the plates were incubated at 28˚C for 24 h. After the incubation period the bacterial colonies were observed and it was further sub-cultured on the same medium to obtain the pure colonies. In our screening, a total of 30 bacterial isolates were isolated and named as SA1 to SA30. It was used for screening of AgNPs synthesis and effective synthesizer strain was further characterized by 16S rDNA sequencing technique.

2.3. Genotypic Characterization of the AgNPs Synthesizing Strain

The morphological and physiological characterizations of the selected isolate were carried out by biochemical tests using the Bergeys Manual of Determinative Bacteriology [14]. Further characterization of isolate was done by means of 16S rRNA gene analyses. The genomic DNA of the isolate was extracted according to standard method. The 16S ribosomal DNA gene was amplified by PCR method with Taq DNA polymerase and primers 27F (5’ AGT TTG ATC CTG GCT CAG 3’) and 1492 R (5’ ACG GCT ACC TTG TTA CGA CTT 3’). The conditions for thermal cycling were as follows: denaturation of the target DNA at 94˚C for four minutes, followed by 30 cycles at 94˚C for one minute, primer annealing at 52˚C for one minute, and primer extension at 72˚C for one minute. At the end of the cycling, the reaction mixture was held at 72˚C for 10 min and then cooled to 4˚C. The PCR product obtained was sequenced by an automated sequencer (Genetic Analyser 3130, Applied Biosystems, USA). The sequence was compared for similarity with the reference species of bacteria contained in

Genbank database, using the NCBI BLAST available at http://www.ncbi.nlm.nih.gov/.

2.4. Biosynthesis of AgNPs

Synthesis of AgNPs was carried out according to the methods described previously [15]-[17]. Briefly, bacteria were grown in a 500 mL Erlenmeyer flask that contained Mueller-Hinton Broth. The flasks were incubated for 21 h in a shaker set at 120 rpm and 37˚C. After the incubation period, the culture was centrifuged at 10,000 rpm and the supernatant used for the synthesis of AgNPs. Two test tubes, the first containing $AgNO_3$ (Sigma, USA, 99.9% pure) without the supernatant and the second containing the supernatant and $AgNO_3$ solution at a concentration of 1mM were incubated at 30˚C. The extracellular synthesis of AgNPs was monitored by visual inspection of the test-tubes for a change in the color of the culture medium from a clear, light-yellow to brown, and by measurement of the peak exhibited by AgNPs in the UV-vis spectra.

2.5. Characterization of AgNPs

2.5.1. UV-Vis Spectroscopy Analysis

Characterization of the synthesized particles was carried out according to the method described previously [17]. The biologically synthesized AgNPs using the cell free supernatant were characterized by UV-vis spectroscopy (Perkin Elmer, Lambda 25) instrument scanning in the range of 200 - 900 nm, at a resolution of 1 nm. All Samples were prepared by centrifuging an aliquot of culture supernatant (1.5 ml) at 10,000 rpm for 10 min and diluted 10-fold for all experiments involving measurement of UV-vis spectra. Cell free supernatant without addition of silver nitrate was used as a control throughout the experiment.

2.5.2. Transmission Electron Microscopy (TEM) Analysis

Samples for TEM analysis were prepared on carbon-coated copper TEM grids. Studies of size and morphology of the nanoparticles were performed by TEM operated at 120 kV accelerating voltage (JTEM-1230, Japan, JEOL) with selected area electron diffraction (SAED). Finally, the obtained images were processed using the software Image J. Image J developed at the National Institutes of Health (NIH), USA is a Java-based public domain image processing and analysis program [18].

2.5.3. Attenuated Total Reflection Fourier Transform Infrared (ATR-FTIR) Analysis

FTIR measurements were carried out using ATR-FTIR Spectrometer (Bruker, Germany, Alpha-P). The instrument was configured with ATR sample cell including a diamond crystal with a scanning depth up to 2 micrometers. Sample powders were applied to the surface of the crystal then locked in place with a "clutch-type" lever before measuring transmittance. Each of the spectra was collected in the range 4000 - 400 cm^{-1} at 2 cm^{-1} resolution. Comparing with the conventional transmission mode, the present technique is faster sampling without preparation, excellent reproducibility and simpler to use.

2.5.4. X-Ray Diffraction (XRD) Analysis

XRD analysis was performed using an automated diffractometer (Philips type: PW1840), at a step size of 0.02˚, scanning rate of 2˚ in 2θ/min and a 2θ range from 10˚ to 70˚. Indexing of the powder patterns and least squares fitting of the unit cell parameters was possible using the software X'Pert High Score Plus.

2.6. Animal Experiment

Three groups of pregnant female albino rat (three animals per group) were used in this study. The first group was taken normal tap water, the second was taken a single dose of AgNPs (0.001 mol of 50 nm AgNPs dispersed in liter ddH_2O) while the third group was subjected to 0.001 mol of sliver nitrate. After 15 days administration, blood samples were obtained from the anesthetized animals. The levels of liver enzymes for alanine transaminase (ALT), aspratae transaminase (AST) and alkaline phosphatase and the kidney marker (creatinine) were determined following the regular clinical kit method. Some blood parameters were also measured.

3. Results and Discussion

3.1. Identification and Characterization of Bacterial Strain

Among the tested organisms, SA2 strain showed rapid synthesis of AgNPs when compared to other isolates and

based on 16S rDNA sequencing, it was found to be *Proteus mirabilis* SA2. The sequences were deposited in GenBank (NCBI) with accession number AB775005. Synthesis of AgNPs was observed by the addition of the selected culture supernatant of *Proteus mirabilis* SA2 to 1 mM aqueous $AgNO_3$ at room temperature. It turns yellow to brown solution with reaction time 2 h indicating the formation of AgNPs (**Figure 1(a)**). The color formation is dependent on the excitation of surface plasmon vibrations of AgNPs [15] [17].

3.2. Characterization of AgNPs Synthesized by *Proteus mirabilis* SA2 Supernatant

The synthesis of AgNPs was confirmed by UV-visible absorption spectroscopy which is widely used for structural characterization of AgNPs. The synthesized AgNPs showed maximum absorbance at 445 nm, implying that the bioreduction of the silver ions has taken place following incubation of the $AgNO_3$ solution in the presence of bacterial supernatants. It is reported that the absorption spectrum of spherical AgNPs presents a maximum between 420 nm and 450 nm [15] [17]. This absorption depends on the particle size, dielectric medium and chemical surroundings [18]. Small spherical nanoparticles (<20 nm) exhibit a single surface plasmon band [20]. The absorption peak (SPR) is obtained in the visible range at 445 nm in (**Figure 1(b)**) with the above mentioned concentration. The stability of AgNPs is observed for 4 months and it shows a SPR peak at the same wavelength.

3.3. TEM Analysis

TEM study showed the morphology and size details of synthesized AgNPs (**Figure 2**). In general, the particles are nano-sized and well dispersed. The formed AgNPs were predominantly spherical in shape with size range of 5 to 45 nm [15] [17]. The crystalline nature of synthesized AgNPs was shown in **Figure 3**. The distinct peaks at 38.23, 44.36, and 64.57 lattice plane value was observed which was indexed h, k, l value are 1 1 1, 2 0 0 and 2 2 0 and planes of face centered cubic silver, which are closely matched with the reported reference values of International Centre for Diffraction Data (ICDD) card number 01-087-0717.

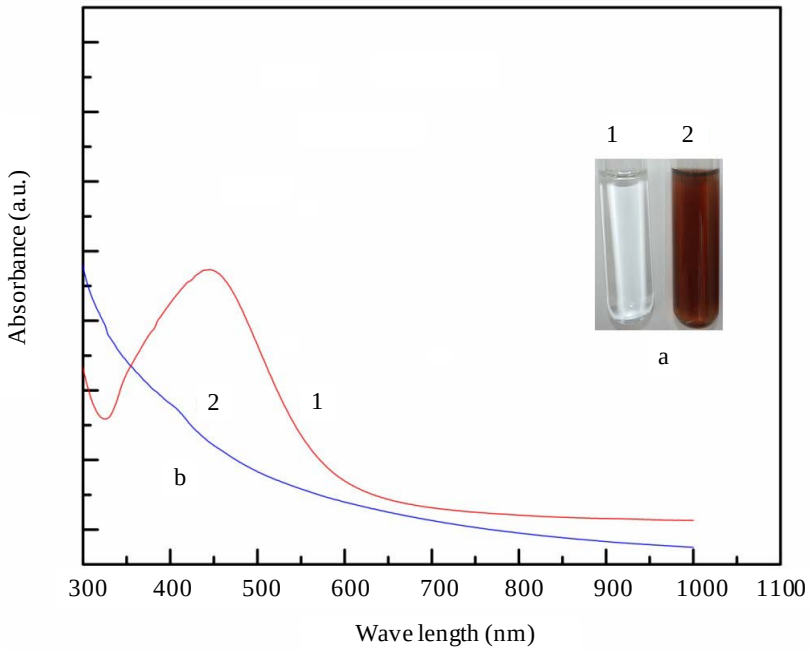

Figure 1. Inset (a) digital photograph of test tubes containing *P. mirabilis* SA2 supernatant reacted with aqueous solution of 1 mM $AgNO_3$. It is observed that the color of the solution turned from colorless to yellow brown after 2 h (tube 2) of the reaction at 30˚C indicating the formation of AgNPs. Blank control (tube 1) contains $AgNO_3$ only; (b) the corresponding absorption spectrum of AgNPs exhibited a strong broad peak at 445 nm and observation of such a band is assigned to surface plasmon resonance of the nanoparticles.

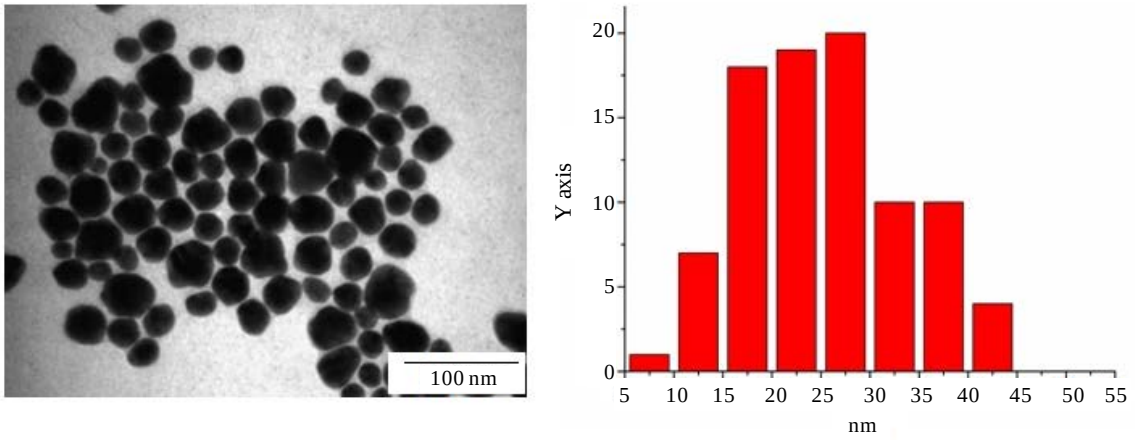

Figure 2. (a) TEM image of the AgNPs produced by the reaction of 1 mM aqueous $AgNO_3$ solution with *P. mirabilis* SA2 supernatant at 30˚C; and (b) its particle size distributions.

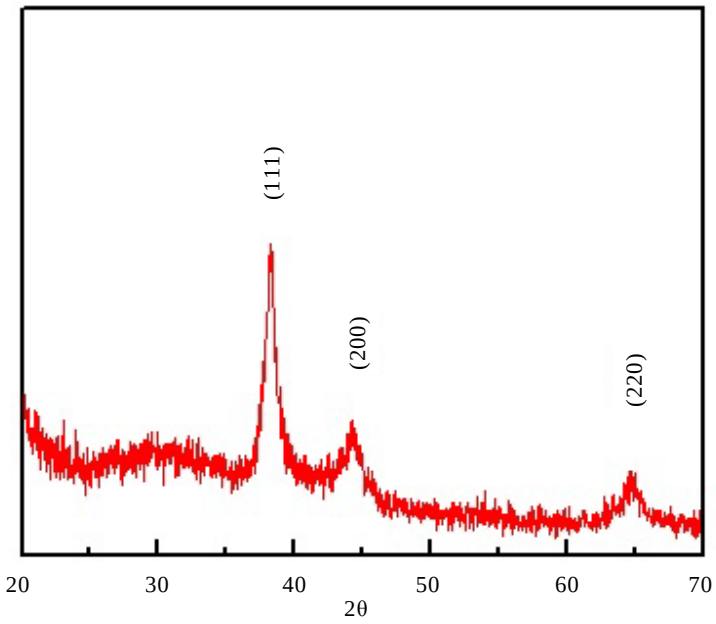

Figure 3. Representative XRD pattern of AgNPs synthesized by the reaction of 1 mM $AgNO_3$ solution with *P. mirabilis* SA2 supernatant s at pH 7.

3.4. ATR-FTIR Analysis

FTIR spectroscopy is a valuable tool to monitor the secondary structural of proteins in various environments [21]. The specific stretching and bending vibrations of the peptide backbone in amide I, II, and III bands provide information about different types of secondary structures such as α-helix, β-sheets, turns, and unordered structures [22]. Aamide I was proven to be the most sensitive probe of protein secondary structure [22]. The ATR-FTIR spectrum (**Figure 4**) of pure protein and that bound to the AgNPs surface showed obvious changes in both the shape and the peak position suggesting the changes in the secondary structure of protein after nanoparticle formation. FTIR results revealed that secondary structure of proteins have been affected as a consequence of binding with AgNPs. The corresponding spectra of AgNPs-protein showed an increase in the intensity of the peak around 1633 cm^{-1} indicating an increase in β-sheet structure during AgNPs formation [23]. The β-sheet structure that occurs in many proteins and consists of two or more parallel adjacent polypeptide chains arranged

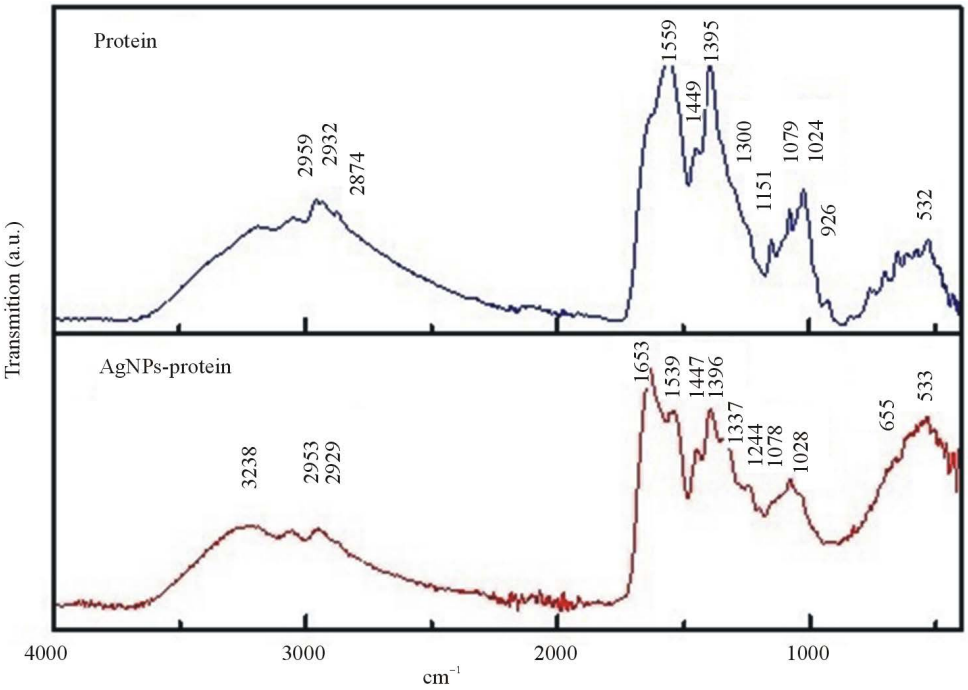

Figure 4. Representative ATR-FTIR spectra pattern of dried powder of native bacterial protein and AgNPs synthesized by the reaction of 1 mM aqueous $AgNO_3$ solution with *P. mirabilis* SA2 supernatant at pH 7.

in such a way where hydrogen bonds can be formed between the chains. Unlike in an alpha helix where the amino acids form a coil, in beta sheets the amino acids are arranged in a zigzag pattern that forms a straight chain. The AgNPs-protein structure is consistent with a β-sheet structure based on the location of peaks at 1635 cm^{-1} and 1539 cm^{-1} which can be assigned to amide I and amide II bands [22].

Mandal *et al*. [24] have confirmed that the secondary structure of proteins change due to self-assembled on nanoparticles with sizes below 20 nm. In the present study, the secondary structure of protein changed to b-sheet in the presence of AgNPs. As a protein molecule interacts with AgNPs surface, the energy of the system is minimized by the relaxation of protein secondary structures. The extent of protein conformational change is strongly dependent on the protein-AgNPs surface interactions via function groups. From the ATR-FTIR spectra, an interaction between AgNPs and protein is farther confirmed by the shift in CH_2 (1449 cm^{-1} to 1447 cm^{-1}) and NH_2 (1395 cm^{-1} to 1397 cm^{-1}) [25].

3.5. The Toxic Effect on Animals

The liver is the target organ obviously affected significantly by the AgNPs. The activity of the liver enzymes for AST and ALP were decreased significantly ($p < 0.05$) in the group treated with AgNPs whereas silver nitrate did not induce any effect compared to the control (**Table 1**). Conversely, the activity of the liver enzyme for ALT increased significantly in the group treated with silver nitrate while AgNPs did not change the enzyme activity as compared to the control. The only estimated kidney marker was creatinine which did not show any remarkable difference among the three groups. With respect to the blood parameters, the only parameter affected by the AgNPs was the mean corpuscular hemoglobin concentration (**Table 2**) which showed significant increase ($p < 0.05$). The biochemical tests revealed that AgNPs caused inhibitory effects on AST and ALP and an excitatory effect on ALT activities. Heavy metals are toxic and therefore they inactivate the endogenous enzymes strongly by interacting with thiol groups of these enzymes [26]. In addition, it is believed that AgNPs bind to functional groups of proteins, resulting in protein deactivation and denaturation [27] [28]. Similar finding were demonstrated by Abbas *et al*. [29]. On the other hands, other studies demonstrated no effect of AgNPs on these enzymes [30], while other studies showed that AgNPs toxicity induced increases in the liver and kidney enzymes [31]. Our results agreed with those supported the enzymes inhibition for AST and ALP and agreed with those

Table 1. Mean ± average deviation for liver and kidney functions in the female pregnant albino rat treated with a single dose of both AgNPs and Silver nitrate for 15 days. Stars referred to the significant differences (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$) among the different treatments based on Student *t-test.*

Treatment	ALT	AST	ALP	Glucose	Creatinine
Control	168.3 ± 3.1	155.7 ± 4.4	8.3 ± 1.3	125 ± 20.4	0.63 ± 0.04
Ag Nitrate	218 ± 10**	157.7 ± 15.5	12 ± 3.3	144 ± 15.3	0.6 ± 0.00
AgNPs	171.7 ± 8.2	117.3 ± 9.1*	5 ± 1.8*	156.7 ± 2.7	0.7 ± 0.04

Table 2. Mean ± average deviation for the blood parameters in the female pregnant albino rat treated with a single dose of both AgNPs and Silver nitrate for 15 days. The significant differences were also referred to as in legend of **Table 1**.

Treatment	WBCs (10^3)	RBCs (10^6)	Hb%	PCV	MCV	MCH	MCHC	Plat.	Lymph. %
Control	8 ± 6.7	7.5 ± 0.16	15 ± 1.3	40.1 ± 2.6	58.8 ± 2.6	21.9 ± 0.44	37.3 ± 1.4	810 ± 2	72.5 ± 11.04
Ag Nitrate	10.1 ± 3.9	7.3 ± 0.44	15.4 ± 0.56	46.2 ± 5.4	59.1 ± 1.8	21.03 ± 0.58	35.6 ± 0.13	893 ± 90.7	54.6*** ± 0.2
AgNPs	7.2 ± 4. 9	7.34 ± 0.15	16.5 ± 0.38	43.2 ± 0.53	58.9 ± 0.62	22.3 ± 0.78	38.3* ± 1	887.7 ± 108.4	74.2 ± 0.8

supported enzyme excitation for ALT enzyme. Meanwhile, the results for kidney function agreed with those did not support any toxic effect of AgNPs on liver and kidney functions. We can interpret this result by that the AgNPs are small enough to be excreted easily by the kidney and therefore the organ did not suffer from its toxicity. We may attribute this slight changes in the blood parameters to that the dose used was very small and the period of administration were also very short. We recommend conducting more investigations using different doses for long period. These results were conducted for pregnant female albino rat where many factors other than AgNPs could induce similar changes. We therefore recommend conducting a similar study on the male animals to be more reliable.

4. Conclusion

In conclusion, AgNPs were synthesized biologically by the bacterium *Proteus mirabilis* and were characterized by several techniques including ultraviolet-visible spectra, FTIR spectroscopy, XRD and TEM. Oral administration of AgNPs by pregnant female albino rat showed hepatic and hematological toxicity. The activity of liver enzymes for AST and ALP was decreased significantly in the group treated with AgNPs. The mean corpuscular hemoglobin concentration showed significant increase.

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