Investigation of Antioxidant and Cytotoxicity Effect of Silver Nanoparticles Produced by Biosynthesis Using *Lactobacillus gasseri*

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Abstract

The biosynthesis of silver nanoparticles (AgNPs) is a new approach in the field of nanotechnology with optimistic implementation in medicine, food control, and pharmacology. The current study was designed to investigate antioxidant and cytotoxicity effect of the AgNPs which were produced by *Lactobacillus gasseri* filtrate. The production of AgNPs was confirmed by the color change from yellow to brown. Characterization of AgNPs was performed using UV visible spectrophotometer, FE-SEM, and Fourier Transform Infrared Measurement (FTIR). The antioxidant activity of AgNPs was tested by DPPH method, and the scavenging test for DPPH showed 19.3%, 32.6%, 47.6%, 72%, 85.3% at concentrations (6.25, 12.5, 25, 52, 100) µg/ml, respectively, as the scavenging percentage increased with increasing concentrations. The effect of AgNPs on the chromosomal pattern was also studied. The results of the experiment of AgNPs against SK-GT-4 cancer cells showed the presence of toxic activity of the used particles against the strains of these human esophageal cancer cells, and no effect on normal cells.

**Keywords:** Nanoparticles, Cancer Cells, Chromosomal Aberrations
1. Introduction

The term "probiotic" was first used in 1965, by Lilly and Stillwell, to describe substances secreted by one organism which stimulate the growth of another. Probiotics encourage the multiplication of other bacteria, which are often known as the normal flora, or they are types of beneficial bacteria that are cultivated in the laboratory and kept alive in special packages that reach millions or billions of bacterial cells in one dose. Probiotics was discovered in 1953 and has proven effective in treating diseases of the digestive system in a special form (1). Probiotics also help to absorb food well and prevent inflammatory bowel diseases, lactose intolerance or lactose sensitivity, especially in infants, prevention of irritable bowel diseases and colon cancer, reducing gas in the stomach and intestines, treatment of constipation and colic, treatment of nutritional deficiencies as it helps to produce several vitamins in the digestive system, reduce fungal infections in the vagina in women, especially those caused by *C.albicans*, which cause many problems, especially in pregnant women. *Lactobacillus* species is one of the most important types of bacteria used in the manufacture of probiotics (2).

*Lactobacillus spp.* are non-spore producing rods that are facultative anaerobic, catalase-negative, (Gram +ve), and grow better under micro aerophilic conditions. The morphology of their Gram stain varies, including short, plump rods, long, thin rods, chains, and palisades. On blood agar, their colonial morphology ranges from small to larger gray colonies with alpha hemolysis. *Lactobacillus* can also be grown on other mediums, such as MRS (Man, Rogosa, and Sharpe) agar, where they appear as white, mucoid colonies. *Lactobacillus* is made up of about 170 species and 17 subspecies, all of which have been officially described and have a solid nomenclatural standing. They are found naturally in the gastrointestinal system and vaginal canal in humans, but they can also be opportunistic pathogens (3).

Silver metal was known since about 4000 B.C. and it was used in many medical uses, even before it was realized that microorganisms are the main cause of infection (4). With the emergence of nanotechnology, it became possible to produce nanoscale silver (5, 6). Silver nanoparticles have been used against reactive oxygen species (ROS), which gave excellent results. It is known that antioxidants have many benefits in scavenging free radicals and ridding the body of many cardiovascular and cancerous diseases (7). Silver nanoparticles have also been used on various types of cancer lines, and it has been observed that tumor progression is inhibited by silver nanoparticles.
and controls the progression of the disease without causing toxicity to normal cells (8). Silver nanoparticles can be synthesized using traditional or unconventional methods, using two different approaches: top-down and down-top, and there are many traditional methods used to obtain Silver nanoparticles, such as chemical reactions / Photochemical, thermal decomposition of various silver compounds, electrochemical, radiation, and the use of microwave-assisted methods (9). Unconventional methods of creating these particles depend on the use of microorganisms such as bacteria, fungi, marine algae, and yeasts or various alcoholic or aqueous plant extracts, as they are considered as a reducing or inhibitory agent (10). Green synthesis methods are considered to be the best in preparing silver nanoparticles due to their many advantages: low cost, environment friendly and does not require high pressure, energy, or the use of chemical reagents (11). The aim of this study performs the biosynthesis of AgNPs using L.gasseri bacterial filtrate with silver nitrate solution and to Study the cytotoxicity effect of silver nanoparticles on cancer cells.

2. Materials and Methods
2.1. Collection of bacterial isolates
Twenty isolates were collected for Lactobacillus sp. from the Microbiology Laboratory at Al-Olwiya Teaching Hospital for Children in Baghdad, where they were collected, diagnosed with VITEK device. Then the isolates were planted on the surface of the slanted bed medium formed from De Man, Rogosa, Sharpe agar (MRS) agar, then incubated and kept at 4°C until use.

2.2. Morphological and microscopic examination
Isolates were grown on MRS agar to determine the color and size of colonies and then stained with Gram stain.

2.3. Maintenance of cell cultures
SK-GT-4 cells were maintained in RPMI-1640 supplemented with 10% Fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin. Cells were passaged using Trypsin-EDTA reseeded at 80% confluence twice a week and incubated at 37 °C (12).

2.4. Biosynthesis and purification of silver nanoparticles by using L.gasseri bacteria
The bacteria were seeded in MRS agar medium for 48 hours, after which the growth medium was discarded by a 4000 rpm centrifuge for 10 minutes, the sediment was removed and the filtrate sterilized with filters of 0.22µm pore dimension and the filtrate was preserved until its use (13). The silver nanoparticles were synthesized using a filtrate of L.gasseri bacteria; 10ml of culture filtrate was
mixed with 90 ml of 5 mM silver nitrate solution and incubated at room temperature for 48 hours. The main discovery of synthesized silver nanoparticles was done in the reaction mixture by detecting the color alteration of the medium from pale yellow to brown as well as observed optical density (13). For purification of AgNPs, the aqueous solution containing AgNPs was placed in test tubes and then placed in a centrifuge at a speed of 10,000 rpm, for 10 minutes. One minute for 10 minutes and the process was repeated 3 times until the filtrate became devoid of any color, and the concentrated precipitate containing silver nanoparticles was placed in an hour bottle, and it was dried in an electric oven at a temperature of 50 degrees Celsius (the thermal drying method) so that the excess water was removed and then collected the precipitate after drying and preserving until use (14).

2.5. Characterization of silver nanoparticles
Take 1 ml of the solution containing silver nanoparticles and add 9 ml of deionized water to it. The aqueous solution was then measured to reveal the nanoparticles formed at wavelengths ranging from 300 to 600 nm. The appearance of the highest absorption (λ max) after the wavelength of 400 nm indicates the formation of silver nanoparticles (15).

FTIR was used to characterize nanoparticles. The bio-produced silver solution was mixed with potassium bromide at a ratio of 1: 100 and then tested using FTIR Infrared Spectrometer within the range 4000-400 cm-1 (15).

Scanning electron microscopy (FE-SEM) was used to determine the particles size and morphology of AgNPs. The sample was dispersed on a smooth surface of carbon base and then was coated with a thin layer of gold and subjected to examination. EDX equipped included with FE-SEM was used to determine the chemical elements of AgNPs.

2.6. Antioxidant activity of AgNPs
Antioxidant activity of AgNPs was measured using stable 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) radicals with minor adjustments according to (16,17). Silver nanoparticles were used at concentrations (6.25µg/ml, 12.5 µg/ml, 25 µg/ml, 25 µg/ml, 100 µg/ml) to investigate the scavenging activity. The samples were mixed with 450 µl of DPPH solution and then complete the volume of mixture to one ml using absolute ethanol. Ascorbic acid was used as a positive control at a concentration of 100 µg/ml. The samples and control are left in dark at room temperature for 30 minutes. The absorbance was measured at 517 nm. Scavenging activity measured according to the equation formula:

$$Scavenging_i\% = \frac{Absorbance \text{ of control} - Absorbance \text{ of sample}}{Absorbance \text{ of control}} \times 100\%$$
2.7. Cytotoxicity Assays of AgNPs

To determine the cytotoxic effect of AgNPs, the MTT assay was done using 96-well plates (18). Cell lines were seeded at 1 × 10^4 cells/well. After 24 hrs. or a confluent monolayer was achieved, cells were treated with tested compounds at different concentrations. Cell viability was measured after 72 h of treatment by removing the medium, adding 28 µL of 2 mg/mL solution of MTT, and incubating the cells for 2.5 h at 37 °C. After removing the MTT solution, the crystals remaining in the wells were solubilized by the addition of 130 µL of DMSO (Dimethyl Sulphoxide) followed by 37 °C incubation for 15 min with shaking (19). The absorbency was determined on a microplate reader at 492 nm; the assay was performed in triplicate. The inhibition rate of cell growth (the percentage of cytotoxicity) was calculated as the following equation (20).

\[
\text{Inhibition rate} = \frac{A - B}{A} \times 100;
\]

where A is the optical density of control, and B is the optical density of the samples (21).

To visualize the shape of the cells under an inverted microscope, the cell was seeded into 24-well micro-titration plates at a density of 1×10^5 cells mL\(^{-1}\) and incubated for 24 h at 37 °C. Then, cells were exposed to AgNPs at IC50 for 24h. After the exposure time, the plates were stained with crystal violet stain and incubated at 37 °C for 10–15 min (22). The stain was washed off gently with tap water until the dye was completely removed. The cells were observed under an inverted microscope at 40× magnification and the images were captured with a digital camera attached to the microscope (23).

2.8. Study effect of silver nanoparticles on the chromosomal pattern

Transplantation was carried out to obtain chromosomes according to the method mentioned in (24) by adding 0.5 ml of peripheral blood to 4.5 ml of complete culture medium (RPMI-1640) prepared with 10 µg/ml of phytohemagglutinin (PHA) stimulator and two concentrations, and two different concentrations of silver nanoparticles prepared biologically using L.gasseri filtrate (0.0, 27, 54) µg/ml, and a positive control tube containing 0.65 µg/ml of methotrexate (MTX) was counted as a positive control, the tubes were incubated tilted at 37 °C for 70 hours, and colchicine solution was added at a final concentration of 10 µg/ml, and the tubes were returned to the incubator at 37 °C 5 for 2 hours, after which the implant was centrifuged at a speed of 2000 rpm for 10 minutes and after that The filtrate was neglected. The precipitate was suspended with 5 ml of a hypotonic calcium chloride solution, which was gradually added with continuous shaking, then the tubes were returned to the incubator for another 45 minutes to get rid of red blood cells by detonating them in the (KCL) solution, and swelling of lymphocytes to be ready to prepare the chromosomes of which. Then the contents of the tubes were centrifuged at a speed of 2000 rpm for 10 minutes to get rid of the filtrate and suspend the precipitate with a cold fixative solution prepared immediately by mixing three parts.
of absolute methanol with one part of glacial acetic acid, adding This solution is in the form of drops on the wall of the tube with continuous shaking until a volume of approximately 5 ml is obtained, after which the centrifugation process is carried out at a speed of 2000 rpm. This process was repeated several times until the solution became colorless, then the precipitated cells were suspended with (1.5 - 1 ml) of the fixative solution, mixed by a clean, dry Pasteur pipette, ready for installation on cool, wet slides. A certain size of the prepared cells was taken from above and at a height of about 30 cm, 7 spaced drops were dropped on the glass slide to obtain a good spread of chromosomes for easy observation of chromosomal changes.

The slides were air-dried in the previous paragraph, and after they were completely dried, they were stained with Giemsa stain for two minutes, then washed with distilled water, during which Blast Index (BI) and Mitotic Index (MI) were calculated according to the equations shown below.

\[
MI = \frac{Mitotic\ cells}{1000\ cell} \times 100\
\]

\[
BI = \frac{stimulated\ cells}{1000cell} \times 100\%
\]

Chromosomal aberrations= summation of total abnormalities within 25 mitotic cells.

2.9. Statistical analysis

The obtained data were statically analyzed using an unpaired t-test with GraphPad Prism 6. The values were presented as the mean ± SD of triplicate measurements (25).

3. Result
3.1. Morphological and microscopic examination

*L. gasseri* bacteria grew on De Man, Rogosa, Sharpe agar (MRS) by streaking method and incubated at 37 °C for 24 hours and the results are as shown in (Figure 1A). And microscopic examination was done using a Gram stain and the cells were examined with a light microscope to identify the shapes of the cells, their colors, and their sizes. The results are as shown in (Figure 1B).
3.2. Biosynthesis of silver nanoparticle

The results of the biosynthesis process were shown by using *L. gasseri* bacteria filtrate with AgNO$_3$ solution at a concentration of 5 mmol, at a pH of 6.5 and a room temperature of 37°C for 48 hours on the synthesis of AgNPs, and the color change of the mixture to a brown color was evidence of positive the synthesis process (Figure 2).

Figure 2. Stages of the synthesis of silver nanoparticles A=at zero time, B=after 48 hours

3.3. Characterization of AgNPs using UV-Visible spectrophotometer

The characterization of the silver nanoparticles created using a UV visible spectrophotometer showed the highest absorbance (λmax) at the wavelength of 424 nm (Figure 3).
3.4. Characterization of AgNPs using Fourier Transform Infrared (FTIR) spectrometer

The samples were examined by FTIR device, the spectral scanning of the samples was conducted within the range (400-4000) cm\(^{-1}\), the spectral absorption results of silver nanoparticles showed the presence of bands at the range (3368.79) on the presence of the O–H bond, and the presence of the bands at the range (2958.69) on the presence of the C–H bond, also indicates the presence of bands at the range (1632.3) on the presence of the C=O the double bond, while indicating the presence of bands at the range (1399.38) on the presence of the C–H bond, and the bands at the range (1060.21) Indicates the presence of the C–N bond, this result agrees with (26,27), as shown in (Figure 4).

The FTIR assay is one of the precise laboratory tests that are used to identify the chemical elements in the compounds under study, where the identity of the chemical compounds is determined based on how the chemical bonds between the compounds absorb infrared radiation, as each compound has its absorption (28).
3.5. Characterization of AgNPs using FE-SEM analysis

The FE-SEM analysis was conducted to evaluate the size, surface morphology, and uniformity of nanoparticles. It is a technique that is used for obtaining qualitative and quantitative information and provides further approaches into the morphology and size details of the nanoparticles (29). Mostly of AgNPs displayed a more obvious structural arrangement, have a spherical and have smooth surfaces with an observation of particles agglomeration and aggregation with size range between (58.06 to 72.91) nm, as shown in (Figure 5).

Figure 5. FE-SEM images for AgNPs A) Average size of AgNPs. B) Top view of AgNPs
EDX results showed that silver atoms contain 71.3% of the total sample components and a small percentage of Cl, O, C, S, Si also noted that peak of Au is present due to coating sample with gold in the examination of FE-SEM, as shown in the (Figure 6).

![Figure 6. EDX spectrum for AgNPs](image)

### 3.6. Antioxidant activity using DPPH radicals scavenging assay

DPPH is a free radical that is stable at room temperature, produces a dark violet color when dissolved in organic solvents, and is responsible for absorbance at the wavelength of 517 nm. DPPH is reduced and turns yellow when silver nanoparticles are present due to the presence of a phenolic hydroxyl group (30). Figure 7 shows the antioxidant activity of silver nanoparticles using different concentrations, and the results, Table 1 showed that the antioxidant activity of silver nanoparticles at a concentration of 100 μg/ml was 85.3%, while the antioxidant activity of silver nanoparticles at a concentration of 6.25 μg/ml it was 19.3%.

**Table 1. Antioxidant activity using DPPH assay.**

<table>
<thead>
<tr>
<th>Silver nanoparticles</th>
<th>Concentration μg/ml</th>
<th>Antioxidant Activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6.25</td>
<td>19.3</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>32.6</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>47.6</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>85.3</td>
</tr>
</tbody>
</table>
Figure 7. Antioxidant activity of AgNPs using DPPH assay. Ascorbic acid represents the positive control.
3.7. Cytotoxicity effect of silver nanoparticles against cancer cell line

The cytotoxic effect of AgNPs against SK-GT-4 cells was studied. The antitumor activity of the AgNPs was tested by studying their ability to inhibit the proliferation of tested cells. The results of this study showed highly significant cytotoxic activity of AgNPs against the cancer cell line, but not normal cell line as showed in figure 8 and figure 9. The results suggest the ability of AgNPs to suppress the growth of cell lines and this effect is a concentration-dependent manner (Table 2).

Figure 8. Control untreated SK-GT-4 cells

Figure 9. Morphological changes in SK-GT-4 cell after treated with AgNPs
Figure 10. Cytotoxic effect of AgNPs in SK-GT-4 cells. IC50=26.92 µg/ml

Table 2. It represents the toxic activity of AgNPs against SK-GT-4 cells at different concentrations

<table>
<thead>
<tr>
<th>Concentration μg/ml</th>
<th>Mean±SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.25</td>
<td>2.33 ±0.882</td>
</tr>
<tr>
<td>12.5</td>
<td>11 ±1.527</td>
</tr>
<tr>
<td>25</td>
<td>30.66 ±2.404</td>
</tr>
<tr>
<td>50</td>
<td>52 ±2.886</td>
</tr>
<tr>
<td>100</td>
<td>79 ±2.309</td>
</tr>
</tbody>
</table>

Standard error mean = SEM

3.8. Effect of silver nanoparticles on the chromosomal pattern

Table 3. shows that the effect of chromosomal aberrations (MI) is inversely proportional to the increase in AgNPs concentrations, which results in the occurrence of chromosomal abnormalities through the appearance of diploid chromosomes and ring chromosomes after treatment with concentrations of silver nanoparticles. This decrease in the indicators of MI and BI came as a result of cell death or stopping at a stage of the interphase and the effect of silver nanoparticles on its division.
Table 3. Chromosomal aberrations induced in peripheral blood lymphocytes (PBLs) treated with AgNPs

<table>
<thead>
<tr>
<th>Concentration µg/mL</th>
<th>BI</th>
<th>MI</th>
<th>TCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>66.22</td>
<td>1.25</td>
<td>0.24</td>
</tr>
<tr>
<td>27</td>
<td>62.12</td>
<td>1.12</td>
<td>0.29</td>
</tr>
<tr>
<td>54</td>
<td>60.51</td>
<td>0.92</td>
<td>0.31</td>
</tr>
<tr>
<td>MTX0.65</td>
<td>16.55</td>
<td>0.11</td>
<td>0.52</td>
</tr>
</tbody>
</table>

BI=Blast Index, MI= Mitotic Index, TCA= Total Chromosomal Aberrations

4. Discussion

In the biosynthesis of silver nanoparticles, the color change of the solution explains the formation of the silver nanoparticles by the enzymes present, such as the nitrate reductase enzyme, which the bacteria secrete out of the cell and which helps in reducing the silver nitrate to nanoparticles (31). The characterization of AgNPs using a UV visible spectrophotometer is one of the most important tests used to verify the formation of silver nanoparticles, and they are detected depending on the extent of the optical absorption between wavelengths (300-900) nanometers to investigate the size of the nanoparticles, which ranges between (2-100) nanometers, as metals of nanoscale have free electrons, and these electrons give (SPR) depending on their size, which is formed as a result of the vibrations of the metal's electrons compared to light waves. And covered by showing (SPR) after the wavelength of 400 nanometers in the process of synthesizing silver nanoparticles using L.gasser filtrate and agree with (32).

was noted from the antioxidant activity of silver nanoparticles that this effect increases with increasing concentration but it remains lower compared to ascorbic acid, this agrees with (33). Plant-mediated AgNPs production has also been investigated, and researchers have shown that antioxidant activity rises gradually as treatment dosages are increased (34). Antioxidants are beneficial in the treatment of illnesses including neurological disorders, cancer, and AIDS because of their scavenging capability (35).

The unique ability of silver nanoparticles in the death of cancer cells has been proven by targeting the structure and function of mitochondria, as silver metal accumulates inside the mitochondria and this affects the respiratory chain of the cell, causing its programmed death (Apoptosis) (36).

Some studies have shown that small-sized silver nanoparticles enter the nucleus, affecting the nuclear chromatin and making the cell dense, which leads to cell shrinkage and death (37). Several studies indicate that the toxic effect on cancer cells results from the active Physico-chemical interaction of
silver atoms with cellular proteins for functional groups in cells, as well as with nitrogenous bases and phosphate groups in DNA, which causes DNA cell death (38).

It has been observed that the increased toxicity of cancer cells is greater than that of normal uninfected cells. Therefore, the use of silver nanoparticles is a typical therapeutic strategy (39).

The decrease in the indicators of MI and BI came as a result of cell death or stopping at a stage of the interphase and the effect of silver nanoparticles on its division, and the reason for this may be the effect of the toxin that binds to cell receptors located in the plasma membrane and leads to the sensitization process and contributes to in the process of responding to these substances and stimulating the systems responsible for the detoxification process (40). The effect of any cleaved material is by activating the genetic material of the cell, as the genes responsible for growth are activated to give orders to enter the mitotic cycle and within a specific period that depends on the cell type and the efficiency of the stimulating material in the presence of silver nanoparticles, the cell will not be able to go through its four phases during the normal period time of division, and it may have exceeded the first mitotic cycle (M1), but it will not be able to pass in the second and third (41).

Conclusions
The present study demonstrated the synthesis of silver nanoparticles using locally L. gasseri. The synthesized silver nanoparticles were characterized by using UV-visible spectrophotometer, FE-SEM, and Fourier Transform Infrared Measurement (FTIR). The antioxidant activity of AgNPswas observed. Silver nanoparticles exhibited cytotoxic activity against SK-GT-4 cancer cells and no effect on normal cells.

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References


