Photo-Biosynthesis and Biological Evaluation of Silver Chloride Nanoparticles Using *Pseudomonas aeruginosa* and *Rhizobium leguminosarum*

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The present investigation reported the photo-extracellular synthesis of silver chloride nanoparticles (AgCl NPs) using *Pseudomonas aeruginosa* NRRL B-800 and *Rhizobium leguminosarum* NRRL B-509 bacterial free supernatants under visible light irradiation at room temperature. AgCl NPs were characterized using UV-Visible Spectroscopy, X-ray diffraction (XRD), Transmission Electron Microscopy (TEM), Scanning Electron Microscopy (SEM), Energy Dispersive Spectroscopy (EDS), Fourier-Transform Infrared Spectroscopy (FTIR). TEM and SEM micrographs revealed the extracellular formation of spherical nanoparticles in size of 42.21 nm and 66.74 nm with *Pseudomonas aeruginosa* and *Rhizobium leguminosarum*; respectively. The synthesized AgCl NPs were evaluated for their antimicrobial activity against 6 food born bacterial pathogens (*E. coli* ATCC 8739, *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 9027, *B. cereus* ATCC 33018 and *S. marcescens* ATCC 13880). The antifungal and antifungal activities of AgCl NPs were evaluated against *Candida albicans* ATCC 10231 and *A. niger* ATCC 16888. AgCl NPs exhibited strong antioxidant and anticancer potential. A possible mechanism has been proposed based on the fast precipitation of AgCl NPs from AgNO3 and NaCl solutions under visible light.

**Keywords:** Extracellular biosynthesis; Silver Chloride Nanoparticles; Photosynthesis; *Pseudomonas aeruginosa* NRRL B-800; *Rhizobium leguminosarum* NRRL B-509; Supernatant

**INTRODUCTION**

Nanoparticles have numerous applications in medicine, agriculture, electronics and industry. For its wide importance and applications, different ways emerged to synthesize nanoparticles. Biosynthesis of nanoparticles is considered a reliable method since it is eco-friendly, economically, non-toxic and relatively simpler comparing with other approaches to synthesize nanoparticles such as physical and chemical methods (Attia et al., 2016). Silver nanoparticles (Ag NPs) research is an important topic since they have a great impact in biomedical applications (Attia et al., 2016; Singh et al., 2017). Ag NPs can be used as powerful antimicrobial agents against several types of microorganisms such as *Salmonella typhi*, *S. aureus* (MRSA), *Staphylococcus epidermidis* (MRSE), *Streptococcus pyogenes*, *Vibrio cholera*, and *Klebsiella pneumonia*, were all sensitive to silver nanoparticles biosynthesized from supernatant of *Staphylococcus aureus*. The toxic effect of the silver nanoparticles was optimal against MRSE, MRSA and *S. pyogenes* (Nanda and Saravanan,
pathogenic bacteria (Bacillus cereus ATCC33018, Staphylococcus aureus ATCC25923, Escherichia coli ATCC 8739, Pseudomonas aerogonosa ATCC 9027 and Serratia marcescens ATCC 13880) along with their antifungal potential against Candida albicans ATCC 10231, antifungal potential against Aspergillus niger ATCC16888 and their antioxidant and anticancer potentials. Utilization of the biosynthesis silver chloride nanoparticles under visible light could add value to the economy of different branches of industrial sectors.

MATERIALS AND METHODS

Chemicals

Silver nitrate (AgNO₃) was purchased from Sigma Aldrich (St. Louis, USA) and media from Oxoid (UK).

Microorganisms

The strains used in the work, are listed in Table 1. Pseudomonas aeruginosa (NRRL B-800) was grown in nutrient broth medium composed of 5 g/L peptone and 3 g/L beef extract, pH adjusted at 7±0.2. Rhizobium leguminosarum (NRRL B-509) was grown in Yeast Extract Mannitol (YEM) medium, which contains 0.1 g/L NaCl, 0.2 g/L MgSO₄, 0.5 g/L K₂HPO₄, 1.0/L g yeast extract and 10 g/L mannitol. pH adjusted at 6.8 ± 0.2. The cultures were grown with shaking at 200 rpm in 250-mL Erlenmeyer flasks at 30 °C. Trypton soy Agar (TSA) medium contains 17 g/L tryptone, peptone 3 g/L, 5 g/L NaCl, 2.5 g/L K₂HPO₄, 2.5 g/L glucose and 6 g/L yeast extract. pH adjusted at 7.3±0.2. TSA was used for studying antimicrobial activity.

Extracellular synthesis of silver chloride nanoparticles (AgCl NPs)

In order to investigate the production of AgCl NPs, Pseudomonas aeruginosa and Rhizobium leguminosarum strains were inoculated in Nutrient broth and Yeast Extract Mannitol, respectively. The cultured flasks were incubated at 25-30°C for 48 hrs in shaking incubator. After the incubation period, the cultures were centrifuged at 10000 rpm for 15 min. The extracts were used for the synthesis of silver chloride nanoparticles (AgCl NPs). A 9 ml of silver nitrate (1mM) was added to a 1 ml of bacterial extract (1 ml) to reach final concentration of approximately 1 mM. The reaction between the extract and silver ions was carried out in the presence of NaCl under visible

Materials and Methods

The present study investigated the synthesis of AgCl NPs using Rhizobium leguminosarum (NRRL B-509) and Pseudomonas aeruginosa (NRRL B-800). In addition, this report evaluates their potential applications as antibacterial compound against a number of 6 foodborne

Additionally, Ag NPs have been found to act as anticancer and antioxidant materials. For example, it was determined that the antioxidant activity of Ag NPs synthesized from Chenopodium murale leaf extract of high effect (Abdel-Aziz et al., 2014) and for in vitro anticancer activity of Ag NPs produced by E. coli VM1 against human lung cancer cell line (A549), human cervical cancer cell line (HeLa) and normal (Vero) cell line (Maharan et al., 2016). Silver chloride (AgCl) is as a resource of silver ions which has potential effect for treating infections. AgCl nanoparticles has a potential toxicity to bacteria because small nanoparticles could pass through cell membranes and the accumulation of intracellular nanoparticles can led to cell malfunction (Morones et al., 2005). Limited methods were applied for the preparation of small size AgCl nanoparticles such as micro-emulsion technique, ultrasound irradiation, mixing silver nitrate (AgNO₃) with hydrochloride acid in the presence of polyvinyl pyrrolidone or matrix-based technique (Abbasi and Morsali, 2013; Bagwe and Khilar, 1997; Kim et al., 2010). The chemical methods may have adverse effects on their applications because it left some potential toxic chemicals adsorbed on the surface (Daniel and Astruc, 2004). Hence, the need to develop environmentally friendly methods like biological extracts to produce safe nanoparticles (Attia et al., 2016). Biological extracts were applied for the synthesis of nanoparticles by adopting simple procedures, involving reduction technique either extracellularly or intracellularly (Rajeshkumar et al., 2013). Therefore, with the purpose of using AgCl as an antibacterial agent, it is necessary to develop a simple method for preparing AgCl nanoparticles. Paulkumar et al., has demonstrated the synthesis of silver chloride nanoparticles by using Bacillus subtilis MTCC 3053 (Paulkumar et al., 2013). Furthermore, the synthesized silver chloride nanoparticles are characterized by XRD, SEM, TEM, EDAX, and FTIR analysis. The enzyme responsible for synthesis of silver chloride nanoparticles is identified by SDS gel electrophoresis. The antifungal activity of silver chloride nanoparticle is examined against Candida albicans Aspergillus niger and Aspergillus flavus.

The present study investigated the synthesis of AgCl NPs using Rhizobium leguminosarum (NRRL B-509) and Pseudomonas aeruginosa (NRRL B-800). In addition, this report evaluates their potential applications as antibacterial compound against a number of 6 foodborne
light irradiation for one hour. The cell free extract without addition of AgNO₃ was used as control.

**Table 1. Bacterial strains used for production of extracellular nanoparticles.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Features</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>Pathogenic bacteria isolated from infection, outer ear, clinical</td>
<td>ATCC - Hegarty, C. P.</td>
</tr>
<tr>
<td>(NRRL B-800)</td>
<td>Gram-negative, rod-shaped bacterium</td>
<td></td>
</tr>
<tr>
<td><em>Rhizobium leguminosarum</em></td>
<td>Symbiotic nitrogen fixing bacteria isolated from pea</td>
<td>Umbreit, W.W. (U Wis)</td>
</tr>
<tr>
<td>(NRRL B-509)</td>
<td>Gram-negative, rod-shaped bacterium</td>
<td>Accession numbers in other collections: Pea 302</td>
</tr>
</tbody>
</table>

The thermal stability of the formed AgCl NPs was studied and measured by UV-Vis spectrophotometer at different temperatures (Room temperature, 40, 50, 60, 70 and 80 °C) at pH 7.0.

**Antimicrobial activity determination**

After 24 hr of production of AgCl NPs by *Rhizobium leguminosarum* and *Pseudomonas aeruginosa* extracts, disk diffusion method was conducted to study the antimicrobial effect of the synthesized extracellular AgCl NPs against Gram-negative bacteria such as *E. coli* (ATCC 8739) and *Pseudomonas aeruginosa* (ATCC 9027) and gram positive bacteria such as *Staphylococcus aureus* (ATCC 25923) and *Bacillus subtilis* (ATCC 6633). Also, the antimicrobial activities were investigated against *Candida albicans* (ATCC 10231) and *Aspergillus niger* (ATCC 16888). Saturated disks (6 mm in diameter) of extracellular AgCl NPs from *Rhizobium leguminosarum* and *Pseudomonas aeruginosa* were used on tryptone soy agar (TSA) medium inoculated with test organisms in triplicate. Plates were incubated for 24 – 48 hrs at the ambient temperature.

**Antioxidant of biosynthesized AgCl NPs**

DPHP method was applied to determine the antioxidant activity of the produced extracellular AgCl NPs. Preparation of different concentration of AgCl NPs samples (40 – 50 – 80 – 100 - 200 µg/ml) according to (Elslimani et al., 2013). The antioxidant potential of the AgCl NPs was determined by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging. The DPPH free radical scavenging potential of AgCl NPs was determined as previously described. Briefly, five different concentrations (40–200 µl/ml) of AgCl NPs and ascorbic acid (ASA) as the standard reference compound were assayed. The absorbance of the reaction mixtures was recorded at 517 nm using the microplate reader and the results were interpreted as the percentage scavenging according to the following equation:

\[
\text{Percentage scavenging} = \frac{\text{Abs}_c - \text{Abs}_t}{\text{Abs}_c} \times 100
\]

where, \(\text{Abs}_c\) is the absorbance of the control and \(\text{Abs}_t\) is the absorbance of the treatment (Gopinath et al., 2013).

**Anticancer of biosynthesized AgCl NPs**

Neutral red dye was used to stain the viable cells in order to determine the viability/cytotoxicity rate. This method relies on the uptake of neutral red into lysosomes of living cells. A549 lung and MCF7 breast cancer cells were grown in 96-well plate for 24 hrs using DMEM media supplemented with bacterial extract (containing AgCl NPs) at 37°C. Cells were then collected and treated with a neutral red-containing medium (neutral red concentration: 40 mg/ml) and incubating overnight. After washing cells with PBS, neutral red was added again and incubated for 1-2 hours at 37°C. Neutral red distaining was carried out after washing the neutral red. Finally, spectrophotometer analysis was performed to calculate the viability/cytotoxicity ratio and was confirmed by microscopic examination (Paulkumar et al., 2013).

**Characterization of AgCl NPs**

UV-Vis spectra of the prepared AgCl NPs were measured using a Perkin Elmer Lambda 40 UV-Visible spectrophotometer using 1 cm path length Hellma quartz cuvettes. Transmission Electron Microscopy (TEM) images were obtained using a JEOL JEM-1400 electron microscope operated at 100 kV. A drop from diluted sample dispersion was deposited onto an amorphous carbon film on 400 mesh copper grids and left to evaporate at room temperature. XRD
measurements were performed using a Philips PW1710 X-ray diffractometer using Cu Ka radiation (\(\lambda = 1.54186 \text{ Å}\)). The XRD patterns were recorded from 20 to 701 with a step size of 0.02012\(^\circ\) and collecting 10 s per step. A halogen lamp (HALOPAR 20 75W 230 V 301 GU10, Italy) was used for the extract preparation. Samples for scanning electron microscope was applied using SEM Model Quanta 250 FEG (Field Emission Gun) attached with EDX Unit (Energy Dispersive X-ray Analyses), with accelerating voltage 30 KV, magnification of 1x up to 1000000x and resolution for Gun.1n). The FTIR spectrum of AgCl NPs was studied using FTIR spectrophotometer Jasco 4100 FTIR. The spectrum was recorded at a resolution of 4 cm\(^{-1}\) in the range of 450–4000 cm\(^{-1}\). Gas Chromatography (GC) analysis was performed using Agilent Technologies 5977A MSD, Agilent Technologies 7890B GC System. The oven temperature was maintained initially at 40°C for 3 min, and then programed to 250°C at a rate of 15°C/min. Total run time was 19 min. Helium was used as the carrier gas at Septum Purge flow rate of 3 ml/min with a split ration of 75:1. Chloroform was used as the solvent in all GC experiments.

**RESULTS AND DISCUSSION**

**Silver chloride nanoparticles production from microbial extracts**

The photo-extracellular synthesis of silver chloride nanoparticles is primarily characterized by UV-vis spectroscopy, which is a favorable technique to analyze the formation of nanoparticles. *Pseudomonas aeruginosa* and *Rhizobium leguminosarum* strains were screened for the production of AgCl NPs using their extracellular (cell free supernatant) of bacterial cultures. Upon adding silver nitrate (1mM) to the supernatant of bacterial strains (*Pseudomonas aeruginosa* and *Rhizobium leguminosarum*) under direct light conditions with halogen lamp for 1 hour at room temperature and neutral pH, change in color was observed from colorless to yellowish brown. The formation of AgCl nanoparticles was monitored by measuring optical density at wavelength range 300 - 800 nm of the solution using UV-vis-spectrophotometer. Figure 1 shows strong band at 423 nm with small peak at 258nm (Figure 1a) and at 428nm with small peak at 281nm (Figure 1b) proved the formation of AgCl nanoparticles in the range size 1-100nm. The presence of chloride ions in the extract might be a responsible source for the formation of silver chloride nanoparticles. Generally, the silver nitrate is used as a precursor for the synthesis of silver nanoparticles, whereas, herein, the chloride ions from sodium chloride in the culture medium bound to the silver metal ions under visible light irradiation for 1 hour and form silver chloride nanoparticles (Jain et al., 2011; Pal et al., 2007). After observation of change of color as indication of production of nanoparticles, GC-MS was performed in order to analyze the compounds of the extract that contributes the formation of AgCl nanoparticles. Eicosane, Heneicosane and Isonipeotic acid N-(cyclohexylcarbonyl) heptyl ester compounds were detected in high concentrations by GC-MS analyses for *Pseudomonas aeruginosa* extract. *Rhizobium leguminosarum* cell free extract was also analyzed by GC-MS, resulting in identification of certain compounds, which were Eicosane, heptadecane, carbonic acid decyl hexadecyl ester and phenol 2,5-bis(1,1-dimethylthyl). These compounds act as capping and reducing agents in the formation of AgCl NPs. Figure 2, FTIR spectrum of *Pseudomonas aeruginosa* extract shows different absorption peaks at 3431.71, 1632.45, 1044.26 and 586.254 cm\(^{-1}\). Hydroxyl group appeared at band 3431.71 cm\(^{-1}\) and distinct peak in the region 1632.45 cm\(^{-1}\) demonstrates secondary amines and aromatic groups of N-H and C=C stretches. Alkyl halides found at bands 1044.26 and 586.254 cm\(^{-1}\) for C-F and C-CI bonds respectively. While in *Rhizobium leguminosarum* extract, FTIR showed also different absorption peaks at 3406.64, 1632.45, 1379.82, 1089.58, 1024.02, and 714.497 cm\(^{-1}\). Peak in 3431.71 cm\(^{-1}\) could be due to the stretching vibration of the C-OH bond of proteins in the bacterial extract. Band appeared at 1632.45 cm\(^{-1}\) indicates C=C and N-H groups of alkenyl stretch and secondary amines. C-H bond showed by band 879.381 cm\(^{-1}\) indicates alkenes and alkyne, and other band appeared at 1089.58 cm\(^{-1}\) specifies C-H and C-O-C groups. Marked peak is at 1024.02 cm\(^{-1}\) indicates C-N bond which can be contributed to aliphatic amines or to a combination of alcohols and phenols. Peak in the range of 700 cm\(^{-1}\) relates to the alkyl halides band especially the carbon-chlorine bond. These structures of the changed active compounds revealed the presence of reducing and stabilizing compounds in the bacterial extracts. From these data, a possible mechanism for the AgCl NPs formation has been proposed based on the photosensitization of the extract compounds in the presence of AgNO\(_3\) solution and Cl ions from the culture media (Attia et al., 2016). TEM and SEM
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Spherical nanoparticles were shown by the images of both TEM and SEM produced by the bacterial extracts.

**Figure 1.** UV-Visible absorption spectra of biosynthesized AgCl NPs extracted from cell-free extract of two different bacterial strains; (A) *Pseudomonas aeruginosa* and (B) *Rhizobium leguminosarum* after 1 hour of direct light.

**Figure 2.** FTIR of cell-free extract of two different bacterial strains; (A) *Pseudomonas aeruginosa* and (B) *Rhizobium leguminosarum*. 
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**Figure 3.** Transmission electron microscope and scanning electron microscope examination of biosynthesized NPs extracted from cell-free extract of two different bacterial strains; (A-B) *Pseudomonas aeruginosa* and (C-D) *Rhizobium leguminosarum*.

Figure (3A and 3B) show that AgCl NPs formed by *Pseudomonas aeruginosa* were in nano size 42.21±2.32 nm by TEM and SEM, respectively. However, Figure (3C and 3D) show that nanoparticles formed by *Rhizobium leguminosarum* were in a range from 66.74±3.60 nm by TEM and SEM, respectively (Jain et al., 2011). The phenomenon of crystalline structure of the dried biosynthesized nanoparticles was identified by X-Ray Diffractometer to confirm the morphology of the as-prepared particles, as shown in figures 4 (A&B). The prepared nanoparticles mainly show XRD patterns located at (2θ= 27.5°, 32.15°, 46.14°, 54.7°, 57.3° and 76.6°) corresponding to the formation of AgCl crystallographic planes {(111), (200), (220), (311), (222) and (420)} (JCPDS 31-1238). The diffraction peaks of the samples are intense and sharp, which confirms that the obtained AgCl nanoparticles are crystalline. The data was compared to the database of Joint Committee on Powder Diffraction Standards (JCPDS) file no. (31-1238) (Gopinath et al., 2013). The average size ranged from (43.231-46.754) nm for *Pseudomonas aeruginosa* and (64.691-69.4764) nm for *Rhizobium leguminosarum* as confirmed by calculation using Scherrer equation.

\[D_{\text{50}} = \frac{K \lambda}{B \cos \theta}\]

Where \(D_{\text{50}}\) is equivalent of particles average core diameter; \(K\) is the grain shape factor; \(\lambda\) is the...
incident X-ray wavelength; $\beta$ denotes the full width at half-maximum (in radians) of the highest intensity 100 powder diffraction reflection, $\theta$ is the corresponding diffraction angle.

Figure 4. XRD pattern of the formed AgCl nanoparticles by using the bacterial extracts under visible light. (A) *Pseudomonas aeruginosa* and (B) *Rhizobium leguminosarum*.

Figure 5. EDX of synthesized AgCl nanoparticles (A) *Pseudomonas aeruginosa* and (B) *Rhizobium leguminosarum*.
Ibrahim et al., Synthesis of AgCl NPs using Pseudomonas aeruginosa and Rhizobium leguminosarum

The chemical composition of AgCl NPs was analyzed by an energy dispersive spectrometer (EDS) spectrum via SEM (Fig. 5 A&B). The results show the peaks of AgCl, Cl and Ag, confirming the high purity of AgCl nanoparticles sample (Gopinath et al., 2013). Thermal stability of the formed AgCl NPs was checked under different temperatures. The change in absorption and color intensity were determined spectrophotometrically at different temperatures (room temperature, 40, 50, 60, 70 and 80 °C) at pH 7.0 as indicated in Figure (6). The thermal stability of the produced AgCl nanoparticles from Rhizobium leguminosarum is greater than that formed from Pseudomonas aeruginosa extract along with the change of temperature.

Biological applications of biosynthesized silver nanoparticles

Antimicrobial activity determination

There are enormous reports available to exemplify the antibacterial and antifungal property of the silver nanoparticles. Very few reports are available to explain the antifungal and antibacterial property of silver chloride nanoparticles. Thus, the present investigation demonstrates the biosynthesis, antibacterial and antifungal property of silver chloride nanoparticle. The AgCl NPs saturated disk displayed admirable antibacterial activity against all 6 foodborne pathogenic bacteria, as indicated by diameter of inhibition zones of 7-14 mm (Table 2 and Table 3). Among the pathogenic bacteria, AgCl NPs produced by Rhizobium leguminosarum were more active against E.coli ATCC 8739 and P. aeruginosa ATCC 9027 (13 and 14 mm inhibition zone, respectively) than AgCl NPs produced by Pseudomonas aeruginosa against E.coli ATCC 8739 and P. aeruginosa ATCC 9027 (7.5 and 12.6 mm inhibition zone, respectively). They displayed potential antifungal and antifungal activities against Candida albicans ATCC 10231 and A. niger ATCC16888 with zones of inhibition ranging from 14.3 and 13 mm in case of AgCl NPs produced by Pseudomonas aeruginosa (Table 2) and from 10 and 14 mm in case of AgCl NPs produced by Rhizobium leguminosarum (Table 3). In that, the Candida albicans and Aspergillus niger are more sensitive to silver chloride nanoparticles. Therefore, synthesized silver chloride nanoparticle is a good source which is easily produced and extensively useful in biomedical application. Gopinath et al., reported the antibacterial activity of silver chloride nanoparticles against Gram-positive bacteria Staphylococcus aureus and Streptococcus pyogenes and Gram-negative bacteria E. coli and Proteus vulgaris (Gopinath et al., 2013). There are no many previous reports available to explain the possible mechanisms of antifungal activity of silver chloride nanoparticles. Only, Kanniah et al., reported the occurrence of cellular proteins in B. subtilis which was extracted by ammonium precipitation method and the molecular weight of the protein is identified by
SDS-PAGE (Paulkumar et al., 2013). In that, lane 1 contains the marker protein and lane 2 loaded with extracellular protein exhibits an intense band with the molecular weight of 37 kDa that might be the nitrate reductase enzyme which is responsible for synthesis of silver chloride nanoparticles.

**Table 2. Antimicrobial effect of silver chloride nanoparticles produced from Pseudomonas aeruginosa NRRL B-800 (Disc diameter: 6 mm)**

<table>
<thead>
<tr>
<th>Test microorganism</th>
<th>Diameter of inhibition zone (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> ATCC 8739</td>
<td>7.5 ± 0.4</td>
</tr>
<tr>
<td><em>B. subtilis</em> ATCC 6633</td>
<td>11.0 ± 0.81</td>
</tr>
<tr>
<td><em>S. aureus</em> ATCC 25923</td>
<td>12.6 ± 0.47</td>
</tr>
<tr>
<td><em>Candida albicans</em> ATCC 10231</td>
<td>14.3 ± 2.62</td>
</tr>
<tr>
<td><em>P. aerogonosa</em> ATCC 9027</td>
<td>12.6 ± 0.47</td>
</tr>
<tr>
<td><em>B. cereus</em> ATCC 33018</td>
<td>7.0 ± 0.0</td>
</tr>
<tr>
<td><em>A. niger</em> ATCC 16888</td>
<td>13.0 ± 0.0</td>
</tr>
<tr>
<td><em>S. marcescens</em> ATCC 13880</td>
<td>8.3 ± 0.47</td>
</tr>
</tbody>
</table>

**Table 3. Antimicrobial effect of silver chloride nanoparticles produced from Rhizobium leguminosarum NRRL B-509 (Disc diameter 6 mm)**

<table>
<thead>
<tr>
<th>Test microorganism</th>
<th>Diameter of inhibition zone (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> ATCC 8739</td>
<td>13.0 ± 1.41</td>
</tr>
<tr>
<td><em>B. subtilis</em> ATCC 6633</td>
<td>8.0 ± 0.0</td>
</tr>
<tr>
<td><em>S. aureus</em> ATCC 25923</td>
<td>12.6 ± 0.94</td>
</tr>
<tr>
<td><em>Candida albicans</em> ATCC 10231</td>
<td>10.0 ± 0.81</td>
</tr>
<tr>
<td><em>P. aerogonosa</em> ATCC 9027</td>
<td>14.0 ± 0.0</td>
</tr>
<tr>
<td><em>B. cereus</em> ATCC 33018</td>
<td>6.8 ± 0.49</td>
</tr>
<tr>
<td><em>A. niger</em> ATCC 16888</td>
<td>14.0 ± 0.81</td>
</tr>
<tr>
<td><em>S. marcescens</em> ATCC 13880</td>
<td>6.2 ± 0</td>
</tr>
</tbody>
</table>

**Table 4. Antioxidant effect of biosynthesized AgCl NPs**

<table>
<thead>
<tr>
<th>Sample I.D.</th>
<th>Sample Concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>40</td>
</tr>
<tr>
<td>Treatment 1</td>
<td>57.767</td>
</tr>
<tr>
<td>Treatment 2</td>
<td>47.960</td>
</tr>
<tr>
<td>Control 1</td>
<td>0</td>
</tr>
<tr>
<td>Control 2</td>
<td>0</td>
</tr>
</tbody>
</table>

Treatment 1 = *Pseudomonas aeruginosa* nanoparticles treatment
Treatment 2 = *Rhizobium leguminosarum* nanoparticles treatment
Control 1 = Only Extracellular supernatant of *P. aeruginosa*
Control 2 = Only Extracellular supernatant of *R. leguminosarum*
DPPH = Chemical compound 2,2-diphenyl-1-picrylhydrazyl

Similarly, Jain et al., has achieved that the presence of 32 kDa reductase enzyme in *Aspergillus flavus* NJP08 acts as a reducing agent for the production of silver chloride nanoparticles from silver ions (Jain et al., 2011; Pal et al., 2007). In addition, the stationary phase is the elongation phase of bacterial cells and it could be considered as an active phase for the production of silver chloride nanoparticles.

**Antioxidant effect of biosynthesized AgCl NPs**

The antioxidant effect of AgCl NPs produced from *Pseudomonas aeruginosa* and *Rhizobium leguminosarum* was measured. Different concentrations (40, 50, 80, 100, 200 µg/ml) of AgCl NPs were tested. The DPPH scavenging potential of AgCl NPs determines the potency of antioxidants. Our data show that increasing the biosynthesized AgCl NPs leads to increase in DPPH % radical scavenging activity (RSA), indicating more effective antioxidants. AgCl NPs produced from *Pseudomonas aeruginosa* show a great potential to be used as a powerful antioxidant material with IC₅₀ 11.562, since 200
µg/ml of AgCl NPs results in 70% RSA. For *Rhizobium leguminosarum* AgCl NPs, 200 µg/ml concentration results in 50% RSA with IC₅₀ 122.529 Therefore, we conclude that AgCl NPs biosynthesized from the two bacterial strains show antioxidant effect, with a greater potential for AgCl NPs biosynthesized from *Pseudomonas aeruginosa* (see figure 7).

Figure 7. Antioxidant analysis of biosynthesized AgCl NPs extracted from cell-free extract of two different bacterial strains; *Pseudomonas aeruginosa* and *Rhizobium leguminosarum*.

A) Lung cancer (A549) cell line

B) Breast cancer MCF7
Anticancer effect of biosynthesized AgCl NPs

The anticancer or cytotoxicity effect of biosynthesized AgCl NPs from *Pseudomonas aeruginosa* and *Rhizobium leguminosarum* extracts were determined. Two cancerous cell lines were used in this experiment; which are lung cancer (A549) cell line and breast cancer (MCF7) cell line. These two cell lines were treated with AgCl NPs extract with different concentrations (75, 100, 200, 300, 400, 500, 600 µg/ml) (See figure 8). Generally, the data showed as expected that the cell viability decreased with increasing AgCl NPs concentration. In A549 cell line, continuous decline of cell viability was observed, when cells were treated with increasing concentrations of AgCl NPs extract of *Pseudomonas aeruginosa*, much faster and efficient than AgCl NPs extract of *Rhizobium leguminosarum*. For instance, cell viability of A549 cell line was zero % at AgCl NPs extract (600 µg/mL) from *Pseudomonas aeruginosa* while it was ~35% at AgCl NPs extract from *Rhizobium leguminosarum* (Fig. 8A). Similarly, in breast cancer MCF7 cell line, AgCl NPs extract of *Pseudomonas aeruginosa* has a significant influence comparing with their counterpart of *Rhizobium leguminosarum*. Almost all cells were severely affected and showed no viability when they were treated with AgCl NPs extract from *Pseudomonas aeruginosa* at a low concentration of 75 µg/ml, while at the same concentration cells show full viability to AgCl NPs extract from *Rhizobium leguminosarum*. This means that AgCl NPs of *Pseudomonas aeruginosa* has a very robust cytotoxic and anticancer effect against MCF7 cells. Furthermore, dramatic change was observed in MCF7 cells treated with AgCl NPs extract of *Rhizobium leguminosarum* when the concentration was increased into 200 µg/ml as cell viability changed from 100% into 11.4% until reaching to maximum cell death at concentration 600 µg/ml by 3.9 % viability (Fig. 8B). This indicated that the cell extract of *Rhizobium leguminosarum* has to be used in a relatively high concentration to show its cytotoxic/anticancer effect on MCF7 cells (Repetto et al., 2008).

CONCLUSION

The AgCl NPs that prepared using this biosynthesized approach exhibited maximum absorbance at 423 nm and revealed the extracellular formation of spherical nanoparticles in size of 42.21 and 66.74 nm with *Pseudomonas aeruginosa* and *Rhizobium leguminosarum* extracts, respectively. The EDS and XRD Spectra were confirming a high purity of AgCl NPs. The thermal stability of the produced AgCl NPs from *Rhizobium leguminosarum* is greater than that formed from *Pseudomonas aeruginosa*. The FTIR spectral analysis revealed the presence of reducing and stabilizing compounds in that bacterial extracts. Among the pathogenic bacteria, AgCl NPs produced by *Rhizobium leguminosarum*...
were more active against *E. coli* ATCC 8739 and *P. aeruginosa* ATCC 9027 (13 and 14 mm inhibition zone, respectively) than AgCl NPs produced by *Pseudomonas aeruginosa* against *E. coli* ATCC 8739 and *P. aeruginosa* ATCC 9027 (7.5 and 12.6 mm inhibition zone, respectively). They displayed high antifungal and antifungal activities 14.3 and 13.0 mm inhibition zone against *Candida albicans* and *A. niger*, respectively. The AgCl NPs exhibited strong antioxidant and anticaner potential. This study is the first report on the phot-biosynthesis of AgCl NPs using cell free extract of *Pseudomonas aeruginosa* and *Rhizobium leguminosarum*. We present a clean, easy and cheap biosynthesis of AgCl NPs using two microbial strains that have the advantages to exhibit anticancer, antibacterial, antifungal, antifungal and antioxidant activities.

**CONFLICT OF INTEREST**

The present study was performed in absence of any conflict of interest.

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**AUTHOR CONTRIBUTIONS**

All authors contributed equally in all parts of this study.

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