SYNTHESIS AND ANTIMICROBIAL ACTIVITIES OF GOLD NANOPARTICLES AGAINST SALMONELLA SER. TYPHI

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Abstract

Salmonella ser. Typhi is common pathogenic bacteria found in the environmental reservoirs, responsible for major disease outbreaks. Prevalence of drug-resistant strains of Salmonella ser. Typhi in environment is alarming for human health. The present study aims to generate drug-resistance profiling of Salmonella ser. Typhi, isolated from potable water of Gwalior and develop an alternative antimicrobial agent. Gold nanoparticles (AuNPs) were synthesized by chemical approach and characterised by UV-visible spectroscopy and Electron Microscopy. The morphology of nanoparticles was found to be quite spherical within the size range of 20-30 nm. Synthesized AuNPs were used as antimicrobial agent against drug-resistant Salmonella ser. Typhi. Study revealed that the synthesized AuNPs were effective against Salmonella ser. Typhi. The study can further be used in development of a potential antimicrobial agent against drug-resistant pathogenic bacteria.

Key words: Salmonella ser. Typhi, Gold nanoparticles, Antimicrobial resistance and Minimum Inhibitory Concentration.

Introduction

Contamination of safe water is a serious public-health problem in rural-urban interface. Deterioration in the microbiological quality of water often leads to water-borne disease outbreaks, which adversely effects human health. Water is said to be regenerative, as it can absorb pollution loads up to the certain levels without affecting its quality, beyond which water is harmful to both human and non-humans (Reynolds et al., 2008, Ercumen et al., 2014, Dickin et al., 2016).

Salmonella ser. Typhi, causative agent of typhoid fever, is one of the prevalent pathogens in potable water. Typhoid results nearly 22 million cases and 216,500 deaths per year in Asia, especially countries like India exhibit high load for typhoid fever (Date et al., 2014). Typhoid is a communicable disease and spreads in India by different ways. The bacterium can spread through typhoid patients, carried in large quantity by stool and vomit. It further travels to water bodies by different means. After consumption of contaminated water, the human population gets exposed to the bacterium and results in typhoid fever. In countries of South Asia, particularly Southeast Asia, typhoid fever is very much prevalent. India falls in the category of zone of typhoid by Salmonella ser. Typhi (CDC, 2017). Outbreaks of Salmonellae infections have been reported due to consumption of contaminated foods including vegetables, egg etc. According to CDC Report of 2014 ciprofloxacin resistance is developing in Salmonella ser. Typhi strains.

Classical methods for the identification of pathogens include pre-enrichment and biochemical assays (Law et al., 2015, Biswas et al., 2016). Polymerase Chain Reaction (PCR), being specific and sensitive have been established as the gold-standard for detection of pathogenic bacteria. Salmonellae isolate harbouring invA gene has been identified in potable water using molecular methods (Jyoti et al., 2010, Agarwal et al., 2017).

The indiscriminate use and easy availability of antibiotics without prescription leads to the dissemination of drug resistance among Salmonellae to most of the available antibiotics (Lima et al., 2013). Combination of antibiotics with non-antibiotic drug seems to be a new choice to overcome bacterial resistance (Farha et al., 2013). As a result, the drug-resistant (DR) water-borne
Salmonella ser. Typhi are prevalent in environment and spread worldwide, resulting in high rate of morbidity and mortality. This leads to necessity for the development of potential new alternative materials in order to combat this problem. Novel and effective antibiotic synthesis and implementation has many roadblocks including higher cost, time and shorter life cycle (Ventola, 2015). The development of antibiotics is indeed expensive, time consuming, risky and is also unattractive because of their short life cycle and rapid bacterial resistance. Therefore, the development of a novel antimicrobial agent which can overcome drawbacks of existing antibiotics and can work against Salmonella ser. Typhi is an urgent need.

Nanomaterials are materials ranging from 1-100 nm (FDA, 2014). They possess exceptional properties as compared to their corresponding bulk materials and have successful impact on biology and medicine (Azam et al., 2012). Smaller dimension and high surface area are the outstanding properties of metallic nanoparticles (NPs) which allow them to actively interact with various recognition elements in biological system (Spivak et al., 2013, Shrivastava et al., 2019). At nano-scale size, the surface electrons become active which results into unique optical and mechanical properties (Guo et al., 2014). Nanoparticles are frequently used in different bio-applications such as therapeutics and also as antimicrobial agents (Ranghar et al., 2014) due to their high bio-availability. The interaction of Silver nanoparticles and their effectiveness are based on the different types of capping agents used (Ganandhas et al., 2013). Several metallic nanoparticles, including Silver and Gold are able to inactive bacterial enzymes. Therefore, nanoparticles in synergy with antibiotics could be more effective as antimicrobial agent (Hari et al., 2014). The additive effect can be seen in antibiotics against several bacterial strains (Bhardwaj et al., 2016).

**Materials and Methods**

**Bacterial Culture**

Potable water samples were collected from pre-identified sites of Gwalior. Each sample was filtered using membrane filtration technique. An aliquot of 500 ml sample from each site was concentrated and re-concentrated and brought up to 500 µL using repetitive centrifugation at 18000 x g for 10 min at 4°C. This was followed by spreading the concentrated sample on Hi-Chrome Improved Salmonella Agar (Hi-media, Mumbai) and incubated for 18 h. Colonies showing pink colour on Hi-Chrome media were preliminary confirmed as Salmonella spp. and were preserved -70°C, as glycerol stock (15% v/v) for further characterization. Salmonella ser. Typhi MTCC 733, procured from Microbial Type Culture Collection (IMTech, Chandigarh) was used as positive control.

**Isolation of Genomic DNA**

Genomic DNA was isolated from the sample by boil prep method. A 500 µL of concentrated water (after repetitive centrifugation) was boiled in a water bath at about 90°C. The debris of lysed cells was removed by centrifugation at 7000 x g for 4 min at 4°C. DNA was precipitated using boil prep method (Jyoti et al., 2010). The extracted DNA was in kept 100 µL TE (pH 8.0) for further use.

**Identification of Salmonella ser. Typhi**

Salmonellae isolated from different sites of Gwalior city were further revived for the molecular characterization. Specific signature genes were selected for the molecular characterization of Salmonellae. The target virulent gene invA was found to be highly conserved among wide Salmonella species and almost in all serovar.

**Primers**

To detect Salmonellae harbouring invA gene in drinking water samples, primers (Table 1) were adopted from Jyoti et al., 2010.

**Detection of Salmonellae using PCR**

Genomic DNA from pure cultures and environmental isolates were prepared and purified. In order to detect the signature gene invA in isolated strains, Polymerase Chain Reaction was performed using specific primers. The PCR assay was performed in 50 µL reaction mixture using 2x Hi-media PCR kit, containing dNTPs (200 µM), Taq polymerase, 10 X reaction buffer, MgCl₂, primer pairs (0.4 µM each) and DNA (5 µl). The optimised temperature conditions were as: initial denaturation for 3 min at 95°C and then 30 cycles at 95°C for 30s, 54°C for 45s, and 72°C for 45s. All the assays were done in triplicate. Purified multigenomic DNA (5 µL) from environmental samples were also used as template. Post PCR the amplicons were run on agarose gel containing ethidium bromide. Salmonella ser. Typhi MTCC 733 was used as the positive control for invA gene.

**Molecular Characterization for identification of Salmonella ser. Typhi**

**Primers**

In order to detect Salmonella ser. Typhi, in drinking water samples (samples were first confirmed for Salmonellae and were then check for Salmonella ser. Typhi), PCR reactions were performed harbouring signature genes-ViaB, FliC-D and Prt. Primer pair for
all the genes were adopted from Kumar et al., 2006.

Detection of Salmonella ser. Typhi Using PCR

Genomic DNA isolated from water samples after biochemical characterization was further identified using PCR. Specific primers of virulent signature gene Via B, Fli-D & Prt genes of Salmonella ser. Typhi were used for the identification of bacteria and were followed by Kumar et al., 2006 with slight modifications. PCR was performed in 50 µL reaction volume containing 0.2 µM of each primer pair (separate for each reaction), 200 µM of each dNTPs, 1 unit of Taq DNA polymerase, 2.5 mM MgCl₂, 1X PCR reaction buffer and 5 µl of template DNA. PCR was run using optimised temperature conditions as: denaturation at 94°C for 30s, annealing temperature was varied in all the cases, (Via B gene: 56°C for 45 s, Flic-D gene 60°C for 45 s and Prt gene 62°C for 45 s and extension at 72°C for 30 s. PCR was performed through 35 cycles in Gradient Thermo Cycler (Bio-Rad Laboratories, Hercules, CA, USA). Amplicon were analysed on 1.8% agarose gel and observations recorded. Salmonella Typhi MTCC 733 was used as positive control for Via B, Fli-D and Prt gene.

Drug resistance profiling for Salmonella ser. Typhi

Drug resistance profiling was done at phenotypic level using disc diffusion method. After confirmation, they were further checked for drug resistance at genotypic level, using antibiotic resistance genes. Antibiotic resistance gene was first checked on NCBI Blast and then they were checked for specificity and selectivity.

Phenotypic Characterization

Drug-resistance profiling was done for the identified isolates of Salmonella Typhi against several antibiotics of major classes as per Clinical and Laboratory Standards Institute (CLSI) guidelines. These are Clindamycin (10 µg/disc), Cefoxitin (30 µg/disc), Ciprofloxacin (5 mcg/disk), Chloramphenicol (30 mcg/disk), Fosfomycin (200 mcg/disk), Aztreonain (30 mcg/disk), Ampicillin/sulbactem (10 mcg/disk), Ertapenem (10 mcg/disk), Meropenem (10 mcg/disk), Amikacin (30 mcg/disk), Doriopenem (10 mcg/disk), Amoxyclav (30 mcg/disk), Trimethoprim (5 mcg/disk), Ceftazidime (10 mcg/disk), Cefuroxime (30 mcg/disk), Cefaxolin (30 mcg/disk). Each test was performed in triplicate. Data for drug-resistance were recorded as resistant (R), intermediate (I) or sensitive (S), based on CLSI guidelines. Overnight culture was spread onto Mueller Hinton (MH) agar medium plate and antibiotics were placed on the MH agar plates followed by overnight incubation at 37 °C.

Synthesis of Gold Nanoparticles

Synthesis of Gold nanoparticles was achieved by reducing auric chloride (HAuCl₄) with sodium borohydride (NaBH₄). In an Erlenmeyer flask, add 18.5 ml of de-ionized water and kept the flask on magnetic stirrer, then add 0.5 ml of 0.01 M HAuCl₄, stir it properly so as to maintain homogeneous mixing. After proper mixing add 0.5 ml of 0.01M Sodium citrate (addition of sodium citrate should be very precise, a small drop rate should be maintained throughout the addition) and then keep it for constant stirring for 30 min at 15°C. During the Au NPs synthetic process, the solution colour changed from yellow to pink then to red wine in colour, indicating the formation of AuNPs. AuNPs were kept for overnight in dark at room temperature, so as to settle the nanoparticles and then collecting then by centrifugation at 15000 rpm for 15 min. A repetitive wash was done with de-ionized water and pellet was collected and particles were dried in oven at 55°C.

Characterisation of Nanoparticles

Synthesised nanoparticles were characterised using various biophysical techniques. Size, concentration, shape and crucial factors for optical properties of gold nanoparticles. UV-Visible spectroscopy was used to observe the spectra of synthesised gold nanoparticles.

Morphology and size of the NPs was determined by Electron Microscope at the 120 KV (JEOL 2000). Samples after preparation and preliminary characterization were sent to sophisticated test and instrumentation centre (STIC), Cochin, Kerala. The size and particle distribution of nanoparticles was confirmed using transmission electron microscopy (TEM). Briefly, the sample was prepared by placing a drop of collected nanoparticles on a carbon coated copper grid and subsequently drying the sample in an oven at 60°C before transferring it into microscope and the size and morphology was characterized by TEM (JEOL 2000, STIC Cochin, Kerala).

In-vitro experiment for antimicrobial activity of nanoparticles against Salmonella ser. Typhi

Salmonella ser. Typhi MTCC 733 was grown at 37°C in Luria Bertani broth. Various concentrations of gold nanoparticles (0, 10, 20, 30, 40, 50 and 100 µg/mL) were added to the grown bacterial culture (~1 x 10⁸ CFU), followed by incubation at 37°C for 16-20 h in shaking incubator at 120 rpm. Bacterial cultures showing poor growth were spread (100 µL aliquots) onto the control MH agar plates (without any antibiotics) to further examine the culturability of culture and bactericidal effect of nanoparticles.

Estimation of Minimum inhibitory concentration for Salmonella ser. Typhi using Agar dilution method

MIC and MBC were evaluated by agar dilution method.
method. Agar dilution was followed using Wiegand et al. 2008. Briefly, the Muller Hinton Agar (MHA) was prepared and sterilized. After autoclaving, the AuNPs in different concentrations were added before the agar solidified. This was followed by mixing the bacterial suspension (~1 x 10^6 CFU/mL). The surface of the agar along with bacterial culture was dried and incubated at 37°C for 20 h. Grown colonies after incubation were counted next day.

Results and Discussion

Isolation of Salmonellae

Environmental water samples were analysed for the presence of Salmonellae. Salmonellae were confirmed primarily on the basis of colour of each individual colony on Hi-Chrome Salmonella Agar.

Molecular Characterisation of Salmonellae

Positive samples were further analysed using molecular method for the presence of Salmonellae. The 147 bp amplicon was observed on gel electrophoresis (Fig. 1). Results revealed that Salmonellae strains isolated from environmental sample exhibited virulent gene invA. The present study suggests the prevalence of Salmonellae in environmental potable water samples. Similar observations were also reported in previous studies (Jyoti et al., 2010 and Agarwal et al., 2015).

Molecular characterisation for identification of Salmonella ser. Typhi

Table 1: Nucleotide sequence of candidate oligomers of invA gene of Salmonellae.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence (5-3)</th>
<th>Tm(°C)</th>
<th>Amplicon</th>
</tr>
</thead>
<tbody>
<tr>
<td>invA</td>
<td>Forward: 5’-CGCACCGTCAAAGGAACC-3’</td>
<td>56.8</td>
<td>147 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-GCCCGATTTTTCTCTGGATGG-3’</td>
<td>56</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Nucleotide sequence of candidate oligomers of via B, FliC-D and Prt genes of Salmonella ser. Typhi.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence (5-3)</th>
<th>Tm(°C)</th>
<th>Amplicon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Via B</td>
<td>5’CACGCACCATCATTTGCGC3’</td>
<td>57</td>
<td>738 bp</td>
</tr>
<tr>
<td></td>
<td>5’ACGTTAATGTCAAGATGCTAC3’</td>
<td>57.5</td>
<td></td>
</tr>
<tr>
<td>FliC-D</td>
<td>5’GGTGAACGGCAGTACCATCG3’</td>
<td>56.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5’GAGCAACGCCGATACCTCG3’</td>
<td>59</td>
<td>587 bp</td>
</tr>
<tr>
<td>Prt</td>
<td>5’CGTTTGAGGTCTCCTTGAGATCACG3’</td>
<td>60.5</td>
<td>369 bp</td>
</tr>
<tr>
<td></td>
<td>5’CCAATATAGGCGCGGGCGAGTTC3’</td>
<td>61</td>
<td></td>
</tr>
</tbody>
</table>

Table 3: Nucleotide sequence of candidate oligomers of via B, FliC-D and Prt gene of Salmonella ser. Typhi.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Amplicon</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Via B</td>
<td>738 bp</td>
<td>+</td>
</tr>
<tr>
<td>FliC-D</td>
<td>587 bp</td>
<td>+</td>
</tr>
<tr>
<td>Prt</td>
<td>369 bp</td>
<td>+</td>
</tr>
</tbody>
</table>

Different sized PCR products corresponding to each gene were observed in gel electrophoresis (Table 3). Our interpretation on virulence markers indicate that the potable water is contaminated by Salmonella ser. Typhi isolates exhibiting Via B, Fli-D & Prt virulent genes. Prevalence and survival of Salmonella ser. Typhi in water has also been reported previously and. Various regulatory genes are expressed while facilitating the survival of pathogen in water. Kingsley et al have reported the role of gene regulatory adaptation in S. Typhi which helps survival in water (Kingsley et al., 2018). Liu et al have discussed the prevalence of S. Typhi in irrigation water and suggested the entry of pathogen in viable but non-culturable (VBNC) state of S. Typhi (Liu et al., 2018).

Phenotypic Characterisation of drug resistance profiling for Salmonella ser. Typhi

Drug resistance profiling was performed using disc diffusion method at phenotypic level. Selected strains showed high resistance against Clindamycin, Aztreonam, Trimethoprim, Ceftazidime, Cefuroxime, Cefaxolin, Oxacillin, Erythromycin, Tetracycline, Co-trimoxazole (Table 4). Similar observations were reported in Salmonella spp., isolated form potable water (Agarwal et al., 2015). However, intermediate to Amikacin Amoxycilav, Tetracycline and Streptomycin were observed among some isolates. Some of the strains were found to be sensitive against Chlorumphenicol, Fosfomycine, Ampicillin/Sulbactam, Ertapenem, Meropenem, Doripenem. Nair et al., have discussed the prevalence of various antibiotic-resistant Salmonella serotypes in food animals and food supply (Nair et al., 2018). Aregbo et al have reported the presence of drug-resistant bacteria in water reservoir (Aregbo et al., 2018). Prevalence of antibiotic-resistant bacteria in water is alarming and crucial for public health.

Synthesis and Characterisation of Gold Nanoparticles

Highly stable Au nanoparticles were synthesized using Sodium citrate as capping agent. Sodium borohydrate was used as reducing agent. Finally, synthesized product was characterized using standard characterization techniques, to check the shape size and morphology of the particle.

Characterization of nanoparticles was done using different methods:

(I) UV-Visible Spectroscopy
Table 4: Antimicrobial susceptibility pattern of *Salmonella* ser. Typhi isolates was determined by disk diffusion assay.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Antibiotics</th>
<th>Zone of Inhibition (mm)</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Clindamycine (CD &lt;sup&gt;30&lt;/sup&gt;)</td>
<td>13</td>
<td>Resistance</td>
</tr>
<tr>
<td>2.</td>
<td>Cefoxitin (CX &lt;sup&gt;30&lt;/sup&gt;)</td>
<td>20</td>
<td>Intermediate</td>
</tr>
<tr>
<td>3.</td>
<td>Ciprofloxacin (CIP &lt;sup&gt;3&lt;/sup&gt;)</td>
<td>10</td>
<td>Intermediate</td>
</tr>
<tr>
<td>4.</td>
<td>Chlorepenicol (C&lt;sup&gt;30&lt;/sup&gt;)</td>
<td>30</td>
<td>Sensitive</td>
</tr>
<tr>
<td>5.</td>
<td>Fosfomycin (FO &lt;sup&gt;200&lt;/sup&gt;)</td>
<td>33</td>
<td>Sensitive</td>
</tr>
<tr>
<td>6.</td>
<td>Aztreonam (AT &lt;sup&gt;30&lt;/sup&gt;)</td>
<td>11</td>
<td>Resistance</td>
</tr>
<tr>
<td>7.</td>
<td>Ampicillin/subbactem (A/S 10/10)</td>
<td>28</td>
<td>Sensitive</td>
</tr>
<tr>
<td>8.</td>
<td>Ertapenem (ETP &lt;sup&gt;10&lt;/sup&gt;)</td>
<td>30</td>
<td>Sensitive</td>
</tr>
<tr>
<td>9.</td>
<td>Meropenem (MRP &lt;sup&gt;10&lt;/sup&gt;)</td>
<td>33</td>
<td>Sensitive</td>
</tr>
<tr>
<td>10.</td>
<td>Amikacin (AK &lt;sup&gt;30&lt;/sup&gt;)</td>
<td>14</td>
<td>Intermediate</td>
</tr>
<tr>
<td>11.</td>
<td>Doripenem (DOR &lt;sup&gt;30&lt;/sup&gt;)</td>
<td>32</td>
<td>Sensitive</td>
</tr>
<tr>
<td>12.</td>
<td>Amoxyclav (AMC &lt;sup&gt;30&lt;/sup&gt;)</td>
<td>18</td>
<td>Intermediate</td>
</tr>
<tr>
<td>13.</td>
<td>Trimethoprim (TR &lt;sup&gt;3&lt;/sup&gt;)</td>
<td>NIL</td>
<td>Resistance</td>
</tr>
<tr>
<td>14.</td>
<td>Cefazidime (CAZ &lt;sup&gt;10&lt;/sup&gt;)</td>
<td>12mm</td>
<td>Resistance</td>
</tr>
<tr>
<td>15.</td>
<td>Cefuroxime (CXM &lt;sup&gt;30&lt;/sup&gt;)</td>
<td>NIL</td>
<td>Resistance</td>
</tr>
<tr>
<td>16.</td>
<td>Cefoxolin (CZ &lt;sup&gt;30&lt;/sup&gt;)</td>
<td>NIL</td>
<td>Resistance</td>
</tr>
<tr>
<td>17.</td>
<td>Oxacilzine (Ox&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>NIL</td>
<td>Resistance</td>
</tr>
<tr>
<td>18.</td>
<td>Erythromycin (E&lt;sup&gt;15&lt;/sup&gt;)</td>
<td>NIL</td>
<td>Resistance</td>
</tr>
<tr>
<td>19.</td>
<td>Tetracycline (TE&lt;sup&gt;20&lt;/sup&gt;)</td>
<td>13</td>
<td>Intermediate</td>
</tr>
<tr>
<td>20.</td>
<td>Streptomycin (STM&lt;sup&gt;30&lt;/sup&gt;)</td>
<td>17</td>
<td>Intermediate</td>
</tr>
<tr>
<td>21.</td>
<td>Co-trimoxazol (Cot&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>12</td>
<td>Resistance</td>
</tr>
<tr>
<td>22.</td>
<td>Norfloxin (NOR&lt;sup&gt;10&lt;/sup&gt;)</td>
<td>21</td>
<td>Sensitive</td>
</tr>
</tbody>
</table>

UV-Visible spectrum was taken in an optical quality quartz cuvette with a 1 cm path length. A visible colour changes from yellow to pink due to Surface Plasmon Resonance (SPR) vibration was observed indicating the formation of nanoparticles. Spectra were seen at room temperature, while double distilled water was used as a blank. The absorption spectrum was recorded from of 200 to 700 nm. The Gold nanoparticles synthesized by chemical method exhibit Surface plasmon resonance spectra at 515 nm.

(II) Transmission Electron Microscopy

Size of Gold NPs was in the range of 20 to 30 nm as evident from Transmission Electron Microscopy. Nanoparticles were prominently spherical in shape (Fig. 2). The selected area diffraction pattern of Au nanoparticles evidenced the crystalline planes of the face-centred-cubic structured gold (Fig. 3), which suggested the crystalline nature of synthesised gold nanoparticles.

*In-vitro experiment for antimicrobial activity of gold nanoparticles against* *Salmonella* ser. *Typhi* and evaluation of MIC

Growth inhibition of *Salmonella* ser. Typhi was examined in broth containing varying concentrations (0, 10, 20, 30, 40, 50 and 100 µg/mL) of AuNPs. Gold nanoparticles were highly effective at concentration ≥ 50 µg/mL. At concentration of 40 µg/ml, the gold nanoparticles began to show modest antimicrobial effect on *Salmonella* Typhi and fewer viable cells were observed as compared to control samples. Further, it was observed that at concentrations ≥ 40 µg/mL.

Fig. 1: PCR amplification of *invA* gene in *Salmonella* isolates
Lane 1: 50 bp DNA ladder; Lane 2 and 3: Positive isolates; Lane 4: Negative control and Lane 5: Positive control.

Fig. 2: TEM micrograph of synthesised Gold nanoparticles.
ml of the nanoparticles the Salmonella ser. Typhi culture lost its culturability. The MIC of gold nanoparticles was evaluated to be 50 µg/mL. Previous reports have demonstrated that NPs of size less than 5 nm can enter human tissues easily and may disrupt the cell normal biochemical environment (Bahadar et al., 2016, Vishwakarma et al., 2010).

Acknowledgement

We wish to express our sincere acknowledgement to Dr. Ashok Kumar Chauhan, President, RBEF parent organization of Amity University Madhya Pradesh (AUMP), Dr. Aseem Chauhan, Additional President, RBEF and Chairman of Amity University Madhya Pradesh, Gwalior, Lt. Gen. V.K. Sharma, AVSM (Retd.), Vice Chancellor of AUMP Gwalior, for providing necessary facilities, their valuable support and encouragement throughout the work. This work was partially supported by MPCST funded project. Financial assistance to M.A (JRF) is also acknowledged to MPCST, Bhopal. We are also thankful to CSIR-CDRI, Lucknow (U.P) for providing laboratory facility.

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