Green synthesis of silver nanoparticles from *Artemisia pallens*, Wall. ex DC. and its cytotoxicity activity as an anti-mycobacterial agent

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ABSTRACT

In present investigation, the synthesis of silver nanoparticles from of *Artemisia pallens*, were studied for Anti-mycobacterial activity against three species as *Mycobacterium tuberculosis* (MTCC-300), *M.pheli* (MTCC-1724), *M.avim* (MTCC-1723). The Anti-mycobacterial activity of silver nanoparticles from *A. pallens* was evaluated by agar cup well plate method using different dilutions such as 10 mg/ml, 20 mg/ml and 30 mg/ml. These extracts showed maximum activity at 30mg/ml. Among the strains, the maximum zone of inhibition was noted against *Mycobacterium tuberculosis* (32.5mm) and *Mycobacterium spegmatis* (29.4mm) and *Mycobacterium pheli* (29.4mm) respectively. The Minimum inhibitory concentration (MIC) of silver nanoparticles for *A.pallens* showed against two species of *Mycobacterium* like *Mycobacterium tuberculosis* as (230 µg /mL) and *Mycobacterium pheli* (230 µg /mL). These results showed that the silver nanoparticles from *A.pallens* is an effective Anti-mycobacterial agent. The cytotoxicity was also done by hemolytic assay.

Key words: *Artemisia pallens* Wall. ex DC (AP), AgNPs, Anti-mycobacterial, MIC, Hemolytic assay

INTRODUCTION

*A.pallens* is an erect, much branched, woody canescent herb, very aromatic and cultivated for offering god and goddess’s. Among diverse nonmaterial silver was focused with much interest due to its unique properties, such as conductivity, stability, catalytic and antibacterial property. Silver nanoparticles are making significant progress in the area of nanotechnology and nanomedicine for last ten years because of their excellent catalytic, optical, electrical and antifungal ,antibacterial, antmycobacterial applications. Briefly, selection of biological entity for synthesis of nanoparticles in term of availability, less time-consuming and novelty were considered to be the third generation. [1]. The work on synthesis of nonmaterials using diverse biological regime has been done on various groups such as plants [2], algae [3], diatoms [4], fungi [5], bacteria [6] and biomolecules [7]. It opens up a wide array of opportunities to explore in various fields like medicine, pharmaceuticals, electronics and agriculture [8]. AgNPs have many applications such as targeted drug delivery and antimicrobials [9], anti-cancerous [10], anti-tuberculosis [11, 12], amoebicidal [13], catalyst [14, 15], biosensor [16], in textiles [17], agriculture [18], management of insects [19, 20], cosmetics [21], tissue regeneration [22] and dye reduction [23].

In previous era TB is found in worldwide distribution. Tuberculosis is an infection caused by *Mycobacterium tuberculosis*, characterized by chronic inflammatory changes with formation of tubercles on lungs. Even though more than 250,000 children develop TB each year, inexcusably most anti-TB agents are not available in suitable pediatric formulations (Swaminathan et al., 2010). Focusing on treatment for active TB, there is paramount needed to develop new and effective anti TB therapeutic regimen, focusing on not only development of the new chemicals, but also repurposing existing anti-tb agents in clinical development. According to world health organization (WHO) report, 8.8 million incident cases of TB were recorded, of which 1.1 million deaths were documented among HIV-
negative group and an additional 0.35 million deaths from HIV-associated TB infections (global tuberculosis control-2011).

MATERIALS AND METHODS

Plant materials:
The plant of *A. pallens* was collected from Kurunda village, Basmat taluka in Hingoli district, Maharashtra India, where it is cultivated as a medicinal plant. The plants were collected during the June-September 2013 while flowering and fruiting. Further the plant materials were dried under shade and after optimum drying, coarsely powdered and stored in well closed container till further use.

Collection of pathogens
The *Mycobacterium* strains such as *Mycobacterium tuberculosis*, *M.pheli* and *M.avim* were obtained from Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh (PB) India and were subcultured and maintained on Lowenstein Jensen media.

Preparation of micro organism:
A loop full culture of pure strains of different *Mycobacterium* species were inoculated into 100 ml of sterile Lowenstein Jensen media and incubated for four days at 37° C for bacterial culture. After four days of incubation, 0.5 ml of broth containing the microorganisms were added into 9 ml of Lowenstein Jensen media. The Ten fold serial dilutions were made in the range of $10^{-1}$ to $10^{-9}$. 100 µl of the dilutions ranging from $10^{-5}$ to $10^{-8}$ were spread on the sterile Lowenstein Jensen media plates and kept at 37° C for four days. The number of colonies forming units were counted and numbers of microorganisms in each ml of stock culture were calculated.

Chemicals
Silver nitrate (99.9%), absolute alcohol (99.9%), Lowenstein Jensen media, DMSO, Rifampcin were purchased from Himedia Pvt. Ltd., India. All chemicals were prepared in double-distilled deionized water.

Preparation of plant extracts
The fresh leaves of *A. pallens*, without any infection were collected and 5 g of the leaves were weighed and washed with double-distilled water before use. The leaves were air-dried for 10 days and were then kept in the hot air oven at 60° C for 24–48 h. The leaves were then cut into fine pieces and 100 ml of double-distilled water was added. The mixture was boiled for 10 min before being decanted, and then was cooled and filtered through Whatman No. 1 filter paper. The boiled extract was refrigerated and used for further experimental procedures.

Biosynthesis of silver nanoparticles
Biosynthesis of silver nanoparticles was done according to the method of Song and Kim [25, 26]. The plant of *A. pallens* was collected from kurunda village. The plants were collected during the June-September period of 2013 and airs dried for 10 days and then were kept in the hot air oven at 60° C for 20 minute. The leaves were ground to a fine powder. 1 mM silver nitrate solution was added to plant extract to make up a final volume of 200 ml and centrifuged at 18,000 rpm for 25 min. The collected pellets were stored at 4 C. The supernatant was heated at 50°–95° C. A change in the color of solution was observed during the heating process [26].

UV-visible spectroscopy analysis
The color change in reaction mixture (metal ion solution + *A. pallens* extract) was recorded through visual observation and Silver nanoparticles were characterized by UV–vis schimadzu1600 spectrophotometer. The bioreduction was monitored and the absorption spectra in 300–700 nm range. The bioreduction of silver ions in aqueous solution was monitored by periodic sampling of aliquots (1 ml) and subsequently measuring UV-Vis spectra of the solution.

Anti-Mycobacterial and by agar cup plate method
The anti-mycobacterial of silver nanoparticles from *Artemisia pallens* was evaluated by cup plate method using different dilutions viz., 10 mg/ml, 20 mg/ml and 30 mg/ml. Sterilized nutrient agar plates were prepared under aseptic conditions. Six mm diameter holes were made in the agar plates using a sterile borer. 0.2 ml of the test organisms was spreaded on Lowenstein Jensen media plates. Samples, standard drug Rifampcin were dissolved with DMSO and the solvent control (DMSO) were added into each hole separately. The plates were maintained at 4° C for 30min to allowed diffusion of solution into the agar medium. The plates were incubated at 37° C for one to four days for bacteria. The zone of inhibition was measured using antibiotic zone reader.
Determination of Minimum Inhibitory Concentration:
The MIC of the plant extract Artemisia pallens were performed using the broth micro dilution assay against the three Mycobacterium species. Tests were performed in sterile 96-well micro plates by dispensing into each well a total volume of 300µl comprising 100µl of standardized suspension of test culture (110⁶ cells/ml) 100µl and incubated up to 48 h at 37°C. MIC was determined by absorbance measurement at 620 nm using thermo make Automatic Ex-Micro plate Reader (M 51118170). The MIC was defined as the lowest concentration of the sample that inhibited the growth of test microorganism.

Hemolytic activity
The hemolytic activities of the test compounds were determined using human red blood cells [24]. Human erythrocytes from healthy persons were collected in tubes containing EDTA (1–2 mg/ml) as anti-coagulant. The erythrocytes were harvested by centrifugation (Heraeus Megafuge 40, Thermo Fisher Scientific Inc., MA) for 10 min at 634 × g at 20°C, and washed three times in PBS. PBS was added to the pellet to yield a 10% (v/v) erythrocytes/PBS suspension. The 10% suspension was diluted 1:10 in PBS. From each suspension, 100 µL was added in triplicate to 100 µl of a different dilution series of AgNPs (or Rifampcin as a standard anti-mycobacterial) in the same buffer in Eppendorf tubes. Total hemolysis was achieved using 1% Triton X-100. The tubes were incubated for 1 h at 37°C and centrifuged for 10 min at 634 × g at 20°C. From the supernatant fluid, 150 µL was transferred to a flat-bottomed micro titer plate (Himedia Ltd., India), and the absorbance was Measured at 450 nm. Percent haemolysis was calculated using following equation;

\[
\%\text{Hemolysis} = \frac{\frac{1}{4} A_{450} \text{of test compound treated sample} - A_{450}\text{of buffer treated sample}}{A_{450}\text{of 1 % Triton-X 100 treated sample} - A_{450}\text{of buffer treated sample}} \times 100\%
\]

RESULTS AND DISCUSSION
The silver nanoparticles of Artemisia pallens was found to be effective against various Anti-mycobacterial species as indicated by the zone of inhibition. Maximum inhibition was obtained against Mycobacterium tuberculosis (30.2mm), M.pheli (28.4mm) and M.avim (27.8mm). The silver nanoparticles of Artemisia pallens was found to be effective against all bacterial strains used in the study at a concentration of 10 to 30 mg/ml. For Mycobacterium tuberculosis (36.2 mg/ml), for M.pheli (48.4 mg/ml) and for M.avim (47.8 mg/ml) was found to be maximum value against silver nanoparticles of Artemisia pallens a 30mg/ml. Mycobacterium tuberculosis (19.1) ,M.pheli (14.2) and M.avim (12.3).

The MIC was calculated by serial dilution method for Mycobacterium tuberculosis (230 µg/ml), M.pheli (230 µg/ml) and M.avim (240 µg/ml)

<table>
<thead>
<tr>
<th>Sr.No</th>
<th>Organism</th>
<th>Diameter of zone of inhibition(mm)</th>
<th>Artemisia pallens AgNPs (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Mycobacterium tuberculosis</td>
<td>19.1 25.1 36.2</td>
<td>23.1</td>
</tr>
<tr>
<td>2.</td>
<td>Mycobacterium pheli</td>
<td>14.2 22.2 48.4</td>
<td>20.6</td>
</tr>
<tr>
<td>3.</td>
<td>Mycobacterium avim</td>
<td>12.3 21.6 47.8</td>
<td>21.3</td>
</tr>
</tbody>
</table>

Table 2: Determination of Minimum Inhibitory Concentration (MIC) of AgNPs by Artemisia pallens

<table>
<thead>
<tr>
<th>Sr.No</th>
<th>Organism</th>
<th>MIC in µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Mycobacterium tuberculosis</td>
<td>230</td>
</tr>
<tr>
<td>2.</td>
<td>Mycobacterium pheli</td>
<td>230</td>
</tr>
<tr>
<td>3.</td>
<td>Mycobacterium avim</td>
<td>240</td>
</tr>
</tbody>
</table>

The in vitro hemolytic assay is a screening tool for developing in vivo toxicity towards host cells [27]. The silver nano partials showed no significant toxicity to human erythrocytes at minimum growth inhibitory concentrations (P = 0.0403 for AgNPs) only 0–12% hemolysis was observed at the tested concentrations, while Rifampcin showed 100% hemolysis at 10 µg/mL.
In future, there has been a mounting interest in the discovery of new antimicrobial compounds due to a startling increase in the rate of infections with antibiotic resistant microorganisms. There is a urgent need to have green nano particles from plants those are non toxic, target oriented, cost effective, novel agent. Tuberculosis (TB) is an infectious bacterial disease that can be transmitted from person to person because of the droplets from the throat and lungs of people suffering from the active respiratory disease. TB has remained a serious problem in developing countries, and the multidrug resistant strains of Mycobacterium tuberculosis are imposing the restrictions on the usage of current antimycobacterial drugs. Alarming data from the World Health Organization (WHO) revealed that TB has spread to every corner of the globe (WHO, 2006). Present investigation shows that the green synthesis of silver nanoparticles from Artemisia pallens are producing novel drug against TB those are responsible for inhibition of Mycobacterium species.

CONCLUSION

In the present study, silver nanoparticles from Artemisia pallens plant showed significant anti-mycobacterial activity against Mycobacterium tuberculosis, M.pheli, and M.avim by agar cup plate method and MIC calculated by broth micro dilution assay. This activity may be attributed to the presence of novel safe, effective, less toxic and target oriented plant drug present in the plant as anti-TB agent. The present work reveals the potentia l application of silver nanoparticles of Artemisia pallens as an anti-TB agent.

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