Green synthesis of cobalt oxide nanoparticles for potential biological applications

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Abstract
Cobalt oxide nanoparticles (Co₃O₄-NPs) have many applications and now a days the green methods of synthesis of these NPs are preferred over other methods because of associated benefits. In this study, Co₃O₄-NPs were synthesized by using leaves extract of Populus ciliata (safaida) and cobalt nitrate hexahydrate as a source of cobalt. The synthesized NPs were analyzed by different techniques such as fourier transform spectroscopy (FTIR), x-ray diffraction (XRD), transmission electron microscopy (TEM) and scanning electron microscopy (SEM). Antibacterial activities of the synthesized Co₃O₄-NPs were evaluated against gram negative and gram positive bacteria and found active against Escherichia coli (E. coli), Klebsella pneumonia (K. pneumonia), Bacillus subtilis (B. subtilis) and Bacillus licheniformis (B. licheniformis). The activity results were analyzed statistically by one-way ANOVA, with ‘Dunnett’s Multiple Comparison Test’. The maximum mean activity (21.8 ± 0.7) was found for B. subtilis and minimum mean activity (14.0 ± 0.6) was observed for E. coli.

Introduction
The nanotechnology employs many physical and chemical processes to fabricate nano materials with unique properties [1]. Through nanotechnology, we can develop new materials with sizes (at least in one dimension) less than 100 nm. These materials have vast range of applications such as nanomedicine, nanoelectronics, biomaterials, energy production and consumer products [2]. Also, these materials have a variety of applications in agriculture, environment, information, communication and heavy industry [3].

Conventionally, nanoparticles were synthesized by different methods such as pyrolysis and abrasion but these methods have some shortcomings such as high cost, low synthesis rate and high energy requirements [4]. The chemical methods such as sol gel technique and chemical reduction are mostly used for synthesis of these materials. But these methods employ the use of toxic chemicals and also result in the production of hazardous side products [4]. Thus, there always remains a quest in the scientific community to develop such methods which are less toxic, ecofriendly, cost effective and clean for the synthesis of nanoparticles [5]. Therefore, recently relevant scientific communities are interested in the synthesis of metal oxide nanoparticles by using different plant extracts. Furthermore, the biogenesis of metal oxide nanoparticles is gaining an increasing attention due to its simple experimental set up and easiness to obtain nano particles with variable sizes and morphologies [6–9].

Recently, Co₃O₄-NPs have gained considerable attention due to their unique and important applications. These NPs have applications in gas sensors, lithium ion batteries, solar selective absorbers, capacitors, field emission materials, energy storage systems, electrochromic thin films, magneto resistive devices and catalysis [10–17]. In recent past, graphene and Co₃O₄ composites have been reported in which cobalt oxide helps in increasing the dimensional stability of substrate [18].
Recently, the synthesis of copper oxide and zinc oxide nanoparticles using *Populus ciliata* leaf extract has been reported [18, 19]. In the present study, we are reporting the synthesis of Co$_3$O$_4$-NPs using *Populus ciliata* leaves extract. This plant belongs to the family (Silicaceae) and this species is mainly distributed in the areas of indo-Pakistan, Nepal and Bhutan. The wood of this plant is used for making many useful items. The bark of this plant is also used for the treatment of many diseases [20]. For these uses, millions of trees are cut down and their leaves remain as bio-waste. Hence, it is imperative to develop procedures and technologies to consume this bio-waste beneficially. For this purpose, we have used *Populus ciliata* leaves as a reducing agent for the synthesis of Co$_3$O$_4$-NPs. According to relevant literature survey, the leaf extract of this plant has not been used for the synthesis of Co$_3$O$_4$-NPs so far [18, 19]. The synthesized Co$_3$O$_4$-NPs have been characterized by various techniques and antimicrobial activities are evaluated against selective gram-positive and gram negative bacterial species.

### Materials and methods

#### Materials

The cobalt nitrate hexahydrate Co(NO$_3$)$_2$.6H$_2$O (98%) was purchased from VWR Chemicals (BDH). Fresh leaves of *Populus ciliata* were collected from Chehla Campus, University of Azad Jammu and Kashmir, Muzaffarabad, Pakistan.

#### Preparation of leaf extract

The leaves extract was prepared with a reported method [19]. The leaves were first washed with tap water followed by distilled water and dried under air at room temperature for 24 h. Then these leaves were chopped properly in mechanical blender. The chopped leaves (10 g) were taken in a glass beaker (250 ml) and distilled water (100 ml) was added and mixture was heated to boiling. After 2 h, the heating was stopped and solution color change was noted from colorless to light brown. The solution was kept to attain room temperature, was filtered and stored in refrigerator for further use.

#### Synthesis of Co$_3$O$_4$ NPs

Co(NO$_3$)$_2$.6H$_2$O was used as a precursor to synthesize the nanoparticles. 1 mmolar solution of this salt was prepared by adding its 2.91 g, into 100 ml distilled and sterilized water. For the synthesis of Co$_3$O$_4$ NPs, 30 ml of cobalt salt solution and pant leaves extract (20 ml) were taken in a glass beaker and were heated at 80 °C for three hours. A color change (from light brown to dark brown) indicated the formation of Co$_3$O$_4$-NPs. The solution was cooled down to room temperature. The resultant solution was centrifuged at 15 000 rpm for 15 min, the supernatant solution was decanted and residue (Co$_3$O$_4$-NPs) was dried at 60 °C in an oven.

#### Characterization

Synthesized, Co$_3$O$_4$ nanoparticles were characterized by different techniques such as FTIR, TEM, XRD and SEM. FTIR analysis (in the range of 400–4000 nm) was carried out with the help of FTIR spectrophotometer (Shimadzu 8400S). XRD analysis was done on instrument (Bruker D8 Advance x-ray diffractometer) by using Cu Kα radiation (λ = 1.5418 Å). Morphology of prepared cobalt oxide nanoparticles was also analyzed by scanning electron microscope (FEINOVA nano SEM 450). To find out the size and morphology of cobalt oxide nanoparticles, TEM analysis was performed by using HITACHI H-7700 TEM at 200 KV.

#### Anti- bacterial activities

Solution of Co$_3$O$_4$-NPs was prepared by mixing its 2, 4 and 8 mg in 1 ml distilled and sterilized water and was placed in the sonicator to properly dissolve the nanoparticles. The proper mixing of nanoparticles was examined by the absence of any suspended particles in the solution. Test organisms were streaked over the fresh prepared nutrient agar in the petri plates and incubated at 37 °C. Then, a loop full of 24 h. old culture was taken and inoculated in the pre autoclaved 0.9% saline solution. This saline solution was placed in the shaker at 37 °C for thorough mixing of the test organism. After that, 100 μl of saline solution was added in the plates with a micropipette. Then, with the help of sterilized cotton swab, test organisms were properly spread over the surface of petri plates. When petri plates were solidified, 50 μl of nanoparticles’ solutions were added in the wells. One well was filled with the water as the control while other wells were filled with commercial bacitracin solution and nanoparticles’ solutions, respectively. The petri plates were incubated at 37 °C for 24 h and then graduated scale was used to measure the zones of inhibition.
Statistical analysis
Data were presented as mean ± SEM and analyzed statistically by one-way ANOVA, with ‘Dunnett’s Multiple Comparison Test’, to identify any significant differences between the means.

Results and discussion

FT-IR analysis was performed to find out the different functional groups that are present in leaf extract of *Populus ciliata* and are helpful for the synthesized nanoparticles and act as capping and stabilizing agents. IR spectrum of plant extract as well as of synthesized NPs is shown in figure 1. Both types of spectra have almost similar peaks except that in case of plant extract containing NPS, there is slight shifting and broadening of peaks. A peak obtained at 3458 cm⁻¹ is the characteristic peak of hydroxyl group of phenolic compounds. Some other major peaks were obtained at 1622 cm⁻¹, 1381 cm⁻¹, 1082 cm⁻¹ and 533 cm⁻¹ can be attributed to the carbonyl group, amide group, C–O of alcohols or phenols and Co₃O₄, respectively.

The confirmation of successful synthesis of cobalt oxide nanoparticles was done by powder x-ray diffraction (XRD). The obtained diffraction pattern do not have any sharp peaks which indicate that the synthesized nanoparticles are not well crystalline (figure 2). However, there are few weak peaks at 2θ values of 30.3, 36.4, 45.0 and 60.0 which correspond to (210), (311), (400) and (511) planes of Co₃O₄, respectively. The particle size was estimated using Scherrer formula and it was found in the range of 40–50 nm.

Transmission electron microscopy (TEM) was performed to know about the structural features of synthesized cobalt oxide nanoparticles (figure 3). TEM image in figure 3(a) showed that the synthesized product possesses single morphology. In the large area TEM image, it can be seen that few nanoparticles are aggregated together in the form of groups. It is believed that these nanoparticles are well dispersed in solution and are aggregated during analyte preparation. In order to know more about morphology of cobalt oxide nanoparticles, TEM images at higher magnifications were captured as shown in figure 3(b). It can be seen that most of the nanoparticles are square shaped and size estimated from the TEM images lies in the range of about 15–35 nm.

Figures 4(a)–(d) show the FE-SEM images of Co₃O₄ nanoparticles at different magnification, which clearly exhibit the nanoparticles like morphology indicate well uniform particles with narrow size distribution lies in the range of 25–35 nm. The surface of as synthesized nanoparticles is very smooth, which facilitates the better contact with the bacterial cell wall and hence increases bacterial killing ability of NPs. Such a behavior of smooth surfaced NPs has already been established in the literature [20]. The elemental composition of the synthesized Co₃O₄-NPs was evaluated from EDX analysis (figure 5). In this figure, the major peaks indicate the Co and O of the synthesized NPs. However, some minor peaks of carbon, calcium, sodium, sulphur and silicon are also present which are attributed to the plant extract used. The elemental composition of the nanoparticles shows 26 weight per cent cobalt and 68 weight per cent oxygen corresponding to cobalt oxide (Co₃O₄). The compositional data from the EDX analysis agree well with theoretically calculated values, indicating a good compositional homogeneity across the nanoparticles. The EDX spectrum shows sharp peaks between 0 and 2 KeV and between 6 and 8 KeV corresponding to crystalline Co₃O₄-NPs.

**Figure 1.** FTIR spectra of cobalt oxide nanoparticles.
Figure 2. XRD pattern of cobalt oxide nanoparticles.

Figure 3. TEM images of cobalt oxide nanoparticles at different magnifications.

Figure 4. SEM image of Co₃O₄ nanoparticles.
Antibacterial assay
According to reported literature of the Co$_3$O$_4$-NPs, these NPs have been employed in catalysis and their biological applications have been less explored. Ali Talha Khalil et al have applied the Co$_3$O$_4$-NPs against gram positive (Staphylococcus aureus, Staphylococcus epidermis and Bacillus subtilis) and gram negative (Klebsiella pneumonia, Pseudomonas aeruginosa and Escherichia coli) bacterial pathogens. These researchers have found that anti-bacterial activity of these NPs increased with increasing their concentration \[21\]. Also, these researchers have used Co$_3$O$_4$-NPs for cytotoxic, antioxidant and enzyme inhibition assays. Marcella Mauro et al have studied the use of impaired and healthy human skin cells for Keratinocytes Toxicity studies \[22\]. Judhit Vijaya et al have synthesized Co$_3$O$_4$-NPs from Azadirachta indica leaves (extract) and applied for the antimicrobial activities against gram negative and gram positive bacteria \[23\]. Elena Boss et al have studied that Co$_3$O$_4$-NPs cross the plasma membrane by a non-endocytosis pathway and thus gain access to the cytoplasm \[23, 24\].

Results of Co$_3$O$_4$-NPs’ antibacterial activities are presented in table 1. Cobalt oxide nanoparticles produced good antibacterial activities against both gram positive and gram negative bacteria. The antibacterial activities of Co$_3$O$_4$-NPs were higher against gram positive bacteria as compared to gram negative bacteria. Among gram positive bacteria, maximum inhibition zone (24.5 $\pm$ 1.3) against B. subtilis was measured while for gram negative bacteria (Klebsiella pneumoniae), maximum inhibition zone (20.4 $\pm$ 0.7) was observed. It is important to note that activity of cobalt oxide NPs against B. subtilis was even higher than Bacitracin (the antibiotic used as a control in these studies). Such results indicate the potential of these NPs against human pathogens. Among both classes of bacteria, minimum zone of inhibition (16.0 $\pm$ 0.8) was found for E. coli. The gram positive bacteria have thick peptidoglycan cell wall but porous in nature with higher permeability as compared to gram negative bacteria and such a structure of cell wall facilitates the maximum absorption of these NPs \[21–23, 25\]. Also, the size of nanoparticles is small which ensures the maximum absorption of NPs by bacterial species and hence resulting into their death. Gram negative bacteria have thin peptidoglycan wall with less permeability \[21–23, 25\]. So based upon the structural characteristics of cell walls of both type of bacteria, we assume an enhanced permeability and hence greater activity of Co$_3$O$_4$-NPs for gram positive bacteria \[21–23, 25\]. Highly reactive oxygen species (ROS) such as hydrogen peroxide formed in the presence of metallic ions, result in a
Table 1. Antibacterial activity of cobalt oxide nanoparticles against selected bacteria.

| Bacterial strains       | Zones of inhibition by control (water) in Millimeter (mm) | Zones of inhibition by bacitracin (known antibacterial agents) in millimeter (mm) at various concentrations | Zones of inhibition by cobalt oxide nanoparticles in millimeter (mm) at various concentrations | Mean activity of cobalt oxide nanoparticles (with 95% CI) in mm |
|-------------------------|----------------------------------------------------------|----------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------|---------------------------------------------------------------|
|                         | 2 mg ml⁻¹ | 4 mg ml⁻¹ | 8 mg ml⁻¹ | Mean activity of bacitracin (with 95% CI) in mm | 2 mg ml⁻¹ | 4 mg ml⁻¹ | 8 mg ml⁻¹ | Mean activity of cobalt oxide nanoparticles (with 95% CI) in mm |
| Gram-negative bacteria  | 0 ± 0     |           |           |                                             | 14.5 ± 0.5 | 19.9 ± 0.8 | 23.5 ± 1.7*** | 19.3 ± 1.0*** | 11.0 ± 0.5 | 15.1 ± 0.6 | 16.0 ± 0.8 | 14.0 ± 0.6 |
| * Escherichia coli      | 0 ± 0     |           |           |                                             | 12.2 ± 0.2 | 24.9 ± 1.3 | 27.2 ± 2.1 | 21.4 ± 1.2*** | 12.8 ± 0.2 | 17.8 ± 0.9 | 20.4 ± 0.7 | 17.0 ± 0.6 |
| * Klebsiella pneumoniae| 0 ± 0     |           |           |                                             | 9.3 ± 0.3  | 21.1 ± 1.1 | 25.3 ± 2.0 | 18.6 ± 1.1 | 19.7 ± 0.4 | 21.2 ± 0.5 | 24.5 ± 1.3 | 21.8 ± 0.7*** |
| Gram-positive bacteria  | 0 ± 0     |           |           |                                             | 13.1 ± 0.2 | 21.2 ± 1.5 | 24.2 ± 1.8 | 19.5 ± 1.2 | 14.1 ± 0.4 | 19.2 ± 1.2 | 22.5 ± 0.9 | 18.6 ± 0.8 |
| * Bacillus subtilis     | 0 ± 0     |           |           |                                             |           |           |           |                                             |
| * Bacillus licheniformis| 0 ± 0     |           |           |                                             |           |           |           |                                             |

Each value represents the mean ± SEM of four replicates. #positive control is commercial bacitracin (a known antibacterial agent). Statistical icon: *** = p ≤ 0.001.
substantial damage to bacterial DNA, cell membrane and cause protein dysfunction. The cobalt ions interact with thiol groups of bacterial enzymes, cause such results indicate the potential of these NPs against human pathogens (figure 6) inactivation and lead to death.

**Conclusion**

Several conclusions have been made with synthesized cobalt oxide nanoparticles. Firstly, cobalt oxide nanoparticles were synthesized by cost effective and eco friendly green method by using Populus ciliata leaf extract and cobalt nitrate hexahydrate. The prepared nanoparticles, were analyzed by various techniques such as FTIR, XRD, TEM and SEM. These techniques revealed the successful synthesis of cobalt oxide nanoparticles. Antibacterial activities of synthesized cobalt oxide nanoparticles were analyzed against gram positive and gram negative bacteria and it was found that by increasing concentration of cobalt oxide nanoparticles, antibacterial activity was increased.

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