Antimicrobial Efficacy of Biogenic Cobalt and Copper Nanoparticles against Pathogenic Isolates

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Abstract: Biogenic synthesis of cobalt (Co) and copper (Cu) nanoparticles (NPs) was performed using the bacterial strains Escherichia coli and Bacillus subtilis. Prepared NPs were confirmed by a color change to maroon for CoNPs and green for CuNPs. The NPs characterization using FTIR showed the presence of functional groups, i.e., phenols, acids, protein, and aromatics present in the Co and CuNPs. UV-vis spectroscopy of E. coli and B. subtilis CuNPs showed peaks at 550 and 625 nm, respectively. For E. coli and B. subtilis CoNPs, peaks were observed at 300 nm and 350 nm, respectively. Antibacterial and antifungal activity of B. subtilis and E. coli Co and CuNPs was determined at 100 mg/mL concentration against two bacterial strains at 5, 2.5, and 1.5 mg/mL against fungal two strains F. oxysporum and T. viridi, respectively. B. subtilis CuNPs showed significantly higher inhibition zones (ZOI=25.7-29.7 mm) against E. coli and B. subtilis compared to other biogenic NPs. Likewise, B. Subtilis CuNPs showed lower MIC (4.3 ± 6.3) and MBC (5.3 mg/mL) values against both tested isolates. Antifungal activity of B. subtilis and E. coli CuNPs and CoNPs showed a concentration-dependent decrease in ZOI. Among all biogenic NPs, B. subtilis CoNPs showed the highest ZOI (25-30 mm) against F. oxysporum followed by E. coli CuNPs with maximum ZOI (20-27 mm) against T. viridi. Again, B. subtilis CoNPs and E. coli CuNPs showed lowest MIC and MFC values against both fungal isolates. In conclusion, the current study showed that biogenically synthesized B. subtilis Cu or CoNPs can be used as effective antimicrobial agents due to their potential antibacterial and antifungal potential.

Key words: nanoparticles, CoNPs, CuNPs, biogenic synthesis, antibacterial, antifungal, MIC, MFC

1 Introduction

Biological systems have evolved as a revolutionary technology for synthesizing nanoparticles (NPs) in recent years. Synthesis of NPs expanded greatly in the past few decades by manipulating their properties which makes the research area to grow bigger1, 2. With 1-100 nm NPs size, they are utilized in medical industries, catalysis, electro-chemistry biosensors, water treatment, and trace substance detection3. They are synthesized by using chemical, physical and biological methods. Depending upon the external environment, NPs may be hollow, spherical, cylindrical, flat and spiral4, 5. Lately, the use of microorganisms for biogenic NPs synthesis has been encouraged. Microbes consist of biomolecules that act as natural bio-laboratory for capping NPs, hence contributing an essential role in forming NPs6.

Recent work on cobalt oxide (CoNPs) has explored their structural, magnetic, electronic, and catalytic properties. CoNPs used to disinfect water bodies confirm their antibacterial activity7, 8. Recently, Dogra et al.9 reported the antibacterial activity of biogenic NPs using various metals salts such as Co, nickel oxide, ZnO NPs, and titanium dioxide NPs against Escherichia coli. Other studies...
reported increased anti-bacterial potential of NPs against bacterial strains with an increase in NPs concentration\(^9\)^\(^{10}\).

Likewise, biogenic synthesis of copper (Cu) NPs is more under consideration than the other methods, using bacteria because of the requirement of mild conditions such as pH, temperature and inherent ability of bacteria to convert toxic metals to non-toxic ones. Biogenically synthesized CuNPs exhibit antimicrobial activity against many Gram-positive and Gram-negative bacteria types. The bacterial growth of *E. coli* and *B. subtilis* was also observed to be retarded by CuNPs. Recent studies have also revealed that CuNPs can act as an anti-cancerous agent and have the potential to be used for waste water treatment\(^10\).

Bacteria have been reported to possess greater potential for nanoparticles production. Bacterial interaction with hazardous metals causes them to convert harmful metal ions to non-toxic metal oxides for their survival. Reportedly, different bacteria generate various essential thiol-containing chemicals that function as a capping agent for NPs, making them table by preventing oxidation, agglomeration, and aggregation. To date, there is no detailed understanding of mechanism behind the nanoscale change. Experimental parameters such as temperature, simple downstream processing, pH, and a short creation period all play important role in bacteria driven NPs synthesis and stability\(^11\).

Owing to increased microbial resistance against antibiotics, promising antibacterial potential of bacterial driven NPs synthesis and little information of about *E. coli* as well as *B. subtilis* basis NPs potential, the current study has been designed to synthesize biogenically Cu and Co NPs using aforementioned bacterial isolates. Following their characterization using FTIR and UV-Vis spectroscopy, the *in vitro* antimicrobial (antibacterial and antifungal) potential of synthesized Cu, and CoNPs, was tested against bacterial and fungal isolates.

2 Materials and Methods

2.1 Microbial strain, metal salts, and culture media

Bacterial isolates (*Bacillus subtilis* and Escherichia coli) and fungal strains (*Fusarium oxysporum* and *Trichoderma viridi*) were obtained from Microbiology Lab, Department of Zoology, Government College University, Lahore, Pakistan. NPs were synthesized using copper sulfate (CuSO\(_4\)) and cobalt sulphate (CoSO\(_4\)), obtained from the Department of Zoology and Chemistry, Government College University, Lahore, Pakistan. Nutrient agar and nutrient broth were prepared and sterilized by autoclaving.

2.2 Synthesis of biogenic cobalt and copper nanoparticles (CoNPs and CuNPs)

Biogenic synthesis of CoNPs and CuNPs was performed extracellularly using a pre-established protocol\(^{12}\). In brief, purified bacterial isolates were inoculated in nutrient broth and incubated for 24 hours at 37°C. Both bacterial cultures were centrifuged at 5000 rpm for 10 minutes under sterilized conditions. Pellet was discarded and the supernatant was collected in separate flasks. 3.5 g (w/v) of CoSO\(_4\) was added into the flask, mixed thoroughly, and kept at 37°C in an incubator for 48 hours until a magenta deposition was seen\(^13\), the same procedure was repeated by using bacterial culture and CuSO\(_4\) until a bluish-green deposition was seen, indicating formation of CuNPs. Control flasks with nutrient broth only were run in parallel and prepared NPs solution was stored at 4°C.

Cu and CoNPs were oven-dried to get powder form following the method\(^{10}\). Cu and Co NP solution was poured into sterilized falcon tubes aseptically and centrifuged at 5000 rpm for 10 minutes. The supernatant was discarded and pellet was stored. Pellet was placed in sterilized Petri dishes and placed in an incubator at 37°C for three days until the Cu and CoNPs were dried completely. The dried Cu and CoNPs were collected in Eppendorfs and stored in refrigerator at 4°C for further use. Prepared Cu and CoNPs were green and pink in color, respectively.

2.3 Characterization of Co and CuNPs

The characterization of Cu and CoNPs was performed using ultraviolet-visible spectroscopy (UV-vis; UV-1700, Shimadzu) and Fourier-transform infrared spectroscopy (FTIR; Bruker Alpha Platinum ATR). For UV-vis spectroscopy, Co and CuNPs solutions were used by absorbing light emitted by substances, thus showing the UV-visible absorption spectrum of Co and CuNPs\(^{14}\). FTIR study was performed using the dry powder form of prepared NPs and showed different functional groups present in the Co and CuNPs\(^{15}\).

2.4 Antibacterial assay of Co and CuNPs

Agar well diffusion method was used to study antibacterial study of Cu and CoNPs\(^{12}\). Muller Hinton Agar (MHA) was prepared and autoclaved. media and Petri plates were incubated for 24 hours at 37°C. Dried CoNPs of both, *B. subtilis* and *E. coli* were prepared in solution form by adding 100 mg of CoNPs and CuNPs, each into 1 mL sterilized distilled water. Rifampicin (100 µg/mL) was used as positive control.

6 mm wells were made in each petri dish containing MHA with the help of a sterilized cork borer. An antibacterial assay was performed against two bacterial isolates, *B. subtilis* and *E. coli*. Using sterilized cotton bud, fresh suspensions of *E. coli* and *B. subtilis* were spread on plates. 100 µL of *E. coli* CoNPs and *B. subtilis* CoNPs were added to the respective wells. 100 µL autoclaved distilled water and rifampicin were added as negative and positive controls, respectively. Same procedure was repeated for
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2.4 Preparation of Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC)

Minimum inhibitory concentration (MIC) is defined as the minimum concentration of any agent that can inhibit the bacteria’s growth. The MIC of both E. coli and B. subtilis CoNPs was done by using NPs solution against E. coli and B. subtilis. In brief, 12 test tubes were taken for each of two E. coli and B. subtilis CoNPs and 1 mL sterilized PDB was added. The optical density (O.D) of each culture was adjusted to 0.5 McFarland turbidity standard. For E. coli CoNPs, 10 µL of F. oxysporum was poured in 6 test tubes and 5, 10, 15, 20, and 25 µL of the E. coli CoNPs solution was added. Likewise, 10 µL of B. subtilis was added into other set of 6 test tubes and 5, 10, 15, 20, and 25 µL of the E. coli CoNPs solution was added. Blanks with bacterial inoculum but without CoNPs were run in parallel for each of the two sets of test tubes. The same process was repeated for B. subtilis and E. coli CuNPs. The test tubes were left in incubator at 37°C for 24 hours under shaking conditions and results were noted.

MBC is defined as the concentration that kills 99.99% of the original inoculum. For this purpose, nutrient agar was prepared, poured in petri plates and allowed to solidify. 10 µL from MIC test tubes with no visible turbidity was spread evenly on plates in replicates. The plates were incubated for 24 hours at 37°C for 24 hours results were recorded.

2.5 Determination of Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC)

Minimum inhibitory concentration (MIC) is defined as the minimum concentration of any agent that can inhibit the bacteria’s growth. The MIC of both E. coli and B. subtilis CoNPs was done by using NPs solution against E. coli and B. subtilis. In brief, 12 test tubes were taken, and 1 mL of sterilized nutrient broth was added to each test tube. Bacterial density was adjusted to 0.5 McFarland turbidity standard and added to test tubes. For E. coli CoNPs, 10 µL of E. coli was poured into the first set of 6 test tubes and 5, 10, 15, 20 and 25 µL of the E. coli CoNPs solution was added. Likewise, 10 µL of B. subtilis was added into other set of 6 test tubes and 5, 10, 15, 20, and 25 µL of the E. coli CoNPs solution was added. Blanks with bacterial inoculum but without CoNPs were run in parallel for each of the two sets of test tubes. The same process was repeated for B. subtilis and E. coli CuNPs. The test tubes were left in incubator at 37°C for 24 hours under shaking conditions and results were noted.

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2.6 Antifungal activity of Co and CuNPs

Antifungal activity of the Co and CuNPs was performed against two fungal strains, i.e., F. oxysporum and T. viridi. Potato dextrose broth (PDB) was prepared, autoclaved and transferred into 2 separate falcon tubes. Already maintained fungal cultures of F. oxysporum and T. viridi were sub-cultured in PDB using an inoculating loop in a laminar airflow. The cultures were placed in incubator at 37°C for 24 hours.

Potato dextrose agar (PDA) was prepared, autoclaved, and poured into four sterilized petri dishes. 6 mm wells were made and fresh cultures of F. oxysporum and T. viridi were spread uniformly on respective petri dishes in replicates. Afterwards, 100 µL of E. coli and B. subtilis CoNPs were added into wells of each plate. Wells containing 100 µL autoclaved distilled water and voriconazole solution were marked as negative and positive controls, respectively. Same procedure was performed for the CuNPs. The plates were left for 10 minutes before transferring them into incubator at 37°C for 24 hours and the results were noted.

2.7 MIC and Minimum fungicidal concentration (MFC) for fungi

The MIC of both E. coli CoNPs and B. subtilis CoNPs against F. oxysporum and T. viridi was performed by preparing NPs solution. In brief, 12 test tubes were taken for each of two E. coli and B. subtilis CoNPs and 1 mL sterilized PDB was added. The optical density (O.D) of each culture was adjusted to 0.5 McFarland turbidity standard. For E. coli CoNPs, 10 µL of F. oxysporum was poured in 6 test tubes and 5, 10, 15, 20, and 25 µL of the E. coli CoNPs solution was added. Likewise, 10 µL of T. viridi was added into the second set of 6 test tubes and 5, 10, 15, 20, and 25 µL of the E. coli CoNPs solution was added into five test tubes. One test tube in each set containing fungal inoculum in PDB but without respective NPs was used as control. Exact process was repeated for B. subtilis CoNPs. These test tubes were left in the incubator at 37°C for 24 hours under shaking conditions and results were noted. PDB was prepared, autoclaved, and poured into plates for MFC determination until solidification occurred 10 µL from the MIC test tubes with no visible turbidity was spread evenly on prepared plates. The plates were incubated for 24 hours at 37°C and results were noted.

2.8 Statistical analysis

Data was analyzed using mean and standard error of mean (SEM). One-way analysis of variance (ANOVA) followed by the Post Hoc Tukey test was used to determine the statistical significance at p ≤ 0.05.

3 Results

3.1 Visual observation (Color change)

Visual characterization showed a visible change in the color from red to magenta. Later these NPs were dried, and their color changed from magenta to light burgundy, proving the formation of CoNPs (Fig. 1a). Cell free supernatant of B. subtilis and E. coli and CuSO4 salt were incubated for 48 hrs. Color change from blue to green confirmed the formation of CuNPs (Fig. 1a).

3.2 Characterization of Co and CuNPs

Following the visual observation, Co and CuNPs were characterized by ultraviolet-visible spectroscopy (UV-Vis) and Fourier transform infrared techniques (FTIR).

3.2.1 UV-Vis Spectroscopy

UV-vis spectroscopy was employed to determine the absorption of Co and CuNPs at room temperature (Fig. 1). The peaks were observed in wavelengths ranging from 200–750 nm. For E. coli CoNPs strong and weak peaks were observed at 300 nm and 650 nm. For B. subtilis CoNPs strong and weak peaks were observed at 350 nm and 683 nm (Fig. 1a). UV-Vis absorption spectrum of E.
Fig. 1  (a) Visual representation of biogenically synthesized nanoparticles. UV-vis spectrum of *E. coli* and *B. subtilis* based biogenic synthesis of (b) CoNPs and (c) CuNPs. Highest peaks for *E. coli* and *B. subtilis* CoNPs were observed at 300 and 350 nm, respectively. UV-Vis spectrum of *E. coli* and *B. subtilis* CuNPs showed peaks at 550 and 625 nm, respectively.

Fig. 2  FTIR spectra of (a) *E. coli* CoNPs, (b) *B. subtilis* CoNPs, (c) *E. coli* CuNPs and (d) *B. subtilis* CuNPs.
**Table 1** Antibacterial assay of *E. coli* and *B. subtilis* CuNPs and CoNPs.

<table>
<thead>
<tr>
<th>Biogenic NPs</th>
<th>Concentrations</th>
<th><em>E. coli</em> Zone of inhibition (ZOI) in mm (Mean ± S.E)</th>
<th><em>B. subtilis</em> Zone of inhibition (ZOI) in mm (Mean ± S.E)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive control (Rifampicin)</td>
<td>100 µg/mL</td>
<td>10.0 ± 0.0&lt;sup&gt;0&lt;/sup&gt;</td>
<td>8.0 ± 0.0&lt;sup&gt;0&lt;/sup&gt;</td>
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<tr>
<td><em>E. coli</em> CoNPs</td>
<td>100 mg/mL</td>
<td>16.3 ± 0.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>19.3 ± 0.7&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td><em>B. subtilis</em> CoNPs</td>
<td>100 mg/mL</td>
<td>15.3 ± 0.7&lt;sup&gt;d&lt;/sup&gt;</td>
<td>16.3 ± 0.3&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>E. coli</em> CuNPs</td>
<td>100 mg/mL</td>
<td>22.7 ± 0.8&lt;sup&gt;d&lt;/sup&gt;</td>
<td>18.7 ± 0.9&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>B. subtilis</em> CuNPs</td>
<td>100 mg/mL</td>
<td>25.5 ± 0.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>29.7 ± 0.5&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Green Synthesized CuNP</td>
<td>1 mg/mL</td>
<td>12.7 ± 0.4</td>
<td>14.2 ± 0.8&lt;sup&gt;10&lt;/sup&gt;</td>
</tr>
<tr>
<td>Green Synthesized CoNP</td>
<td>25 mg/mL</td>
<td>20.0 ± 0.1</td>
<td>22.0 ± 0.2&lt;sup&gt;10&lt;/sup&gt;</td>
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**Table 2** MIC and MBC of *E. coli* and *B. subtilis* CoNPs and CuNPs.

<table>
<thead>
<tr>
<th>Bacterial isolates</th>
<th>Positive control (Rifampicin) (100 µg/mL)</th>
<th>B. subtilis CoNPs</th>
<th><em>E. coli</em> CuNPs</th>
<th>B. subtilis CuNPs</th>
<th><em>E. coli</em> CoNPs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MIC</td>
<td>MBC</td>
<td>MIC</td>
<td>MBC</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>8.0 ± 0.0&lt;sup&gt;0&lt;/sup&gt;</td>
<td>6.7 ± 0.3</td>
<td>8.7 ± 0.3</td>
<td>6.3 ± 0.5</td>
<td>5.3 ± 0.5</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>10.0 ± 0.0&lt;sup&gt;0&lt;/sup&gt;</td>
<td>4.7 ± 0.3</td>
<td>5.7 ± 0.3</td>
<td>7.3 ± 0.5</td>
<td>8.3 ± 0.5</td>
</tr>
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</table>

**3.2.2 Fourier transform infrared techniques (FTIR)**

The physical and chemical composition of the CoNPs was studied by Fourier transform infrared (FTIR) spectroscopy. The FTIR spectra were recorded between 500 and 4000 cm<sup>-1</sup>. For *E. coli* CoNPs (Fig. 2a), the notable peaks were observed at 827, 1072, 1398, 1558, and 3261 cm<sup>-1</sup>. Peaks at 827, 1072, and 1398 cm<sup>-1</sup> showed the –OH bond, C-N, and C-O bond stretching in amino acid. The peak at 1558 cm<sup>-1</sup> showed the –OH bond stretch. A peak at 3261 cm<sup>-1</sup> showed the presence of the OH group. Notable peaks observed in the FTIR graph for *B. subtilis* CoNPs were at 827, 1072, 1398, 1558, and 3261 cm<sup>-1</sup>, as shown in Fig. 2b. The peaks at 1031 and 1155 cm<sup>-1</sup> showed the presence of aromatic groups. The peak at 1398 cm<sup>-1</sup> showed the stretching due to the vibration of the nitrogen group. The peak at 1558 cm<sup>-1</sup> showed the formation of the alkene group, the peak at 2320 cm<sup>-1</sup> indicated carbonyl group presence and the peak at 3295 cm<sup>-1</sup> showed the –OH bond at the NPs surface.

FTIR showed different peaks for *E. coli* CuNPs observed 669, 827, 1072, 1338, 1398, 1558, 1573, 1633, 3061, 3261, 3447 and 3566 cm<sup>-1</sup> via FTIR (Fig. 2c). A peak at 3447 cm<sup>-1</sup> was due to the presence of N-H and O-H groups. The peak at 1633 cm<sup>-1</sup> indicated the N-H group. Peak at 1558 cm<sup>-1</sup> showed the presence of carboxyl group. Peak observed at 1398 cm<sup>-1</sup> confirmed presence of C-N group. C-H bond due to presence of glucose residues was confirmed by peak at 1072 cm<sup>-1</sup> (Fig. 2c). *B. subtilis* showed CuNPs different peaks at 668, 825, 1337, 1391, 1456, 1471, 1506, 1558, 1683, 1734, 1867, 2177, 2358, 3564, 3585, 3447, 3851 cm<sup>-1</sup> for *B. subtilis* CuNPs (Fig. 2d). Peak at 1558 cm<sup>-1</sup> indicated presence of carboxyl group. Broad peak at 3564 cm<sup>-1</sup> showed the presence of hydrogen-bonded groups of alcohol (-OH), phenols and amines (N-H) of amide. 1643 cm<sup>-1</sup> peak showed presence of amide carbonyl groups. The peak at 668 cm<sup>-1</sup> gave information about presence of aromatic groups (Fig. 2d).

**3.3 Antibacterial assay of Co and CuNPs**

High surface-to-volume ratios and nano scale sizes are distinguishing characteristics of biogenic NPs, which improve their reaction with pathogenic microbes. *E. coli* and *B. subtilis* CoNPs were more efficient against Gram-positive (*B. subtilis*) than Gram-negative bacteria (*E. coli*) showing ZOI 19.3 ± 0.7 mm and 16.3 ± 0.3 mm, respectively. *B. subtilis* CoNPs showed ZOI of 16.3 ± 0.3 mm against *B. subtilis* and 19.3 ± 0.7 mm against *E. coli* (Table 1).<sup>14, 15</sup> *E. coli* and *B. subtilis* CuNPs showed higher ZOI against Gram-negative (*E. coli*) than Gram-positive strain (*B. subtilis*). *E. coli* CuNPs showed 22.7 ± 0.8 and 18.7 ± 0.9 mm ZOI against *E. coli* and *B. subtilis* bacterial strains, respectively. Overall, *B. subtilis* CuNPs were more potent and showed significantly higher ZOI (25.5-29.7 mm) against both pathogens in this study (Table 1).

**3.4 MIC and MBC of Co and CuNPs**

MIC and MBC of biogenically synthesized Co and CuNPs were tested against both Gram-positive and Gram-negative strains at various concentrations (5-25 mg/mL). In general, *B. subtilis* CoNPs were more susceptible to *E. coli* and
Vice versa. Likewise, *E. coli* CoNPs were more susceptible to *B. subtilis*. *B. subtilis* was the least susceptible to the biogenic *B. subtilis* Co as well as CuNPs with MIC (6.3-8.7 mg/mL) and MBC values of 8.3-8.7 mg/mL (Table 2). Similarly, *E. coli* was least susceptible to *E. coli* Co and CuNPs with MIC (7.3-7.5 mg/mL) and MBC values 8.3-8.8 mg/mL and MBC value of 6.7 mg/mL. Generally, *B. subtilis* CuNP were observed to be more effective with lowest MIC (4.3-6.3 mg/mL) and MBC (5.3 mg/mL) values against both isolates (Table 2).

### 3.5 Antifungal activity of biogenically synthesized Co and CuNPs

Antifungal activity of *E. coli* and *B. subtilis* Co and CuNPs was studied at three concentrations 5.0, 2.5, and 1.5 mg/mL against *F. oxysporum* and *T. viridi*. Antifungal activity of the CoNPs against fungal strains *F. oxysporum* and *T. viridi* was shown in Table 3. Overall, a concentration-dependent decrease in antifungal activity of NPs was observed against fungal strains. *B. subtilis* CoNPs showed strong inhibition of *F. oxysporum* with significantly \( p \leq 0.05 \).
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0.05) bigger ZOI (30.7 ± 0.3) at 5.0 mg/mL concentration. *E. coli* CuNPs were stronger with larger ZOI (27.6 ± 0.0) against *T. viridi* at same low concentration. In general, among the two tested fungal isolates, *T. viridi* was more susceptible to prepared NPs at low concentration (Table 3).

*B. subtilis* CuNPs showed slight growth with inhibition (S.G) for each of three tested concentration against *F. oxysporum* while 25.6 ± 0.5 ZOI was observed against *T. viridi* at 5.0 mg/mL concentration. Overall, *B. subtilis* CoNP and *E. coli* CuNPs showed affective antifungal activity against both isolates (Table 3).

### 3.6 MIC and MFC of Co and CuNPs

The prepared biogenic Co and CuNPs were tested for MIC and MFC values against two fungal strains (*F. oxysporum* and *T. viridi*). MIC and MFC values of *E. coli* and *B. subtilis* Co and CuNPs are listed in Table 4. MIC values of *B. subtilis* and *E. coli* CoNPs against *F. oxysporum* ranged from 25-26 and 116-150 µg/mL and against *T. viridi* were 20-25 and 118-125 µg/mL, respectively. MFC values of *B. subtilis* and *E. coli* CoNPs showed that *B. subtilis* CoNPs were more susceptible against both fungal isolates (116-118 µg/mL and 125-150 µg/mL) (Table 4). Similarly, the MIC value of *E. coli* and *B. subtilis* CuNPs against *F. oxysporum* were 20-21 and 125-126 µg/mL while against *T. viridi* ranged from 20-23 µg/mL. MFC values of *E. coli* and *B. subtilis* CuNPs ranged from 21-23 and 125-128 µg/mL (Table 4). Overall, *B. subtilis* Cu and *E. coli* CoNPs were most effective against both tested fungal isolates with lowest MIC and MFC values (Table 4).

### 4 Discussion

Microbial synthesis of metal nanoparticles can take place either intracellularly or extracellularly. For intracellular synthesis of NPs, additional steps such as reactions with appropriate detergents and ultrasound treatment is required to release the synthesized NPs. However, extracellular biosynthesis is comparatively cheap and requires simpler downstream process leading to large-scale production of NPs with wider potential applications. Therefore, current study focused on extracellular synthesis of metal NPs using bacterial isolates [18], such as *B. subtilis* and *E. coli*. Previous study has reported the greater potential of *B. subtilis* for synthesis of metal NPs. Likewise, El-Shanshory [18] showed that *E. coli* bacteria reaction with highly reactive metal oxide NPs results in significant increase in membrane permeability, causing the bacteria incapable of regulating transport through the membrane leading to ultimate cell death. Further, metal depletion may form irregular pits in bacterial cell membrane affecting its permeability, primarily caused by progressive release of membrane proteins and LPS molecules.

Biologically synthesized CoNPs and CuNPs have been proved to be very effective and valuable compounds. They have excellent antimicrobial and antiviral activity. Biogenic synthesis of CoNPs and CuNPs is a more cost-effective and eco-friendly approach. Maximum absorption peaks of *E. coli* and *B. subtilis* CoNPs observed at 300 and 350 nm via UV-Vis spectroscopy were similar to the studies by Rahman et al. [19]. The authors showed an absorption spectrum at 319 nm as the characteristic absorption peak of CoNP. UV-Vis absorption spectrum exhibited distinct absorption peaks at 625 nm for *B. subtilis* and 550 nm for *E. coli* CuNPs. Similarly, UV-Vis spectrum observed for biogenic (algal-based) CuNPs from *Botryococcus braunii* showed peak at 258-490 nm by Rahman et al. [19], who showed maximum absorption peak at 258 nm. Likewise, Noman et al. [20] biosynthesized *Escherichia* based CuNPs and showed UV-Vis absorption spectrum in the range of 300-800 nm.

The FTIR results of *E. coli* and *B. subtilis* CoNPs showed the presence of functional groups i.e., phenols, acids, protein, and polypeptides which confirmed the biogenic synthesis of CoNPs. Previously, Iqbal et al. [21] also showed the presence of –CH and OH groups in prepared CoNPs and reported that they play an essential role in their stabilization and reduction. Iqbal et al. [21] reported Peaks at 2919 and 1729 cm⁻¹ resulted in stretching of C-H and C=O alkane and alkene groups respectively that are involved in the formation of CoNPs. FTIR spectrum of *E. coli* CuNPs confirmed peak at 3447 cm⁻¹ due to the presence of N-H and O-H groups. Peaks at 1633 cm⁻¹, 1585 cm⁻¹, 1072 cm⁻¹ and 1398 cm⁻¹ were due to the presence of N-H group, carboxyl group, C-H bond and C-N group, respectively. In another study by Slavin et al. [22], biogenic (plant-based) CuNPs from *Bougainvillea* plant flowers extract showed FTIR peaks at 3365, 1643, and 602 cm⁻¹ showed groups of alcohol (-OH), phenols, amines (-N-H), carbonyl group and aromatic groups, respectively.

Antibacterial activity of prepared *E. coli* and *B. subtilis* CoNPs showed inhibition zones against *B. subtilis* and *E. coli* at 100 mg/mL concentration. These results are in accordance with the studies by Stanic et al. [23], who reported that CoNPs showed 6 mm ZOI against *E. coli*. The significant MIC values of both *E. coli* and *B. subtilis* CoNPs showed that *B. subtilis* CuNPs showed effective MIC and MBC values compared to other NPs where a higher MIC as well as MBC values were observed. The MBC values for both *E. coli* and *B. Subtilis* were in the range of 4.3-8.8 µg/mL.

Antifungal activity showed that both *E. coli* CuNPs and *B. subtilis* CoNPs were better antifungal agents. These results corroborate with findings by Athawale et al. [24]. Authors studied the antifungal activity of CoNPs and observed 12 mm ZOI against *C. albicans*. In a study done by

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Rehman et al.\textsuperscript{24}, the antifungal assay of CoNP against \textit{C. albicans} at 1 mg/mL concentration showed promising antifungal potential. Likewise, the growth inhibitory potential of Gram-positive \textit{B. subtilis} and \textit{E. coli} CuNPs against Gram-negative \textit{E. coli} and \textit{B. subtilis} bacterial strains corroborate with findings by Amer et al.\textsuperscript{25}. Similarly, Daniel et al.\textsuperscript{26} reported 9 mm ZOI at 10 \(\mu\)g/mL concentration of biogenic CuNPs prepared from \textit{Dodonaea viscosa} extract against \textit{E. coli}.

MIC and MFC values of \textit{E. coli} and \textit{B. subtilis} Co and CuNPs against fungal strains showed MIC values ranged from 20-26, while MFC values ranged from 116-150 \(\mu\)g/mL. Previously, Tahvilian et al.\textsuperscript{27}, biogenically synthesized (plant based) CuNPs from \textit{Allium saralicum} leaves and reported 4 mg/mL MIC against \textit{E. coli} and 1 mg/mL against \textit{B. subtilis}. Likewise, Ashajyothi et al.\textsuperscript{28} biogenically prepared (microbial based) CuNPs from \textit{Enterococcus faecalis} and checked their antifungal activity against \textit{C. albicans} as well as \textit{C. neoformans}. The authors observed 30, 32 mm ZOI against \textit{Candida albicans} and \textit{C. neoformans}, respectively. In another study, Rasool et al.\textsuperscript{29} studied MIC and MFC of CuNPs against Fluconazole-resistant \textit{C. albicans} and reported values of 2.5 and 2 mg/mL, respectively. Recently, Zhao et al.\textsuperscript{30} also reported MIC and MFC of biologically synthesized CuNPs against \textit{C. guilliermondii} as 2 and 4 mg/mL, respectively. Additionally, Tahvilian et al.\textsuperscript{27} published MIC and MFC of 2 and 8 mg/mL by biologically synthesized CuNPs against \textit{C. albicans}.

5 Conclusion

Nanoparticles (NPs) have remarkable properties that make them useful in fields of medicine, agriculture, environment, and energy\textsuperscript{31}. It was observed that NPs are more efficient against bacteria and fungi as compared to antibiotics and antifungal drugs. Owing to bacterial resistance to specific antibiotics, NPs have gained attention as more efficient therapeutic approach against drug-resistant bacteria and fungi\textsuperscript{32}. Regarding antibacterial potential, among two types of biogenically synthesized NPs (\textit{E. coli} and \textit{B. subtilis}), results showed that \textit{B. subtilis} CuNPs showed significantly higher antibacterial potential with bigger ZOI and lowest MIC-MBC values. Antifungal assay showed that \textit{B. subtilis} CoNPs and \textit{E. coli} CuNPs were better antifungal agents with lowest MIC and MFC values. Overall, \textit{B. subtilis} Co, CuNPs showed significant \((p<0.05)\) antimicrobial potential. The current study concluded that biogenically synthesized NPs are effective antimicrobial agents compared to antibiotics and could be investigated further for toxicity evaluation for various environmental and biomedical applications. Also, the CuNPs and CoNPs in this study have to clarify low cytotoxicity by cell experiments for future practical use.

Author Contributions

IL designed and supervised the study. RA performed the experimental work and wrote the first draft. UH, AL, AB helped in data analysis. SS, SN, MU, MM and FR helped in revision. IL corrected the final draft. All authors approved the final version of manuscript.

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Biosynthesis and Antibacterial activity of Co-Cu Nanoparticles against Microorganisms

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