ABSTRACT
Calcium phosphate nanoparticles (CPNPs) have been synthesized by chemical precipitation method and were characterized by UV-visible spectroscopy (UV-vis), Fourier transform infrared spectroscopy (FTIR), and scanning electron microscopy (SEM). The antibacterial activity against multi-drug resistant (MDR) gram-negative bacteria *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Klebsiella pneumonia* (*K. pneumonia*) was performed by well diffusion method, using different concentrations of CPNPs and different combinations of CPNPs with ciprofloxacin (CIP) (CIP-CPNP100, CIP-CPNP50, and CIP-CPNP25). The minimum inhibitory concentration (MIC) and minimum bacterial concentration (MBC) were evaluated by the broth dilution method and optical density. Cytotoxicity of nanoparticles was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on polymorphonuclear cells. Results indicated that synthesized CPNPs sized 28.02 ± 3.2 nm in diameter as average, with distorted spherical shape appears as agglomerates. CPNPs showed no antibacterial activity against MDR bacteria, but combining them with CIP recorded antibacterial activity represented by inhibition zone against MDR bacteria. It was found that the inhibition zone increases when the concentration of CIP and particle size decreases. The MTT assay reveals the acceptable toxicity of the synthesized nanoparticles. The present study can be helpful to formulate nano-drug conjugates as antimicrobial agents in various fields of medical research.

Keywords: Antibacterial, Calcium phosphate, Hydroxiapatite, Multi drug resistant, Nanoparticles.

INTRODUCTION
The new field of nanotechnology, viz., nanomedicine, introduces a rapid change in the medical field by opening a new version that improves human health. Nowadays, nanotechnology is a promising area for solving various problems in health care and medicine. It is still evolving as a new field of medicine that uses nanoparticles in diagnostics, imaging, genes, and drug delivery. Increasing antibiotic resistance in bacterial strains is a serious and growing threat to human health as MDR bacteria cause millions of infections each year. The antibiotic resistance of bacteria is a global health problem that is continually expanding and is recognized as a medical problem that increases morbidity and mortality rates, which implies the length of hospital stays, as well as, cost and bad prognosis. In fact, the speed at which bacteria are establishing resistance to current antibiotics is faster than the development of new molecules with antimicrobial features. Unfortunately, it is very difficult to identify new bacterial targets that can be used to develop new classes of antimicrobial agents that are safe and effective. Nanotechnology opens new possibilities, allowing new solutions with old resources. Nanoparticles have emerged as novel antimicrobial agents owing to their effectiveness in small doses large surface area to volume ratio, minimal toxicity, and lack of side effects. Numerous studies have applied the nanoparticles (NPs) as the drug carriers, some features that nanocarriers can incorporate in drug delivery systems promoted dissolution of drugs, improved solubility and stability, enhanced absorption of drugs, increased drug targeted performance, controlled release capability of drugs, and reduced side effects. As an important inorganic mineral, calcium phosphate (CP) is a natural biomineral, and therefore, possesses excellent biocompatibility due to its chemical similarity to human hard tissue (bone and teeth). Calcium phosphate has been extensively studied throughout the last few decades for their role in bone and teeth mineralization, as well as, in pathological calcifications. Recently, CPNPs have been widely used in biomedical applications due to their good biocompatibility and bioactivity, such as, non-viral gene, drug delivery vectors, and a carrier in biological systems.

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MATERIAL AND METHODS

Preparation of CPNPs using Chemical Precipitation

CPNPs were synthesized using a chemical precipitation method. A 100 mL of 0.6 M Ca(NO\textsubscript{3})\textsubscript{2}•4H\textsubscript{2}O (Thomas Baker) was vigorously stirred at room temperature, then 100 mL of 0.4 M of (NH\textsubscript{4})\textsubscript{2}HPO\textsubscript{4} (Thomas Baker) was added dropwise as a reducing agent to Ca(NO\textsubscript{3})\textsubscript{2}•4H\textsubscript{2}O solution for 40 minutes (2.5 mL/min). The pH of the final mixture reaction was adjusted to 10 by adding 200 mL of 0.1 M of sodium hydroxide (Thomas Baker), which acts as a precipitating agent through the stirring process. The mixture was left under a magnetic stirrer overnight. A white precipitate appeared in the beaker. The precipitate was vacuum dried and washed by distilled water five times, then it was further dried in an electric oven at a temperature of 40°C for 5 hours.

Characterization of CPNPs

By UV-Vis Spectrophotometer Spectroscopy

The preliminary detection of the formation of CPNPs was done in UV-visible spectrophotometer (Shimadzu UV-1800, Japan), by scanning the absorbance spectra of the solution in the range 285 to 600 nm.

By Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR spectroscopy (Alpha Platinum-ATR) is a widely used approach that identifies functional groups of the NPs with the use of infrared radiation beams. An infrared spectroscopy measures infrared radiation absorption, which is made with every one of the bands in the molecule, thus, provides a spectrum that is applied as percent transmittance (%) vs. wavenumber (cm\textsuperscript{-1}); infrared spectra used in the wave range of 400–4,000 cm\textsuperscript{-1}.

By SEM

The SEM (LEO 982 SEM) is utilized to determine the size, shape, and morphology of the produced nanoparticles through the magnification power of 200.kx.

Preparation of NPs-Antibiotic Combinations

The CIP solution prepared as 25 mg/mL with distilled water, CIP-CPNP combinations were prepared in different weight ratios (WR) of 1:4, 1:2, and 1:1, for CIP-CPNP100, CIP-CPNP50, and CIP-CPNP25, respectively. The reaction was conducted under continuous magnetic stirred for 3 hours at room temperature, then the solutions left undisturbed overnight. Those samples were subjected to characterization by SEM and FTIR by adding a few drops of these solutions on the slide and left to dry at room temperature.

Antibacterial Assay

The bacterial isolates were collected from Al-Imamain Alkadhimain Medical City, Baghdad, Iraq. The two bacterial species were identified as MDR, depending on antibiotic susceptibility testing, using the disk diffusion method. The antibacterial activity of bare CPNPs and CIP-CPNP (100, 50, and 25) were tested against gram-negative bacteria P. aeruginosa and K. pneumoniae by well diffusion method.
incubated overnight at 37°C. 100 µL of CPNPs (25, 15, 10, and 5 mg/mL), and CIP (CIP-CPNP100, CIP-CPNP50, and CIP-CPNP25) were added to the first well of each row and incubated at 37°C/48 hours. To each well, 28 µL of 2 mg/mL of MTT solution was added, cells were incubated at 37°C for 1.5 hours. The MTT solution was aspirated, crystals remain in wells were solubilized by adding 130 µL of dimethyl sulphoxide (DMSO), followed by incubation at 37°C/15 minutes, with shaking. The cell viability was detected by measuring OD at 492 nm using a spectrophotometer. The cell viability was calculated by using the following equation:

\[
\text{Cell viability} \% = (\frac{\text{OD sample}}{\text{OD control}}) \times 100
\]

Statistical Analyses
Statistical analyses were performed using SPSS software version 16.0 (SPSS, Chicago, IL, USA). Analysis of variance (ANOVA) test was used to compare means between different groups. A p-value of 0.05 or less was considered statistically significant.

RESULTS
CPNPs Characterizations
UV-Vis Spectrophotometer Spectroscopy Results
The prepared solution of CPNPs was analyzed using scanning UV-vis spectroscopy, with wavelength values ranging from 285 to 600 nm. Results showed that the maximum absorbance of synthesized CPNPs was 0.916 at wavelength 296 nm, indicating that maximum λ is observed within the UV-vis region (Figure 1).

FTIR Data Analysis
The chemically synthesized CPNPs were analyzed by Alpha Platinum-ATR FTIR, UK. The FTIR spectra showed the characteristic absorption peaks of CPNPs (Figure 2). The sample spectra showed the characteristic bands; the band was observed at 3,499.21 cm⁻¹ to the stretching mode vibration of the hydroxyl group (OH⁻), respectively, bands at 1,020.98, 599.23, and 560.44 cm⁻¹, are attributed to the phosphate group (PO₄³⁻). Three bands at 3,383.75, 1725.22, and 1645.49 cm⁻¹ are attributed to water molecules. In addition, carbonate content was seen, CO₃²⁻ peaks were detected at 1,521.83 cm⁻¹, indicating the presence of carbonate apatite. According to this spectra, it is clear that the chemically synthesized powder is certainly nano-hydroxyapatite (n-HAP).

Scanning Electron Microscopy
The synthesized CPNPs were analyzed by LEO 982 SEM to determine size, shape, and morphology. Observations on magnification power 200 kx and 1.04 μm view field showed the presence of distorted spherical NPs ranging in size 23.81 to 32.74 nm in diameter, with a mean size of 28.02 ± 3.2 nm. Agglomerates consisting of several NPs were observed; it is obvious that CPNPs are coarse and exhibited protuberances on their surfaces (Figure 3).

CIP-CPNPs Combinations Characterizations
Combinations of CIP and CPNPs were prepared in three different WR, i.e., 1:1, 1:2, and 1:4, viz., CIP-CPNP25, CIP-CPNP50, and CIP-CPNP100, respectively, and were
Antibacterial activity of CPNPs

FTIR Spectroscopy of CIP-CPNP

The interaction of CIP with CPNPs was studied by FTIR analysis. The typical FTIR of CIP, CIP-CIPNP100, CIP-CIPNP50, and CIP-CIPNP25 are presented in Figure 4. Regarding the FTIR spectra of CIP, visible peaks appear at a frequency of 3,330.02 cm⁻¹, ascribed to hydroxyl group stretching mode vibration, and 1,271.71 cm⁻¹ assigned to bending vibration of the hydroxyl group. The characteristics peaks at 1,630.13 and 1,029.28 cm⁻¹, ascribed to the stretching vibration of the quinoline group and stretching vibration of the fluorine group, respectively. While, the chrematistics peak at 1,490.53 cm⁻¹ was assigned to stretching vibration of the carbonyl group. Regarding the FTIR spectra of CIP-CPNP100, CIP-CPNP50, and CIP-CPNP25, the characteristic peaks are almost the same as that of CIP.

SEM of CIP-CPNP

Qualitative analysis of NPs behavior and morphology was studied by SEM. Results reveal that CIP-CPNP100 in 1:4 WR gave the smallest size, mean size 29.98 ± 9.8 nm, followed by CIP-CPNP50 in 1:2 WR and CIP-CPNP25 in 1:1 WR, 32.69 ± 6.1 and 42.3 ± 5.9 nm, respectively. That means increment in CIP concentration will increase the size of the nanoparticles. A significant difference was detected in NPs’ size between CIP-CPNP100 and CIP-CPNP25 p value < 0.05. Also, there is a significant difference between bare CPNP and bare CIP-CPNP25, p-value < 0.05, while, no significant differences were detected among other studied groups, p-value < 0.05. Regarding the morphology of CPNPs conjugated to CIP, it is obvious that those conjugated with CIP (100, 50, and 25) showed to appear in bigger agglomerates, and particle shape looks less distorted as compared to bare CPNPs (Figure 5).

Antibacterial Activity

Antibacterial Activity of different Concentrations of Bare CPNPs

The two isolates of bacteria were identified as resistant to CIP, depending on antibiotics susceptibility testing, using the disk diffusion method. The antibacterial activity of different concentrations of bare CPNPs (25, 15, 10, and 5 mg/mL) was evaluated against the two MDR isolates, P. aeruginosa, and K. pneumoniae, using the well diffusion method. Results revealed the absence of antibacterial activity

characterized by FTIR and SEM to verify the conjunction success.
Antibacterial activity of CPNPs

The three CIP-CPNP combinations 1:1, 1:2, and 1:4 (i.e., CIP-CPNP25, CIP-CPNP50, and CIP-CPNP100), respectively, were verified for their antibacterial activity against gram-negative bacteria *P. aeruginosa* and *K. pneumoniae*, using the well diffusion method. Regarding gram-negative bacteria, *P. aeruginosa* showed 22.33 ± 0.58, 21.67 ± 0.58, and 15.67 ± 0.58 mm inhibition zone for CIP-CPNP100, CIP-CPNP50, and CIP-CPNP25, respectively (Figure 6A). While, *K. pneumoniae* showed 25.56 ± 0.58, 25.33 ± 0.58, and 16.67 ± 0.58 mm inhibition zone for CIP-CPNP100, CIP-CPNP50, and CIP-CPNP25, respectively (Figure 6B).

Moreover, CIP and CPNPs don’t have antibacterial activity, only when they delivered together. From the results mentioned above, a highly significant difference in antibacterial activity was detected in CIP-CPNP25 comparing to treated CIP-CPNP100, and CIP-CPNP50, p < 0.001, for the two studied isolates. While, no significant difference in antibacterial activity was detected between CIP-CPNP100 and CIP-CPNP50 for the two studied isolates, p > 0.05 (Table 1).

**Minimum Inhibitory Concentration (MIC) and Minimum Bacterial Concentration (MBC)**

The CIP-CPNP100 was diluted by the double dilution method in five dilutions (1/2, 1/4, 1/8, 1/16, and 1/32), final CIP-CPNP100 concentration in each dilution was 12.5-50, 6.25-25, 3.12-12.5, 1.56-6.25, and 0.78-3.12 mg/mL, respectively. After 24 hours of incubation at 37 ºC, results revealed that 1.56-6.25 mg/mL of CIP-CPNP100 is the MIC for *P. aeruginosa* and *K. pneumoniae*. The MBC was determined to be 3.12-12.5 mg/mL for *P. aeruginosa* and *K. pneumoniae*. Cytotoxicity of CPNPs to Polymorphonuclear Cells

It was attempted to determine the CPNPs’ cytotoxic effect on human polymorphonuclear cells. The MTT assay was applied to detect cell viability by measuring the absorbency on a microtiter plate reader at 492 nm wavelength.

The non-treated polymorphonuclear cells represented the control sample. While, other samples are represented by cells treated with four different concentrations of bare CPNPs (25, 15, 10, and 5 mg/mL), and other cells treated with CIP-CPNP100, CIP-CPNP50, and CIP-CPNP25. It was found that cells viability for non-treated cells was 100% , cell viability percentage were found of other samples as 85, 85, 84, and 84% for polymorphonuclear cells treated with CPNPs (25, 15, 10, and 5 mg/mL), respectively. Also, cell viability was 84% for polymorphonuclear cells treated with different combinations of CIP-CPNP (CIP-CPNP100, CIP-CPNP50,

**Table 1:** Inhibition zone for CIP-CPNP100, CIP-CPNP50, and CIP-CPNP25 against two bacteria studied

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>CIP-CPNP100</th>
<th>CIP-CPNP50</th>
<th>CIP-CPNP25</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em></td>
<td>22.33 ± 0.58a</td>
<td>21.67 ± 0.58a</td>
<td>15.67 ± 0.58b</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>25.56 ± 0.58a</td>
<td>25.33 ± 0.58a</td>
<td>16.67 ± 0.58b</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Note: Different small letters (a and b) noticed in Table 1, indicate exiting significant differences between the above three groups
Antibacterial activity of CPNPs

and CIP-CPNP25). Those results indicate that CPNPs are biocompatible material and possesses no cytotoxic effect. Also, the addition of ciprofloxacin did not increase or decrease its cytotoxic effect (Figure 7).

**DISCUSSION**

CPNPs are good candidates for drug delivery since they are easy to synthesize and inexpensive. They are also recognized by good biocompatibility and biodegradability. In the present work, CPNPs are prepared in a chemical precipitation method. This method is very simple, produces nanoparticles in large quantities, and with high reproducibility. For the characterization of the prepared CPNPs, the following techniques were used, viz., UV-vis, FTIR, and SEM. The maximum absorbance of prepared CPNPs in the present work was recorded at wavelength 296 nm that was compatible with 295 nm that was recorded by Al-Ubaidi in 2018, who prepared colloidal CPNPs using chemical methods also.

The size of the synthesized CPNPs was performed using SEM. The SEM analysis revealed that the prepared CPNPs lie in the nanoscale level with an average particle size 28.08 ± 3.2 nm. This size is comparable to that produced by Mndal and her coworkers, 23.15 ± 2.56 nm in diameter, using the same preparation method. Another study conducted by Chandrasekar and his colleagues in 2013 prepared CPNPs with size 50 nm, using the same preparation method.

The functional group of CPNPs, observed by FTIR spectra, generated one characteristic peak of stretching mode of the hydroxyl group at 3,499.22 cm⁻¹. The stretching mode peak is similar to stretching mode 3,497 cm⁻¹ of the hydroxyl group of HAP NPs, which were prepared by Nithya and Sundaram in 2015, using the precipitation chemical method. The band presented at 1,020.98 cm⁻¹ in FTIR spectra are corresponding to the stretching mode of the phosphate group, while bands at 599.23 and 560.44 cm⁻¹ are found due to the bending mode of the phosphate group. Peaks of the phosphate group were comparable with peaks at 1,020, 600, and 560 cm⁻¹, introduced by Kojima and his group in 2018. The presence of the above functional group, hydroxyl group, and phosphate group, show the formation of CPNPs in the HAP phase according to Kojima. The observed peak at 1,521.83 cm⁻¹ corresponding to the carbonate group is due to the absorption of CO₂ from the atmosphere. On the contrary, the peaks at 1,645.49, 1,725.22, and 3,383.75 cm⁻¹ are attributed to water molecules due to the water adsorption during the synthesis process. Regarding the FTIR spectra of CIP, the characteristics peaks at 3,330.02 and 1,271.71 cm⁻¹ ascribed to stretching mode vibration and bending mode vibration of the hydroxyl group, respectively. The characteristics peaks at 1,630.13 and 1,029.28 cm⁻¹ ascribed to the stretching vibration of the quinoline group and stretching vibration of the fluorine group, respectively. While, the chromatistics peak at 1,490.53 cm⁻¹ was assigned to the stretching vibration of the carbonyl group. The presence of the above functional group of CIP is comparable with the functional group of CIP, reported by Sahoo and his team group in 2011 (Sahoo et al., 2011). In the FTIR spectra of CIP-CPNP combinations, the characteristic peaks were almost similar to that of CIP, or very slight shifting of these peaks occurred. This confirms the presence of CIP in the CPNPs system with no major peak shifts in the fingerprint region of the CIP FTIR spectra; this is thought to be due to the adsorption of CIP on the surface of CPNPs, which further strengthens the claim that the antibiotics are mostly physisorbed on the NPs’ surface.

Regarding CIP-CPNP combinations (CIP-CPNP100, CIP-CPNP50, and CIP-CPNP25), it was found that the particle sizes are 29.98 ± 9.8, 32.69 ± 6.1, and 42.3 ± 5.9 nm in diameter, respectively, indicating that increment in CIP concentration results in increment in CPNPs’ size. This finding is in accordance with other researches. For instance, silver nanoparticles, 51 nm in diameter, were increased in size 61 and 62 nm, when combined with amikacin and vancomycin, respectively. Maleki Dizaj and his group in 2017, reported an increment in the size of calcium carbonate NPs from 89.64 to 116.09 nm, when CIP was added.

Regarding resistance of bacteria to CIP, it may arise as a result of alterations in the target enzymes (DNA gyrase and topoisomerase IV), and/or changes in the efflux system. In the present study, results showed no inhibition zone for all concentrations of CPNPs. This indicates the absence of antibacterial activity of CPNPs. Most researchers verified the absence of such activity for both gram-negative and gram-positive bacteria. However, Addition CIP to the CPNPs resulted in both bacterial growth inhibition and bactericidal effect, against both gram-negative and gram-positive bacteria. It seems that CPNPs enhance the CIP entrance to the bacterial cell. This is due to the fact that binding sites of CPNPs are substantially found in the calcium ions (Ca²⁺), and because the bacterial cell surfaces are negatively charged due to the phosphoryl and carboxylate groups located on the macromolecules of the outer cell envelope. This difference in charge will lead to electrostatic interaction between the CIP-CPNP and the bacterial surface. Thus, the CPNPs will enhance the CIP entrance into the bacteria cell, that CIP will act on the target enzymes (DNA gyrase and topoisomerase IV). The antibacterial activity result is comparable with the antibacterial activity result introduced by Pan and his team in 2018, who was able to enhance the antibacterial activity of gentamicin against MDR bacteria, by using calcium carbonate NPs.

In more detail, it is found that low CIP:CPNPs ratios, i.e., 1:4 (CIP:CPNP100) and 1:2 (CIP:CPNP50), gave higher antibacterial activity, comparing to the third combination that encompasses higher concentration of CIP 1:1 (CIP:CPNP25). This is thought to be due to the fact that the first and second combinations, i.e., 1:4 and 1:2, posses significantly smaller size of CPNPs, 29.98 ± 9.8, and 32.69 ± 6.1 nm, respectively, comparing to the third combination, 1:1, 42.3 ± 5.9 nm. It is speculated that this size smallness will result in an increment in surface/volume ratio, which is one of the most important advantages of NPs that will eventually lead to enhancing the adsorption capacity, and thus, will facilitate CIP entrance.
into the bacteria cell. Our results may lead to speculation that antibiotic resistance of the three studied bacteria is mostly due to the alteration in the efflux transporters not in the CIP target enzyme, especially, that the recorded resistance is for multiple antibiotics, each one posses different target. By using MTT assays to detect CPNPs cytotoxicity, polymorphonuclear cells treated with CPNPs showed 85 and 84% cell viability even when treated with CPNPs of 25 mg/mL concentrate. Also, it is found that the addition of CIP to CPNPs does not affect the cell viability even at the height:weight ratio 1:4. Results revealed the cell viability of 84% of the cell treated with CIP-CPNP (100, 50, and 25). The results of the cell viability of CPNPs and CIP-CPNP (100, 50, and 25) are in good agreement with the result reported by Zhang and his group in 2012, who found the cell viability of the treated cell with vancomycin-HAP NPs is more than 80% even at the material concentration up to 100 mg/mL. 33 According to biological evaluation of medical devices—Part 5: Tests for in vitro cytotoxicity (ISO 10993-5:2009), if cell viability of the material is less than 70%, then it has a cytotoxic potential. However, CPNPs and CIP-CPNP exhibit cell viability greater than 80% indicating that the as-synthesized samples are cytocompatible with primary cell polymorphonuclear. 27

CONCLUSIONS

CPNPs in the phase of HAP could be successfully synthesized using the wet chemical precipitation method that was confirmed and FTIR. The size and morphology of the CPNPs were characterized by SEM analysis. The distorted spherical shape and average size of 28.02 ± 3.2 nm were confirmed through the SEM analysis. It is observed that 1:4 and 1:2 WR of CIP-CPNPs showed higher antibacterial activity against MDR bacteria, as compared to a 1:1 WR, indicating that the CIP-CPNP’s antibacterial activity increases with the decrement in CPNPs size and CIP concentration. Since the MTT assay of the bare CPNPs and CPNPs, in combination with CIP, recorded cell viability more than 80% for polymorphonuclear cells, CPNPs possess good bioactivity and acceptable toxicity that will make them a potential target for further medicinal and therapeutic application.

REFERENCES


