COMPARATIVE ASSESSMENT OF ANTIBACTERIAL AND ANTIFUNGAL ACTIVITIES OF METALLIC ION BASED FORMULATIONS

Nguyen Thi Kieu Oanh¹, Nghiem Thi Ha Lien², Nguyen Ha Nhung³, Dang Khanh Phuong⁴
¹University of Science and Technology of Hanoi - VAST
²Institute of Physics - VAST

KEYWORDS
Antibacterial activity
Antifungal activity
Copper (II) chelates
Silver(I) nanoparticles
Zinc (II) chelates

ANTIFUNGAL ACTIVITIES OF METALLIC ION BASED FORMULATIONS

The threat posed by antibiotic resistance has become particularly critical in recent years because multiple and extended resistant bacteria have become more prevalent, leading to more common hard-to-treat infections worldwide. There is an urgent need to develop new antimicrobial agents against drug-resistant strains. The present study aims to investigate the potential of the new formulations including silver (I) nanoparticles as well as copper (II) and zinc (II) chelates in their separated and complex forms in the treatment of infectious diseases. The capacity of metallic ion based systems to suppress the development of microorganism often seen on wounds, such as Gram-negative bacteria (Klebsiella pneumonia ATCC 700603, Pseudomonas aeruginosa ATCC 27853), Gram-positive bacteria (Staphylococcus aureus ATCC 29213), and fungi (Candida albicans ATCC 10231) were evaluated by microdilution assay. We showed the inhibition percentage against each bacterial strain, which can be used to calculate the minimum inhibitory concentration (MIC) of these metal ions formulations. The positive results suggested that these formulas thus could be developed for wound care in therapy.

Published: 16/5/2023
Email: jst@tnu.edu.vn
DOI: https://doi.org/10.34238/jst.7341

* Corresponding author. Email: nguyen-thi-kieu.oanh@usth.edu.vn

http://jst.tnu.edu.vn
1. Introduction

Antibiotic resistance, which is defined as the capacity of bacteria to withstand antibiotics, is one of the most severe worldwide public health issues of our day. Since the discovery of penicillin more than 90 years ago, germs have developed new types of resistance against even our most potent drugs. A crucial need is effective solutions or alternative medications to address the existing drug resistance situation and against microorganisms, particularly resistant bacteria.

Metal ions have been utilized as antibacterial treatments since ancient times due to their high toxicity to bacteria and yeast, but they do not affect human cells [1]. Some specific inorganic ions that are required to construct cell membranes, DNA and proteins in human cells [2], such as zinc and copper, have been well-known as antimicrobial metals [3]-[5]. Furthermore, non-essential metals like silver (Ag) are particularly toxic to most bacteria and have microbicidal action at shallow doses. Antimicrobial metal compounds, such as metallic surfaces and coatings, chelates, and nanomaterials, now have a wide range of uses in industry, agriculture, and healthcare. These advancements were made feasible by the finding that some metals suppress antibiotic-resistant biofilms, have synergistic bactericidal effects with other biocides, selectively block metabolic pathways, and kill multidrug-resistant bacteria.

The project aims to examine the potential of the metal ions, including silver, copper and zinc and their complex under the new formulations as active agents in sanitizing and wound healing. The fundamental idea is to test the antibacterial effectiveness of silver(I) nanoparticles, zinc(II) and copper(II) chelates/complexes on microorganisms that have often been found on wounds. They include gram-negative bacteria (Klebsiella pneumonia ATCC700603 and Pseudomonas aeruginosa ATCC 27853), gram-positive bacteria (Staphylococcus aureus ATCC 29213), and fungi (Candida albicans ATCC10231). The microdilution assay was applied to test the antimicrobial activities of these formulations against four microorganism. The optical density (OD) data at 600 nm of a 96-well plate were gathered to calculate the % inhibition of metal nanoparticle complex against bacterial strains as well as the minimum inhibitory concentration (MIC). Through this study, we also answered whether there is a synergetic effect of zinc and copper chelates with the ratio 10:1, respectively, against these four microbial strains. This is a preliminary investigation to open the way for the development of new metals ions-based products in wound care.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

AgNO₃, CuSO₄.5H₂O, ZnCl₂, urea (N₂H₄CO), disodium ethylenediaminetetraacetate dehydrate (C₁₀H₁₄N₂Na₃O₈, EDTA.2Na.2H₂O), ethyleneglycol, polyvinylpyrrolidone were purchased from Xilong Chemical company, China. Tryptic soy broth (TSB) was provided by Sigma Aldrich, Germany.

For bioassay, ciprofloxacin with the concentration of 30 µg/ml was used as positive control for antibacterial evaluation while nystatin with the concentration of 40 µg/ml was subjected to be positive control in antifungal test. DMSO was considered as negative control at concentration of 2.5%. These chemicals were provided by Sigma Aldrich, Germany.

2.1.2. Bacterial strains

Bacterial strains including gram-negative bacteria (Klebsiella pneumonia ATCC 700603, Pseudomonas aeruginosa ATCC 27853), gram-positive bacteria (Staphylococcus aureus ATCC 29213), and fungi (Candida albicans ATCC 10231) were provided by LMI-DRISA (Laboratory Mixte International – Drug Resistance in Southeast Asia).
2.2. Methods

2.2.1. Preparation of metallic ion based formulations

Silver nanoparticles were synthesized by reducing AgNO\textsubscript{3} 500 mg/L using ethylene glycol in polyvinylpyrrolidone. Zinc chelates solution was achieved by mixing urea (1 M), disodium ethylenediaminetetraacetate dehydrate (0.05 M), and ZnCl\textsubscript{2} (0.5 M). Copper chelates was prepared by mixing urea (1 M), disodium ethylenediaminetetraacetate dehydrate (0.05 M) and CuSO\textsubscript{4}.5H\textsubscript{2}O (0.05 M). Copper-Zinc chelate complex was created by combining urea (1 M), disodium ethylenediaminetetraacetate dehydrate (0.05 M), CuSO\textsubscript{4}.5H\textsubscript{2}O (0.05 M) and ZnCl\textsubscript{2} (0.5 M). The preparation protocol and method for evaluating obtained nanoparticles were performed following the procedure from Nguyen et al. [6].

2.2.2. Bioassays

The antimicrobial capacity was characterized by inhibition percentage and MIC values using a micro-broth dilution assay in 96 well plates, following a method of Le et al. [7]. To do this assay, the bacterial/fungal strains were sub-cultured on tryptic soy broth (TSB) agar plates and incubated at 37°C for 24h. After bacterial/fungal colonies grew, the same morphological type colony was picked and put in a flask containing TSB. The flasks were incubated at 37°C overnight, and then the OD of bacteria was measured by iMark microplate reader (Bio-Rad, California, USA). For diluting bacterial solution, the turbidity was adjusted to be equivalent to OD\textsubscript{600nm} = 0.4, corresponding to 10\textsuperscript{9} CFU.

A series of stock solution/suspension of Ag (I), Cu (II), Zn (II), Cu (II) - Zn (II) were prepared at 10-times concentration as mentioned in the Table 1 by distilled water. The control solutions including ciprofloxacin 300 µg/ml, nystatine 400 µg/ml and DMSO 25% were made in parallel with tested samples. 20 µl of each metal-based stock solution/suspension, negative/positive control and blank were put in the well, then adding 180 µl of microbial suspension so that the volume in each well was 200 µl.

After filling 96 well culture plates, these plates were incubated at 37°C for 24h. During the incubation, the OD\textsubscript{600nm} in different time points T\textsubscript{0} (t=0), T\textsubscript{3} (t = 3), T\textsubscript{16} (t=16), and T\textsubscript{24} (t = 24) were automatically measured by pre-setting on the iMark microplate reader (Bio-Rad, California, USA). To guaranteed the result's consistency, each sample must do three well, and the experiment must do at least two times.

2.2.3. Data analysis

All experiments were done in triplicate. The obtained data were analysed using GraphPrism 9.0 software for statistical analysis. The raw data from the machine were processed by calculating in Excel to find OD\textsubscript{real} = OD\textsubscript{sample} – OD\textsubscript{blank}. The average OD\textsubscript{real} of three replicates was taken to draw the growth curve. The inhibition percentage of each dilution and positive controls was calculated as (OD\textsubscript{negative control} – OD\textsubscript{sample})\textsuperscript{*100%}/ OD\textsubscript{negative control}.

3. Results

3.1. Metallic ion-based formulation and characterization

The obtained AgNPs suspension was subjected to measure their morphology, size, UV absorbance spectra and zeta potential. As can be seen in the morphology of being spherical with a smooth surface and high uniformity, the average size of approximately 15-20 nm, and the zeta potential of 4.28 mV with the small zeta deviation, that was suitable for the purpose of designing the optimal nanoparticles for therapeutic use.

Cu (II) chelates, Zn (II) chelates and Cu (II) - Zn (II) complex solutions subjected to measure the UV-VIS-NIR properties. The spectra showed that these agents were well dissolve in these solutions and thus ready for the evaluation of antibacterial activities (6).
3.2. Antibacterial and antifungal activities of metal nanoparticles and chelates

To observe the growing progress of bacteria or fungi, and how metallic ions affect, the growth curve of the cell was established for each microorganism at four time points T₀, T₃, T₁₆, and T₂₄. From each stock solution of Ag (I) 500 ppm, Cu (II) 50 mM, Zn (II) 500 mM or Cu (II) 50 mM – Zn (II) 500 mM complexes, four dilutions of 2, 5, 10 and 20 times were performed. The growth curves (data not show) were obtained for each dilution to see the kinetics of the growth and to calculate the inhibition percentage of each dilution against four tested microbial strains (Table 1). From the inhibition percentage of dilutions, the MIC value which is defined as the lowest concentration of an antibacterial agent necessary to inhibit visible growth was proposed for each metallic ion (Table 2).

Table 1. The inhibition percentage (%) of each dilution against 4 microbial strains.

<table>
<thead>
<tr>
<th></th>
<th>K. pneumoniae</th>
<th>P. aeruginosa</th>
<th>S. aureus</th>
<th>C. albicans</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag (I) 25 ppm</td>
<td>1.25</td>
<td>101.24</td>
<td>44.73</td>
<td>63.3</td>
</tr>
<tr>
<td>Ag (I) 50 ppm</td>
<td>5.57</td>
<td>101.36</td>
<td>98.39</td>
<td>73.4</td>
</tr>
<tr>
<td>Ag (I) 100 ppm</td>
<td>8.76</td>
<td>101.71</td>
<td>100.26</td>
<td>82.4</td>
</tr>
<tr>
<td>Ag (I) 250 ppm</td>
<td>16.79</td>
<td>105.76</td>
<td>100.95</td>
<td>100.5</td>
</tr>
<tr>
<td>Cu (II) 2.5 mM</td>
<td>33.53</td>
<td>11.19</td>
<td>6.72</td>
<td>4.5</td>
</tr>
<tr>
<td>Cu (II) 5 mM</td>
<td>38.1</td>
<td>20.14</td>
<td>75.58</td>
<td>10.7</td>
</tr>
<tr>
<td>Cu (II) 10 mM</td>
<td>49.09</td>
<td>83.59</td>
<td>93.62</td>
<td>39.3</td>
</tr>
<tr>
<td>Cu (II) 25 mM</td>
<td>100.86</td>
<td>101.55</td>
<td>100.5</td>
<td>52.8</td>
</tr>
<tr>
<td>Zn (II) 25 mM</td>
<td>95.49</td>
<td>3.42</td>
<td>100.7</td>
<td>80.4</td>
</tr>
<tr>
<td>Zn (II) 50 mM</td>
<td>100.1</td>
<td>4.55</td>
<td>100.5</td>
<td>85.2</td>
</tr>
<tr>
<td>Zn (II) 100 mM</td>
<td>98.32</td>
<td>21.67</td>
<td>95.6</td>
<td>87.1</td>
</tr>
<tr>
<td>Zn (II) 250 mM</td>
<td>100.77</td>
<td>42.73</td>
<td>100</td>
<td>100.2</td>
</tr>
<tr>
<td>Cu (II) 2.5 mM - Zn (II) 25 mM</td>
<td>97.62</td>
<td>96.17</td>
<td>89.65</td>
<td>85.6</td>
</tr>
<tr>
<td>Cu (II) 5 mM - Zn (II) 50 mM</td>
<td>97.75</td>
<td>98.93</td>
<td>101.1</td>
<td>100.4</td>
</tr>
<tr>
<td>Cu (II) 10 mM - Zn (II) 100 mM</td>
<td>100.74</td>
<td>100.56</td>
<td>100.4</td>
<td>100.2</td>
</tr>
<tr>
<td>Cu (II) 25 mM - Zn (II) 250 mM</td>
<td>99.92</td>
<td>99.18</td>
<td>100.7</td>
<td>100.7</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>99.68</td>
<td>99.53</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Nystatin</td>
<td></td>
<td></td>
<td></td>
<td>77.26</td>
</tr>
</tbody>
</table>

3.2.1. Klebsiella pneumoniae

Against K. pneumoniae ATCC 700603 which is a Gram-negative, isolated clinically and resistant to ceftazidime and other oxymino-β-lactams, all 4 diluted series of Ag (I) nanoparticles are ineffective, represented by the low value of inhibition percentages. This resistant phenomenon was consistent with previous investigation which revealed the development of this bacterial strain to silver nanoparticles [7]. In contrast, Zn (II) and Cu (II) - Zn (II) chelates exhibited to show the strong antimicrobial activity while Cu (II) chelate was found to be only effective at the concentration of 25 mM compared to Ciprofloxacin, the positive control in all dilution series.

The MIC value of Cu (II) chelate on K. pneumoniae was determined at 15 mM while those values were less than 25 mM for Zn (II) chelate and 2.5 mM/25 mM for Cu (II) - Zn (II) complexes. These two formulations showed excellent antibacterial activity on the Gram-negative bacteria. Interestingly, the antibacterial potential of Cu (II) - Zn (II) chelates was mainly due to the contribution of Zn (II) chelates, which is similar to previous research on the activity of Zn (II) [8].

3.2.2. Pseudomonas aeruginosa

The new metallic ions formulations of Ag (I), Cu (II), Zn (II) and Cu (II) - Zn (II) complex were tested on the antimicrobial capacity against P. aeruginosa ATCC 27853—an encapsulated, gram-negative, aerobic–facultatively anaerobic, rod-shaped bacterium that can cause disease in many living organisms. As a result, Ag (I) showed the strong inhibition percentage at four tested
dilutions. Cu (II) chelate dilutions of 20 and 10 times were ineffective; bacterial wells contained Cu chelate and grew at a pace comparable to wells grown on TSB medium. However, the bactericidal action of Cu (II) chelate is demonstrated at dilutions of 10 and 25 mM. Notably, Zn (II) chelates did not represent any potential inhibition against P. aeruginosa while Cu (II) - Zn (II) complexes demonstrated significant antibacterial activity at every dilutions. This suggested that there was a synergistic effect of Cu (II) and Zn (II) chelates against this bacterial strain.

The MIC values of Cu (II) and Zn (II) chelates on P. aeruginosa were 10 mM and more than 250 mM while those values of Ag (I) nanoparticles and Cu (II) - Zn (II) were 5 ppm and 1.25 mM/12.5 mM, respectively.

3.2.3. Staphylococcus aureus

Both Ag (I) nanoparticle and Cu (II) chelate had moderate antimicrobial activity against S. aureus ATCC 29213 bacteria. Cu (II) chelate samples almost had no inhibitory effect on this strain at the concentration of 5 mM. Different from Cu (II) chelate, the Cu (II) – Zn (II) complex sample with a dilution of 20 times had an inhibitory level.

The MIC values of Cu (II) chelate on S. aureus was determined as 5 mM, and Ag (I) nanoparticle was 40 ppm. This result suggested the suitable concentration of these nanoparticles for preparation of therapeutic products.

3.2.4. Candida albicans

All four tested concentrations of the Cu (II) chelates did not show strong inhibition potential against C. albicans. The Ag (I) nanoparticles suspension represented the antifungal activity at the concentration of 50, 100 and 250 ppm. Both Zn (II) chelate and Cu (II) – Zn (II) complex demonstrated the same potent against this species at all four dilution concentrations. This was similar to the research hypothesis when ZnO nanoparticles showing antifungal abilities on C. albicans [9].

The MIC value of Ag (I) nanoparticles for C. Albicans was 50 ppm while this value of Zn (II) chelates was 25 mM. The complex Cu (II) – Zn (II) chelates with EDTA and urea showed the MIC value of 2.5 mM – 25 mM.

<table>
<thead>
<tr>
<th></th>
<th>K. pneumoniae</th>
<th>P. aeruginosa</th>
<th>S. aureus</th>
<th>C. albicans</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag (I)</td>
<td>&gt; 250 ppm</td>
<td>5 ppm</td>
<td>40 ppm</td>
<td>50 ppm</td>
</tr>
<tr>
<td>Cu (II)</td>
<td>15 mM</td>
<td>10 mM</td>
<td>5 mM</td>
<td>&gt;25 mM</td>
</tr>
<tr>
<td>Zn (II)</td>
<td>20 mM</td>
<td>&gt; 250 mM</td>
<td>15 mM</td>
<td>25 mM</td>
</tr>
<tr>
<td>Cu (II) – Zn (II)</td>
<td>1.25 mM-1.25 mM</td>
<td>1.25 mM-1.25 mM</td>
<td>2.5 mM – 25 mM</td>
<td>2.5 mM – 25 mM</td>
</tr>
</tbody>
</table>

The microbial inhibition of each metal nanoparticle can vary depending on its target. Overall, the Cu (II) - Zn (II) complex is the only formulation that showed potential antimicrobial activities for all tested strains. Ag (I) nanoparticles worked effectively against P. aeruginosa. Under the particular form, Zn (II) chelates witnessed high potency against K. pneumoniae and S. aureus, while Cu (II) chelates demonstrated the potential effect on the former strain, with a lower effect compared to Zn (II). The metallic ion-based formulations mostly showed stronger sensitivity against gram-negative rather than gram-positive bacteria. This result can be explained by the fact that the cell membrane of gram-negative bacteria is covered by lipopolysaccharides, which bring a negative charge. These molecules have a stronger binding for the positive ions of metal nanoparticles or chelates [10]. From a more applied point of view, despite the worrying escalation of drug resistance, only five new classes of antibiotics have been marketed since 2000, and most of them do not work against gram-negative pathogens [11]. Therefore, there is an urgent need to develop new antibacterial agents, especially for gram-negative bacteria. These proposed metallic ions formulations could be good suggestions to create new products corresponding to each microbial species. This strategy brings an undeniable advantage for
therapeutic use because personalised medicine is often designed explicitly based on quick and exact identification of bacterial strains in wound infection.

In addition, there was always the synergetic effect of Cu (II) and Zn (II) chelates. Our previous study showed a similar tendency in the antibacterial activity of the combination of Cu (II) and Zn (II) in chelation with EDTA and urea compared to either Cu (II) or Zn (II) alone against gram-negative Escherichia coli and gram-positive Staphylococcus aureus bacteria. This study enlarged the power of these formulations on four microbial strains and then confirmed the potential of the Cu (II) - Zn (II) complex as raw materials to develop wound care products.

4. Conclusion

In this study, Ag (I) nanosuspension, Cu (II), Zn (II) and Cu (II) – Zn (II) chelates solutions were prepared with suitable physic-chemical properties for therapeutic use. The antimicrobial activity of these formulations was examined by the microdilution method. The Cu (II) – Zn (II) complex exhibits stable inhibitory activity against different microorganisms. Meanwhile, the antibacterial activity of Ag (I) nanoparticles was found positive on P. aeruginosa. The antibacterial ability of Cu (II) and Zn (II) chelate is also present but not as strong as Cu (II) - Zn (II) complex, suggesting that there is a synergistic effect of Cu (II) and Zn (II) chelates. This study provides an excellent indication to develop metallic ions-based products specific for each microorganism which can be applied widely in wound care therapy.

Acknowledgment

This project was funded in the framwork of Emerging Reseach Group program for BEAM – Biopharma-Environmental Assessment & Monitoring of the University of Science and Technology of Hanoi.

REFERENCES