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Synergistic Antimicrobial Mechanisms of Antimicrobial Peptide-Coated Silver Nanoparticles for the Targeted Treatment of Multidrug-Resistant (MDR) Infections

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ABSTRACT: The emergence of multidrug-resistant (MDR) pathogens poses a critical challenge to global health, necessitating innovative antimicrobial solutions. This study focuses on the synthesis, characterization, and evaluation of antimicrobial peptides (AMP)-coated silver nanoparticles (AgNPs) as a novel approach to combating MDR infections. AMPs, known for their broad-spectrum activity and unique mechanisms, were isolated from *Lacticaseibacillus casei* and successfully precipitated using ammonium sulfate precipitation. The AMPs were conjugated with AgNPs to improve their stability, bioavailability, and antimicrobial efficacy.

AMP-coated AgNPs were synthesized and characterized using UV–visible spectrophotometry, confirming the successful formation of nanoparticles. Antimicrobial and antifungal activities were assessed against a broad range of pathogens using the agar well diffusion method. The AMP-coated AgNPs exhibited enhanced activity compared to AMP alone, with significant inhibition zones observed for both bacterial and fungal strains. Synergy studies revealed that the combination of AMP-coated AgNPs with conventional antibiotics improved therapeutic efficacy, even at reduced dosages. Hemolysis assay evaluated the biocompatibility of the nanoparticles, indicating potential cytotoxicity of the silver nanoparticles at higher concentrations.

These findings underscore the promise of AMP-coated AgNPs as potent, broad-spectrum antimicrobial agents. However, the cytotoxic effects highlight the need for further research into optimizing biocompatibility. This study paves the way for developing advanced therapeutic strategies targeting MDR pathogens, offering an effective alternative to conventional antibiotics in critical healthcare applications.

KEYWORDS: Antimicrobial peptides, AMP-coated silver nanoparticles, multidrug-resistant (MDR) pathogens, synergistic antimicrobial therapy, antimicrobial resistance (AMR), and nanotechnology.

INTRODUCTION

The rapid emergence of multidrug-resistant (MDR) pathogens presents a profound and escalating global health threat, rendering current antimicrobial therapies increasingly ineffective. This alarming trend is largely attributed to the widespread misuse and overuse of antibiotics, which has accelerated the development of resistance among bacterial and fungal pathogens(1). The rise of MDR pathogens not only undermines decades of progress in treating infectious diseases but also escalates the risk of severe clinical and economic consequences. Addressing this critical issue requires the development of innovative and sustainable antimicrobial strategies that can effectively counteract resistant strains and reduce reliance on traditional antibiotics(1) (2) (3).

Among the array of promising alternatives, antimicrobial peptides (AMPs) have emerged as a significant focus of interest in recent years. AMPs are naturally occurring molecules produced by a wide range of organisms, including humans, plants, and microorganisms(4). They play a crucial role in innate immunity, protecting their hosts against a broad spectrum of microbial infections. AMPs possess several intrinsic advantages over conventional antibiotics, which make them particularly appealing for modern therapeutic applications. They are rapid-acting, broad-spectrum agents capable of targeting a diverse array of bacteria, fungi, and even viruses(1). Additionally, their natural origins reduce the risk of adverse side effects commonly associated with synthetic antimicrobials. Unlike conventional antibiotics that target specific metabolic pathways, AMPs often exert their effects through mechanisms such as membrane disruption or interference with vital intracellular processes, making it more challenging for pathogens to develop resistance. This unique mode of action is especially beneficial in the fight against MDR pathogens(4) (5).

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However, despite their numerous advantages, the clinical and industrial application of AMPs remains limited due to several challenges. One of the primary hurdles is their poor stability under physiological conditions, which leads to rapid degradation and diminished efficacy in vivo. Furthermore, the high costs associated with the production and purification of AMPs hinder their large-scale adoption. Issues related to suboptimal bioavailability also limit their potential, as AMPs often require innovative delivery methods to achieve effective concentrations at the site of infection. Addressing these limitations is paramount to unlocking the full potential of AMPs in combating MDR pathogens(5) (6).

To overcome these obstacles, significant attention has been given to the incorporation of AMPs into nanocarrier systems. Nanotechnology has revolutionized various scientific domains by offering innovative solutions to existing challenges, particularly in the biomedical field. Among the various types of nanomaterials, silver nanoparticles (AgNPs) have gained considerable prominence in antimicrobial therapy. AgNPs have been extensively studied because of their inherent antimicrobial properties, biocompatibility, and ease of synthesis. These properties make AgNPs a formidable weapon against MDR pathogens, either as standalone agents or in combination with other therapeutics. The hybridization of AMPs and silver nanoparticles offers a synergistic solution to many of the limitations associated with AMPs. By incorporating AMPs onto the surface of silver nanoparticles, it is possible to increase their stability, protect them from rapid enzymatic degradation, and achieve targeted delivery to infection sites(2). This strategy not only preserves the bioactivity of AMPs but also increases their antimicrobial efficacy through the dual-action mechanism of the hybrid system. Furthermore, the conjugation of AMPs with AgNPs helps reduce systemic toxicity, ensuring that the therapeutic intervention remains safe for host tissues while retaining its potency against pathogens. Engineered properties such as controlled release and sustained antimicrobial activity further enhance the potential of AMP-coated silver nanoparticles, making them suitable for various critical healthcare applications, including the treatment of MDR infections, wound healing, and implant-related biofilm prevention(7).

This study focused on evaluating the antimicrobial efficacy of AMP-coated silver nanoparticles, highlighting their dual-action properties derived from both AMPs and silver nanoparticles. The synthesized nanoparticles were subjected to rigorous testing against a diverse panel of bacterial and fungal pathogens. These pathogens, which are often implicated in severe clinical infections, present significant challenges in healthcare because of their ability to rapidly develop resistance to existing therapies. The results highlighted the broad-spectrum activity of these hybrid nanoparticles, underscoring their potential as next-generation antimicrobial agents(2) (7).

In addition to investigating their standalone antimicrobial properties, the synergistic effects of AMP-coated silver nanoparticles in combination with conventional antibiotics were explored through in vitro studies. Synergistic approaches in antimicrobial therapy are gaining increasing traction for their ability to enhance therapeutic efficacy while reducing the required dosages of drugs. By combining AMP-coated AgNPs with traditional antibiotics, the combined therapeutic potency can be amplified, reducing the likelihood of resistance development and minimizing the degree of toxicity associated with high drug concentrations. Such combinations represent a critical step forward in extending the functional lifespan of existing antibiotics and mitigating the challenges posed by MDR pathogens(8).

MATERIALS AND METHODS

1. Microbial Strains Used

The AMP-producing bacterial strain *Lacticaseibacillus casei* (formerly *Lactobacillus casei*) was isolated from the probiotic fermented milk product *Yakult*. The test pathogens used for the antibacterial assays included *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, and *Klebsiella pneumoniae*. For antifungal assays, *Candida albicans* and *Aspergillus niger* were used. Chloramphenicol was used as the antibiotic control.

2. Isolation and identification of AMP-producing bacteria

MRS agar was prepared and sterilized as a selective medium to isolate AMP-producing bacteria. Two loopfuls of *Yakult* (a commercially available probiotic fermented milk product) were aseptically streaked onto agar plates. The plates were incubated at 37°C for 48 hours(9). After incubation, isolated colonies displaying distinct growth characteristics were observed and selected for further analysis. The selected colonies were subjected to comprehensive identification procedures, combining morphological, microscopic, and biochemical analyses(10) (11).

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3. Extraction, quantification, and characterization of antimicrobial peptides (AMPs)

3.1. Extraction of AMPs

Antimicrobial peptides (AMPs) were extracted from the isolated *Lacticaseibacillus casei* strain through a series of systematic steps. The bacterium was cultured in MRS broth under static conditions at 37°C for 24 hours to facilitate optimal growth and AMP production. Following incubation, the bacterial culture was centrifuged at 5000 rpm for 15 minutes to separate the bacterial cells from the supernatant(12). The collected supernatant, containing the secreted AMPs, was subjected to ammonium sulfate precipitation to concentrate the peptides. The resulting peptide was recovered and stored at -20°C for further analysis(13).

3.2. Quantification and characterization of AMPs

The concentration of antimicrobial peptides (AMPs) in the extracted samples was quantified via the Folin-Lowry method. The absorbance of the samples was measured at 660 nm, and peptide concentrations were determined by comparison with a standard curve prepared using bovine serum albumin (BSA)(13).

For characterization, UV–visible spectroscopy was employed to analyse the AMP samples. The absorbance was scanned in the UV range (190–400 nm), identifying characteristic peaks corresponding to aromatic amino acid residues such as tryptophan and tyrosine, indicating peptides' presence. This analysis confirmed the proteinaceous nature of the extracted AMPs(13).

4. Synthesis and characterization of AMP-coated silver nanoparticles (AMP-coated AgNPs)

Silver nanoparticles (AgNPs) were synthesized by reducing silver nitrate with trisodium citrate under controlled conditions(7). The synthesized AgNPs were coated with AMPs by incubating them with AMP solutions at 4°C, resulting in the formation of stable AMP-coated AgNPs. These nanoparticles were characterized via UV–visible spectroscopy, with the absorbance ranging from 200–500 nm. The characteristic peaks confirmed the successful synthesis of the AgNPs and their coating with AMPs, indicating their hybrid nanoparticle structure(2) (13).

5. Antimicrobial and antifungal assay

The antimicrobial and antifungal activities of the AMPs and AMP-coated AgNPs were assessed via the agar well diffusion method. Mueller–Hinton agar (MHA) and potato dextrose agar (PDA) were utilized for the antibacterial and antifungal tests, respectively. Zones of inhibition around wells containing the test samples were measured to evaluate activity(14).

6. Synergistic studies with antibiotics

Combination samples of AMPs and AMP-coated AgNPs with chloramphenicol were prepared in specific ratios to assess synergistic antimicrobial effects(14) (15). The combinations were tested via the agar well diffusion method against all selected test pathogens.

7. Hemolysis Assay

The biocompatibility of the test samples was determined through a hemolysis assay using freshly collected human red blood cells. The absorbance at 450 nm was recorded to calculate the percentage hemolysis, reflecting the cytotoxicity of the samples(16) (14).

RESULTS

1. Isolation and identification of AMP-producing bacteria

The sterile MRS agar plates inoculated with the probiotic drink (Yakult) were incubated at 37°C for 48 hours, resulting in the formation of distinct, well-separated bacterial colonies. This finding indicates the successful isolation of AMP-producing bacteria with no signs of contamination (Fig. 1). The colony characteristics (Table 1) and morphological characteristics (Fig. 2) were subsequently studied.

Table 1 – Colony characteristics

Characteristics	Observation
Size	1.1 mm
Colour	White
Shape	Circular
Margin	Regular
Elevation	Convex

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Opacity	Opaque
Texture	Smooth
Gram nature	gram-positive
Arrangement	Single rods

Fig. 1 – Isolated colonies of Lacticaseibacillus casei on MRS agar

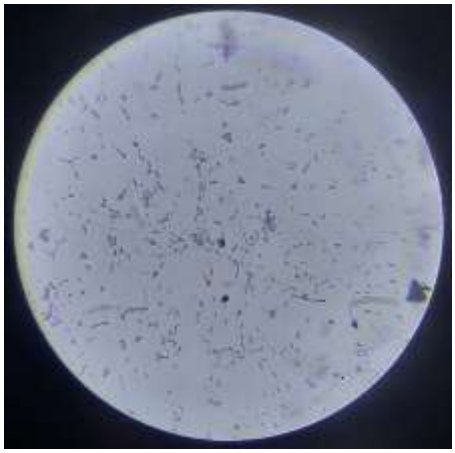

Fig. 2 – Gram nature and arrangement of bacteria under microscope (100X magnification)

Biochemical characterization of the isolated colonies was conducted to confirm that the organism was *Lacticaseibacillus casei* (refer to Table 2).

Table 2 – Biochemical characterization of isolated colonies

Biochemical test	Observation	Inference
Catalase test	No bubble formation was observed	Catalase negative
Dextrose fermentation	Media colour changed from pink to yellow	Dextrose fermentation positive
Maltose fermentation	Media colour changed from pink to yellow	Maltose fermentation positive
Lactose fermentation	Media colour changed from pink to yellow	Lactose fermentation positive
Starch hydrolysis	Clear zone around the colonies after treating with iodine	Starch hydrolysis positive
Indole test	No pink ring formation observed	Indole negative
Methyl red test	Yellow colour observed	Methyl red negative
Voges- Proskauer test	Yellow colour observed	Voges- Proskauer negative
Citrate utilization test	Slant colour changed from green to blue	Citrate utilization positive
Urease test	Yellow colour observed	Urease negative
Nitrate reductase test	Yellow colour observed	Nitrate reductase negative

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The results from the morphological and biochemical characterization confirmed that the isolated bacterium was *Lacticaseibacillus casei*.

2. Ammonium sulfate precipitation and quantification of antimicrobial peptides (AMPs)

The cell-free supernatant from the MRS culture broth grown for 24 hours was precipitated with a 70% ammonium sulfate solution and incubated. The resulting precipitate was centrifuged, and the pellets were collected and resuspended in PBS. The quantification of AMPs was then carried out via the Folin–Lowry method.

Folin-Lowry assay – Quantification of AMP extraction:

Table 3 – Absorbance of peptides obtained through Folin-Lowry assay

Tubes	Concentration of Standard BSA (mg/mL)	OD at 660 nm
Blank	0	0.084
T1	0.03	0.165
T2	0.06	0.270
T3	0.12	0.318
T4	0.24	0.460
Unknown	-	0.155

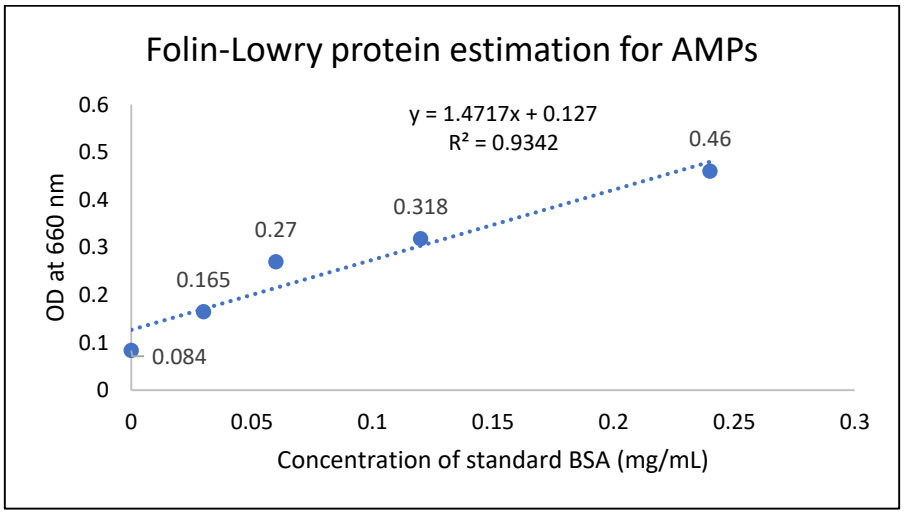

Fig. 3 – Standard curve for Folin-Lowry assay

The absorbance values (Table 3) obtained were used to plot the standard calibration curve (Fig. 3), from which the following values were obtained: slope (m) = 1.4717 and intercept (c) = 0.127. Using the linear equation $y = mx + c$, the concentration of the unknown sample was calculated, where y represents the absorbance at 660 nm. The measured absorbance (y) for the sample without AMP was 0.155. The values are substituted into the equation:

$$0.155 = 1.4717x + 0.127$$

Rearranging to solve for x, the concentration of the unknown sample is:

$$x = \frac{0.155 - 0.127}{1.4717} = 0.0190\ mg/mL$$

Thus, the concentration of AMPs in the unknown sample was calculated to be approximately 0.0190 mg/mL.

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3. Characterization of AMPs by UV–visible spectrophotometry

The characterization of the AMPs was carried out via UV–visible spectrophotometry. The sample was analysed across the wavelength range of 190–400 nm to detect and observe the characteristic absorption peak (refer to Table 4).

Table 4 – Absorbance values of AMPs by UV-Vis Spectrophotometry

Wavelength (nm)	OD obtained
190 nm	0.895
200 nm	1.764
220 nm	1.680
240 nm	0.555
260 nm	0.716
280 nm	0.946
300 nm	0.392
320 nm	0.332
340 nm	0.275
360 nm	0.229
380 nm	0.220
400 nm	0.194

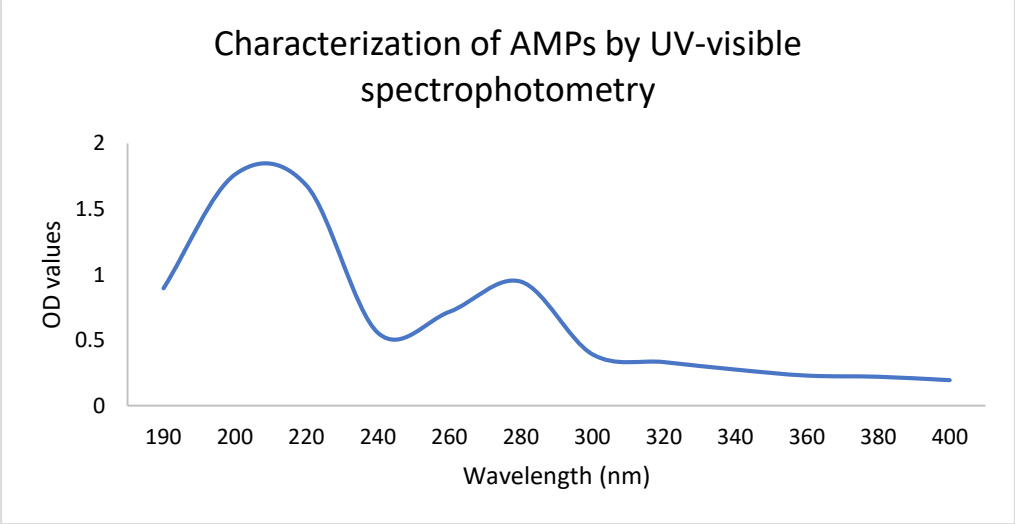

Fig. 4 – Absorption peak exhibited by AMPs

The UV–visible absorption spectrum revealed two prominent peaks (Fig. 4). The first peak, observed between 200 nm and 230 nm, indicates the presence of peptide bonds, as this wavelength range is characteristic of their detection. The second peak, observed between 270 nm and 280 nm, confirms the presence of peptides, as this range corresponds to the absorbance of aromatic amino acids such as tyrosine, tryptophan, and phenylalanine.

4. Synthesis and characterization of AMP-coated silver nanoparticles

The synthesis of AMP-coated silver nanoparticles was successful. These nanoparticles were characterized via UV–visible spectrophotometry, and their absorption spectra were analysed over the wavelength range of 200–500 nm to detect and observe the absorption peak (refer to Table 5).

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Table 5 – Absorbance values of AMP-coated AgNPs by UV-Vis Spectrophotometry

Wavelength (nm)	OD obtained
200 nm	0.171
220 nm	0.175
240 nm	0.167
260 nm	0.176
280 nm	0.196
300 nm	0.156
320 nm	0.133
340 nm	0.114
360 nm	0.096
380 nm	0.093
400 nm	0.168
420 nm	0.158
440 nm	0.136
460 nm	0.125
480 nm	0.115
500 nm	0.097

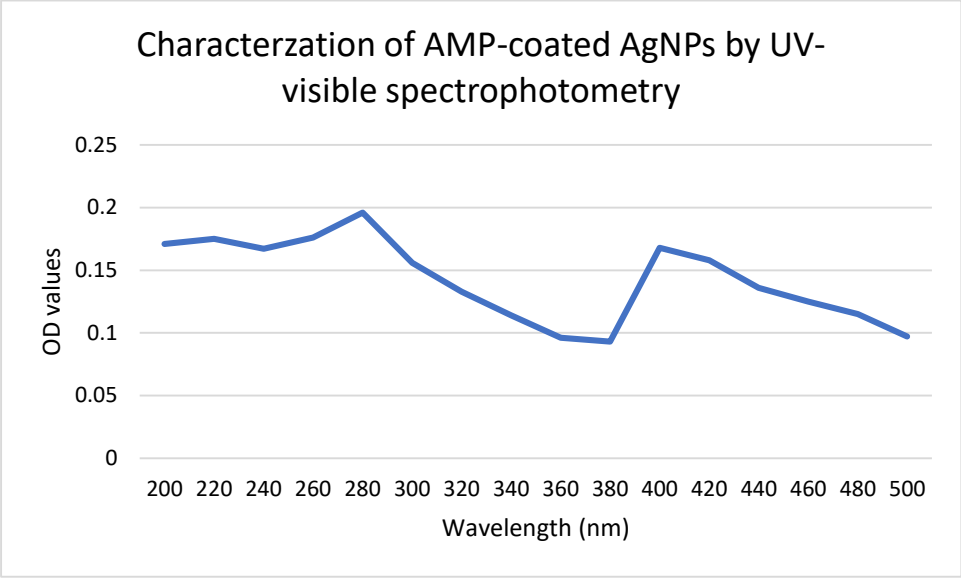

Fig. 5 – Absorption peak exhibited by AMP-coated AgNPs

The UV–visible absorption spectrum exhibited two distinct peaks (Fig. 5). The first peak, observed at approximately 280 nm, is not a characteristic peak for silver nanoparticles but is likely attributed to the AMP coating on the nanoparticles. The second peak, observed between 400 nm and 420 nm, is a characteristic absorption peak for silver nanoparticles, confirming their presence. Together, these peaks verify the successful synthesis of AMP-coated silver nanoparticles, indicating the presence of both AMPs and nanoparticles.

5. Antimicrobial assay via the agar well diffusion method

An antimicrobial assay was conducted against four test organisms: *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa,* and *Klebsiella pneumoniae*. The test samples included the following: (1) AMP solution, (2) AMP-coated AgNP solution,

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(3) a combination of AMP and chloramphenicol antibiotic solution, and (4) a combination of AMP-coated AgNP and chloramphenicol antibiotic solution. A standard chloramphenicol antibiotic solution served as the positive control, while distilled water was used as the negative control (refer to Table 6).

Table 6 – Zone of inhibition obtained for Antimicrobial assay

Sr No	Samples	Quantity of sample	Zone of inhibition (mm)			
			Escherichia coli	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Klebsiella pneumoniae*
1	AMP solution	100 L	16 mm	No zone	No zone	No zone
2	AMP-coated AgNP solution	100 L	22 mm	36 mm	23 mm	21 mm
3	AMP - chloramphenicol solution	50 L	19 mm	19 mm	21 mm	36 mm
4	AMP-coated AgNP - chloramphenicol solution	50 L	14 mm	18 mm	21 mm	27 mm
5	Positive control - antibiotic	50 L	35 mm	39 mm	45 mm	31 mm
6	Negative control – distilled water	100 L	No zone	No zone	No zone	No zone

6. Antifungal assay via agar well diffusion

An antifungal assay was conducted against two test organisms: *Candida albicans* and *Aspergillus niger*. The test samples included the following: (1) AMP solution, (2) AMP-coated AgNP solution, (3) a combination of AMP and chloramphenicol antibiotic solution, and (4) a combination of AMP-coated AgNP and chloramphenicol antibiotic solution. A standard chloramphenicol antibiotic solution served as the positive control, while distilled water was used as the negative control (refer to Table 7).

Table 7 – Zone of inhibition obtained for Antifungal assay

Sr No	Samples	Quantity of sample	Zone of inhibition (mm)	
			Candida albicans	*Aspergillus niger*
1	AMP solution	100 L	No zone	No zone
2	AMP-coated AgNP solution	100 L	29 mm	19 mm

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3	AMP - chloramphenicol solution	50 L	20 mm	32 mm
4	AMP-coated AgNP - chloramphenicol solution	50 L	11 mm	13 mm
5	Positive control - antibiotic	50 L	32 mm	39 mm
6	Negative control – distilled water	100 L	No zone	No zone

7. Hemolysis assay

A hemolysis assay was conducted to evaluate the effects of the samples on red blood cells (RBCs). The absorbance values obtained from the assay are mentioned in Table 8 :

Table 8 – Absorbance for hemolysis assay

Samples	OD at 450 nm
AMP solution	0.220
AMP-coated AgNP solution	1.139
Positive control	1.458
Negative control	0.426
Blank	0.205

The percentage hemolysis was calculated from the absorbance values obtained during the hemolysis assay via the following formula:

$$Percentage\ Hemolysis = \frac{(Sample\ Aborbance - Negative\ control\ Absorbance)}{(Positive\ control\ Absorbance - Negative\ control\ Absorbance)} \times 100$$

For the AMP solution,

$$Percentage\ Hemolysis = \frac{(0.220 - 0.426)}{(1.458 - 0.426)} \times 100 = -\frac{0.206}{1.032} \times 100 = (-)19.96\%$$

For the AMP-coated AgNP solution,

$$Percentage\ Hemolysis = \frac{(1.139 - 0.426)}{(1.458 - 0.426)} \times 100 = -\frac{0.713}{1.032} \times 100 = 69.08\%$$

The results indicate that the AMP solution exhibited a negative percentage hemolysis of -19.96%, suggesting a protective effect on RBCs (less than 5% hemolysis). In contrast, the AMP-coated AgNP solution resulted in 69.08% hemolysis, indicating significant lysis of the RBCs.

DISCUSSION

The findings from the antimicrobial and antifungal assays presented here provide valuable insights into the efficacy and potential applications of AMP-coated silver nanoparticles (AgNPs) as a novel antimicrobial strategy. This study aimed to evaluate the broad-spectrum antimicrobial activity of these hybrid nanoparticles against a range of bacterial and fungal pathogens, with particular emphasis on their synergistic effects when combined with conventional antibiotics(17).

The results of the antimicrobial assay revealed a significant improvement in activity when AMP-coated AgNPs were tested against *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa,* and *Klebsiella pneumoniae*. While the AMP solution alone demonstrated limited activity, with a zone of inhibition of 16 mm against *E. coli* and no zone of inhibition against other organisms, the AMP-coated AgNP solution exhibited notable inhibition against all tested pathogens. The inhibition zones ranged from 21 mm for *Klebsiella pneumoniae* to 36 mm for *Staphylococcus aureus*, highlighting the broad-spectrum potential of the AMP–AgNP combination. This enhanced antimicrobial activity can be attributed to the synergistic interaction between AMPs and silver nanoparticles, where the latter's membrane-disrupting action enhances the ability of AMPs to destabilize pathogen cell walls, increasing their vulnerability to antimicrobial assault.

The combination of AMP and chloramphenicol also demonstrated promising results, with moderate inhibition observed across the bacterial strains. This synergy indicates that AMPs and antibiotics, when used together, can offer a potent therapeutic approach by increasing efficacy and reducing the required dosage of antibiotics, ultimately mitigating the risk of resistance(17). However,

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compared with the combination of standard antibiotics alone, the combination of AMP-coated AgNPs with chloramphenicol yielded slightly reduced inhibition zones. This reduction may be due to the slower diffusion of nanoparticles within the agar medium, a factor that needs to be addressed in future formulations to optimize their application(15).

In terms of antifungal activity, the AMP-coated AgNPs also exhibited improved efficacy compared with the AMP solution alone, which showed no antifungal activity against *Candida albicans* or *Aspergillus niger*. The AMP-coated AgNP solution produced inhibition zones of 29 mm for *Candida albicans* and 19 mm for *Aspergillus niger*, which indicates that the incorporation of silver nanoparticles effectively enhances the antifungal potential of AMPs. These results confirm previous studies in which AgNPs have demonstrated antimicrobial activity against various fungi, suggesting that silver nanoparticles can serve as excellent adjuncts to AMPs in combating fungal infections. The combination of AMP and chloramphenicol also displayed moderate antifungal efficacy, although it was lower than that of the AMP-coated AgNP combination(18).

Despite these promising antimicrobial effects, the hemolysis assay revealed significant concerns about the cytotoxicity of AMP-coated AgNPs. The AMP solution exhibited a negative hemolysis percentage of -19.96%, indicating no/least hemolytic activity, suggesting its therapeutic safety for RBCs. In contrast, the AMP-coated AgNP solution exhibited a hemolysis rate of 69.08%, indicating significant cytotoxic potential, which is likely due to the interaction of silver nanoparticles with red blood cell membranes, leading to membrane disruption and cell lysis. This result underscores the need for caution when using AMP-coated AgNPs at relatively high concentrations or prolonged exposure times in clinical applications. Further studies on surface modification and dose optimization could improve the biocompatibility of these nanoparticles, enhancing their safety for biomedical use.

One noteworthy aspect of this research is the reduced quantity of samples required in the combination study of AMP- and AMP-coated AgNPs with antibiotics, which still showed comparable or enhanced antimicrobial activity. This synergistic effect is a significant advantage in antimicrobial therapy, as it could lead to the development of more efficient and cost-effective treatments with lower dosages and fewer side effects(15).

CONCLUSION

AMP-coated silver nanoparticles exhibit significant antimicrobial and antifungal activity, highlighting their potential as effective agents against multidrug-resistant pathogens. The synergistic effects observed with antibiotics further suggest their ability to enhance therapeutic efficacy while reducing the dosage. However, the cytotoxicity associated with higher concentrations of silver nanoparticles calls for further optimization to improve biocompatibility. Future research should focus on developing surface modifications to mitigate toxicity, exploring sustained-release systems for controlled delivery, and conducting in vivo studies to validate the clinical applicability of AMP-coated AgNPs as promising alternatives in antimicrobial therapy.

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article.

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