

Unexpected Efficacy of Albumin-Bound Glycerol Monolaurate Against Multidrug-Resistant Bacterial Isolates: A Time-Kill Assay Study

Mona A Alrabiah^{1,*}, Amina A Hassan², Murad A Mubarak³, Ahmed M Albarrag⁴,
Mohamed A Dkhil⁵, Ali M Somily⁶, Denis Delic⁶, Taghreed A Hafiz^{3,*}

¹Microbiology and Immunology Department, King Khaled University Hospital, Riyadh, 12372, Saudi Arabia; ²National Center for Radiation Research and Technology (NCRRT), Egyptian Atomic Energy Authority (EAEA), Cairo, Egypt; ³Clinical Laboratory Sciences Department, College of Applied Medical Sciences, King Saud University, Riyadh, 12372, Saudi Arabia; ⁴Pathology Department, College of Medicine, King Saud University, Riyadh, 12372, Saudi Arabia; ⁵Department of Zoology and Entomology, Faculty of Science, Helwan University, Cairo, Egypt; ⁶Department of Translational Medicine & Clinical Pharmacology, Boehringer Ingelheim Pharma, Biberach, Germany

*These authors contributed equally to this work

Correspondence: Taghreed A Hafiz, Clinical Laboratory Sciences Department, College of Applied Medical Sciences, King Saud University, Riyadh, 12372, Saudi Arabia, Email thafiz@ksu.edu.sa

Background: The rise of antibiotic resistance is a significant threat to global health, necessitating the exploration of novel antimicrobial agents. Glycerol monolaurate (GML) is known for its antimicrobial properties, primarily against Gram-positive bacteria, with limited evidence of efficacy against Gram-negative pathogens.

Methods: This study evaluated the antibacterial activity of GML alone and in combination with human serum albumin (HSA) against clinical isolates of carbapenem-resistant and vancomycin-resistant bacteria using MIC and time-kill assays.

Results: Contrary to previous reports, we demonstrate that GML exhibits significant antibacterial activity against Gram-negative bacteria, including strains resistant to conventional antibiotics. It inhibited carbapenem-resistant isolates with MIC values ranging from 25 to 100 µg/mL for *E. coli*, *K. pneumoniae*, and *E. cloacae* and showed bacteriostatic and bactericidal activity. The combination of HSA and GML enhanced this effect, showing potent bactericidal properties across all tested concentrations.

Conclusion: Current findings suggest that HSA-bound GML could be developed as a novel broad-spectrum antimicrobial agent targeting multidrug-resistant pathogens. Future research should focus on formulation optimization, in vivo efficacy studies, and preclinical evaluations to determine its therapeutic potential in clinical settings.

Keywords: Carbapenem-resistant, human serum albumin, MIC, time-kill assay, clinical isolates

Introduction

The emergence of antibiotic resistance in both Gram-positive and Gram-negative bacteria poses a significant threat to public health. Carbapenem-resistant and vancomycin-resistant pathogens are of particular concern, resulting in substantial health, environmental, and economic costs to the global healthcare system.^{1,2} The World Health Organization has developed an international policy to combat antibiotic resistance by restricting antibiotic usage in animals, encouraging better hand hygiene in hospitals, and prohibiting antibiotic prescriptions for viral infections. Importantly, in the last decade, Saudi Arabia's Ministry of Health has set antibiotic use and prescribing restrictions.^{3,4} Furthermore, one of the current actions in addressing this growing issue is the investigation of alternative compounds and substances. Pharmaceutical companies and healthcare institutions have tried to generate novel antibiotics or modify natural antimicrobial medicines.^{5–8}

Glycerol monolaurate (GML) is a naturally occurring surfactant molecule abundant in human milk. It possesses antimicrobial activity against bacteria, fungi, and viruses. It has been utilized as a therapeutic agent in various clinical applications, including wound dressing, to inhibit the growth of exotoxin-producing bacteria.^{9,10}

Human serum albumin (HSA) is a macromolecular monomeric plasma protein with antioxidant and antimicrobial activity used as a therapeutic agent to treat several diseases.^{11,12} HSA has a high ligand-binding capacity and can affect the pharmacokinetic properties of many drugs, including warfarin, chlorpromazine, naproxen, and ibuprofen.^{13–16} As reported in later research, HSA tended to reduce the action of GML when linked together.¹³ This study aims to evaluate the antibacterial activity of HSA coupled with GML against carbapenem-resistant, vancomycin-resistant, and methicillin-resistant isolates. The isolates were collected from immunocompromised patients with cancer and diabetes, patients with wound infections, and ICU patients with pulmonary edema.

Materials and Methods

Bacterial Strains

This investigation employed eleven bacterial isolates, including American Type Culture Collection (ATCC) strains. The two types of bacteria used were Gram-negative bacteria strains susceptible to Gentamicin and strains resistant to it and Gram-positive bacteria strains sensitive to Vancomycin and resistant to it. All bacteria strains have been collected from King Khalid University Hospital in Riyadh in 2019 (Table 1). The isolates were cultured and identified by the Micro Scan Walk-Away[®] system (Beckman Coulter Inc). For antibiotic susceptibility, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 29213 were used to confirm the quality of MIC values according to the Clinical Laboratory Standards Institute (CLSI) and to check the growth condition and sterility of the media.¹⁷

Preparation for Bacterial Suspension

The strains were sub-cultured and revitalized by streaking on blood agar plates and incubated overnight at 37°C. Then, direct saline suspension of isolated colonies was prepared from an 18–24-hour incubated blood agar plate. The turbidity of the suspension was adjusted to a 0.5 McFarland standard (1×10^8 CFU/mL). To prepare the colony count to approximately 5×10^6 CFU/mL, the suspension was diluted 1:20 times in Muller Hinton Broth (MHB).

Preparation of Stock and Working Solutions for Antibiotics, GML and HSA

Two different antibiotics were used: Gentamicin and Vancomycin (Sigma–Aldrich, St. Louis, USA). The pure antibiotic powder was dissolved in water to the desired concentration.¹⁷ The stock solution of this antibiotic was prepared with a concentration of 1600 µg/mL. Then, it was diluted to the desired starting concentration, 64 µg/mL. Glycerol

Table 1 The Clinical Details of the Bacterial Isolate's Origin

Serial Number	Clinical Isolate name	Specimen source	Clinical data
MRSA-19-82	Methicillin-Resistant <i>Staphylococcus aureus</i>	Sputum	5 Years F. PICU, cardiopulmonary failure.
MRSA-19-86	Methicillin-Resistant <i>Staphylococcus aureus</i>	Wound	11 Years F. Inpatient, Wound infection.
MRSA-19-87	Methicillin-Resistant <i>Staphylococcus aureus</i>	Body fluid	4 Years F. Inpatient.
VRE-19-01	Vancomycin-resistant <i>Enterococcus faecalis</i>	Body fluid	89 Years M. Inpatient, (Recto-sigmoid cancer)
VRE-19-02	Vancomycin-resistant <i>Enterococcus faecalis</i>	Urine	64 Years F ICU (breast cancer), diabetic
KPC-19-02	Carbapenem-Resistant <i>Escherichia coli</i>	Body Fluid	49 Years F Inpatient (cholangiocarcinoma)
KPC-19-03	Carbapenem-Resistant <i>Escherichia coli</i>	Tissue	22 Years M Inpatient (Kidney stone with UTI)
KPC-19-07	Carbapenem-Resistant <i>Klebsiella Pneumoniae</i>	Blood	54 Years M ICU (Pulmonary edema, diabetic)
KPC-19-16	Carbapenem-Resistant <i>Enterobacter cloacae</i>	Wound	65 Years M Inpatient, Diabetic.

Monolaurate (purity $\geq 99\%$) and Human serum Albumin were purchased from (Sigma–Aldrich, St. Louis, USA). Pure powder of GML was dissolved in absolute ethanol. The stock solution of GML was prepared by dissolving 1 mg of GML in 1 mL of ethanol to get a concentration of 1000 $\mu\text{g/mL}$.¹⁸ Then, the stock solution was diluted in MHB to get the desired 400 $\mu\text{g/mL}$ concentration. However, HSA was dissolved in Muller Hinton broth at 5 mg/mL in a water bath at 37°C for 30 min to allow protein binding. The HSA bound to GML stock was prepared using a stock solution of GML and HSA in equal volume (1:1) and allowed to stand overnight to form the complex.

Minimum Inhibitory Concentration (MIC)

The Minimum inhibitory concentration was determined with standard antibiotics (Vancomycin and Gentamicin), GML alone, and HSA linked with GML by broth micro dilution method using sterile 96-well Polystyrene Cell Culture plates. The micro dilution plate was prepared by adding 100 μL MHB from well two to well twelve, the first well containing 200 μL of standard antibiotic or GML or HSA linked to GML, and serial double dilution from well one to tenth was performed by transferring 100 μL from each well. Finally, ten μL of bacterial suspension was added to all the wells except well 12. Eleven was kept as a positive control containing MHB and bacterial suspension. At the same time, well 12 was marked as a negative control containing only MHB. Next, the plates were incubated at 37°C for 18–24 h. Similarly, we repeat the above mentioned process for HSA bound to GML (HSA+GML).¹⁹ After incubation, a freshly prepared MTT reagent in sterile water at 0.5 mg/mL stock solution of volume 40 μL was added to each well and incubated for 30 min. Plates were measured at an absorbance of 600 nm in a microplate reader (SpectraMax plus 384). The experiment was done in triplicate.^{19,20} The IC_{50} has been calculated using the Quest Graph™ IC_{50} Calculator, which is based on the four-parameter logistic regression model; MLA“Quest Graph™ IC_{50} Calculator” (AAT Bioquest, Inc., <https://www.aatbio.com/tools/ic50-calculator>).²¹

Time-Kill Assay

The time-kill kinetics assay was analyzed using the MIC values evaluated in the above assay. The test bacterial strain in the late logarithmic growth phase was diluted 1.5×10^8 cfu/mL. One hundred microliters of the antibiotics, GML, HSA, and HSA bound to GML samples containing the highest MIC at 1xMIC, 2xMIC, and 4xMIC concentration was put into each well. These wells have 100 μL of NB and 20 μL of each diluted bacterial cell suspension. Incubation was done at 37°C. Then, 10 μL was aspirated at different time intervals (0, 2, 4, 8, 16, and 24 hours) to identify the effects of antibiotics, GML, and HSA bound to GML of different concentrations on the bacterial population. The colonies were counted and compared with the control regarding cfu/mL. After incubation, colonies were counted and recorded on an Excel sheet to plot the Time-Kill curve. Bactericidal activity is defined as a decrease in colony-forming units (surviving bacteria) greater than three log10-fold, equivalent to 99.9% death of the inoculum.¹⁹

Statistical Analysis

Statistical analysis has been performed using IBM SPSS Statistics for Windows, Version 19.0 (IBM Corp., Armonk, NY, USA). The paired *t*-test was performed to determine the difference in the antibacterial pattern of GML alone and HSA bound to GML on the selected bacterial strains.

Ethical Consideration

The study was designed and conducted in accordance with the Helsinki Declaration and approved by the Institutional Review Board of the College of Medicine (Ref. No. 19/0473/RB). The data was anonymized properly before it was accessed.

Results

Minimum Inhibitory Concentration of Vancomycin and Gentamicin for Clinical Isolates

The MIC of Vancomycin and Gentamicin against clinical bacterial isolates (*MRSA*, *E. faecalis*, *E. coli*, *K. pneumoniae*, and *E. cloacae*), *S. aureus* ATCC 29213 and *P. aeruginosa* ATCC 27813 were determined. The antibiotic sensitivity pattern has been assessed as sensitive, moderate, or resistant based on CLSI recommendations. *S. aureus* ATCC

29213 had a MIC of 2 µg/mL for Vancomycin, while *P. aeruginosa* ATCC 27853 had a MIC of 0.5 µg/mL. Three strains of MRSA had MICs of 4 µg/mL, indicating a moderate sensitivity to vancomycin, in contrast to *E. faecalis*, which was found to be resistant and had MICs of 64 µg/mL. Whereas the MIC of Gentamicin was determined to be 2 µg/mL for *E. coli*, 64 µg/mL for *K. pneumoniae*, and *E. cloacae* found resistant to Gentamicin (Table 2).

Minimum Inhibitory Concentration of GML Alone and HSA Bound to GML (HSA+GML)

The MIC of GML against *S. aureus* ATCC 29213 and *P. aeruginosa* ATCC 27853 was found to be 25 µg/mL and 50 µg/mL, respectively. The MIC for MRSA strains was found to be between 12.5 and 25 µg/mL, which was similar to the MIC for *E. faecalis* samples at 12.5 µg/mL and for strains of *K. pneumoniae*, *E. cloacae*, and *E. coli* showed MIC at 100 µg/mL. Exceptionally, the *E. coli* (KPC-19-03) isolate showed MIC at 25 µg/mL.

The minimum inhibitory concentration for HSA bound to GML (HSA+GML) showed less difference than GML alone. The standard bacterial strains *S. aureus* ATCC 29213 and *P. aeruginosa* ATCC 27853 showed similar MIC of 50 µg/mL. The MIC for clinical isolates of MRSA varied between 12.5 and 100 µg/mL, whereas *E. faecalis* showed the highest concentration, 100 µg/mL, compared with GML alone. Moreover, *E. coli* strains showed the highest concentration of MIC values, 50 to 200 µg/mL. In contrast, gentamicin-resistant strains *K. pneumoniae* and *E. cloacae* were inhibited at higher concentrations of 200 µg/mL and 400 µg/mL, respectively (Table 3). Based on the statistical analysis, the effects of GML alone and HSA bound to GML were found to be significant with a *p*-value of 0.021, which means the HSA bound to GML showed a highly significant difference in antibacterial activity in comparison with GML alone among all the bacterial isolates.

IC₅₀ Values of GML Alone and HSA Bound to GML (HSA+GML)

E. faecalis (VRE-19-01) and *E. coli* (KPC-19-03) showed the lowest IC₅₀ value, 3.82 and 8.59 (µg/mL) with GML, whereas MRSA (19-86) exhibited IC₅₀ value of 11.91 (µg/mL) with HSA bound to GML (Table 4). *E. coli* (KPC-19-02) exhibited the highest IC₅₀ value, 215.07 (µg/mL) with GML, whereas *E. faecalis* (VRE-19-01) showed the highest IC₅₀ value, 195.72 (µg/mL) with HSA bound to GML (Table 4). The statistical analysis results showed a significant *p*-value of 0.026, which means the effects of GML alone and HSA bound to GML were significantly different in the antimicrobial effects on all the bacterial isolates.

Table 2 The Minimum Inhibitory Concentration of Vancomycin and Gentamicin for Clinical Isolates

Clinical Isolates	Antibiotic µg/mL	
	Vancomycin	Gentamicin
<i>Staphylococcus aureus</i> ATCC 29213	2 (S)	N/T
<i>Pseudomonas aeruginosa</i> ATCC 27853	N/T	0.5
MRSA-19-82	4 (I)	N/T
MRSA-19-86	4 (I)	N/T
MRSA-19-87	4 (I)	N/T
<i>Enterococcus faecalis</i> VRE-19-01	64 (R)	N/T
<i>Enterococcus faecalis</i> VRE-19-02	64 (R)	N/T
<i>Escherichia coli</i> KPC-19-02	N/T	2 (S)
<i>Escherichia coli</i> KPC-19-03	N/T	2 (S)
<i>Klebsiella pneumoniae</i> KPC-19-07	N/T	64 (R)
<i>Enterobacter cloacae</i> KPC-19-16	N/T	64 (R)

Notes: Interpretive Categories and MIC Breakpoints (µg/mL) according to CLSI. *Staphylococcus aureus* ATCC 29213 Vancomycin 0.5–2 µg/mL. *Pseudomonas aeruginosa* ATCC 27853 Gentamicin 0.5–2 µg/mL. *Enterobacteriaceae* Gentamicin, ≤ 4 sensitive, = 8 Intermediate, ≥ 16 Resistance. *S. aureus* Vancomycin ≤ 2 sensitive, 4–8 Intermediate, ≥ 16 Resistance. *Enterococcus* ≤ 2 Sensitive, 4–8 Intermediate, ≥ 32 Resistance. (S) sensitive, (I) intermediate, (R) resistant. N/T (not tested).

Table 3 Minimum Inhibitory Concentration of GML and HSA Bound to GML for Clinical Isolates

Clinical Isolates	GML)mg/mL (HSA+GML)mg/mL (
<i>S. aureus</i> (ATCC 29213)	25	50
MRSA (19–82)	12.5	12.5
MRSA (19–86)	25	25
MRSA-19-87	25	100
<i>E. faecalis</i> (VRE-19-01)	12.5	100
<i>E. faecalis</i> (VRE-19-02)	12.5	100
<i>P. aeruginosa</i> (ATCC 27853)	50	50
<i>E. coli</i> (KPC-19-02)	100	200
<i>E. coli</i> (KPC-19-03)	25	50
<i>K. pneumoniae</i> (KPC-19-07)	100	200
<i>E. cloacae</i> (KPC-19-16)	100	400
Mean	45.83	131.94
SD	40.98	120.72

Note: t-test ($p \leq 0.021$).

Abbreviations: GML, Glycerol monolaurate; HAS, Human serum Albumin.

Table 4 IC₅₀ Values of GML and HSA Bound to GML for Clinical Isolates

Clinical Isolates	IC ₅₀ (µg/mL)	
	GML	HSA+GML
MRSA (19–82)	76.79	103.36
MRSA (19–86)	76.59	11.91
MRSA-19-87	31.97	142.72
<i>E. faecalis</i> (VRE-19-01)	3.82	195.72
<i>E. faecalis</i> (VRE-19-02)	170.84	194.48
<i>E. coli</i> (KPC-19-02)	215.07	282.84
<i>E. coli</i> (KPC-19-03)	8.59	59.53
<i>K. pneumoniae</i> (KPC-19-07)	61.97	159.00
<i>E. cloacae</i> (KPC-19-16)	48.94	282.84
Mean	77.17	159.156
SD	71.54	92.31

Note: T-test ($p \leq 0.026$).

Abbreviations: Gent, Gentamicin; Vanc, Vancomycin.

Time-Kill Assay

MRSA (MRSA-19-82)

The isolate was intermediate susceptible to vancomycin with a MIC of 4 µg/mL. Using different concentrations equal to 1xMIC, 2xMIC, and 4xMIC (4 µg/mL, 8 µg/mL and 16 µg/mL) showed a bactericidal effect, a decline in the number of colonies over time until there were none (Figure 1A). GML had a similar impact on MRSA and exhibited a bactericidal effect, reducing the initial log CFU/mL by more than three logs over time (Figure 1B). Furthermore, HSA bound to GML (HSA+GML) had similar effects (Figure 1C). Importantly, HSA alone had no antibacterial effect against MRSA (Figure 1D).

E. faecalis (VRE-19-02)

The isolate showed resistance towards vancomycin with different concentrations (1xMIC, 0.25xMIC, and 0.5xMIC) as the log CFU/mL remained unchanged across time (Figure 2A). However, GML exhibited a bactericidal effect, reducing the starting log CFU/mL over time by greater than 3 logs (Figure 2B). HSA+GML had a similar effect to GML but was quicker than GML alone (Figure 2C). HSA had no antibacterial effect (1x, 2x, and 4xMIC) (Figure 2D).

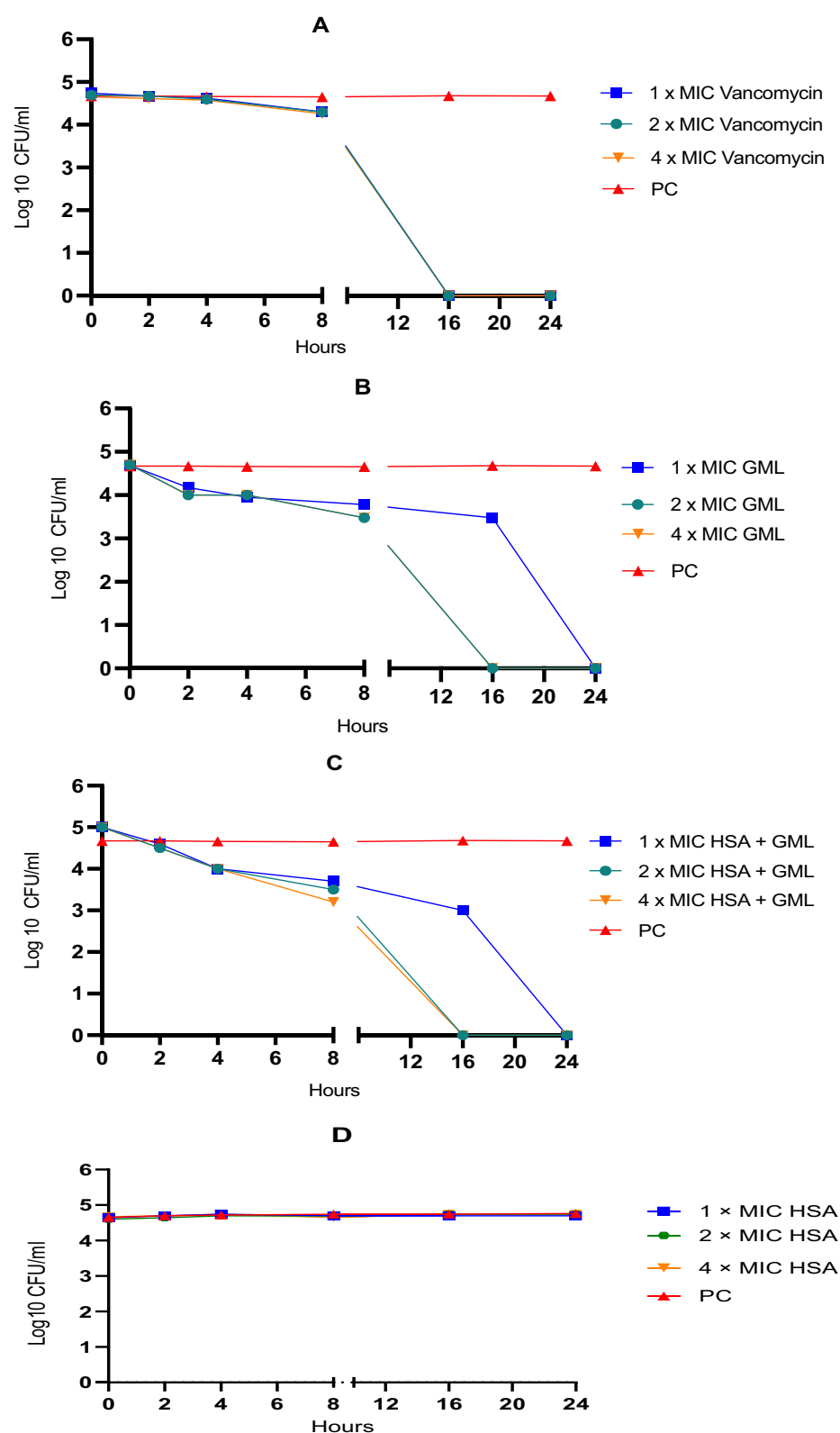


Figure 1 Time-kill assay of MRSA (MRSA-19-82) isolate. **(A)** Gentamicin (1x,2x,4xMIC) presents bactericidal effect; **(B)** GML (2xMIC,4xMIC) shows similar effect to Gentamicin but slower bactericidal effect with 1xMIC; **(C)** HSA+GML (1x,2x,4xMIC) effect is comparable to GML; **(D)** HSA had no antibacterial effect (1x,2x,4xMIC). **Abbreviation:** PC, positive control for bacterial growth.

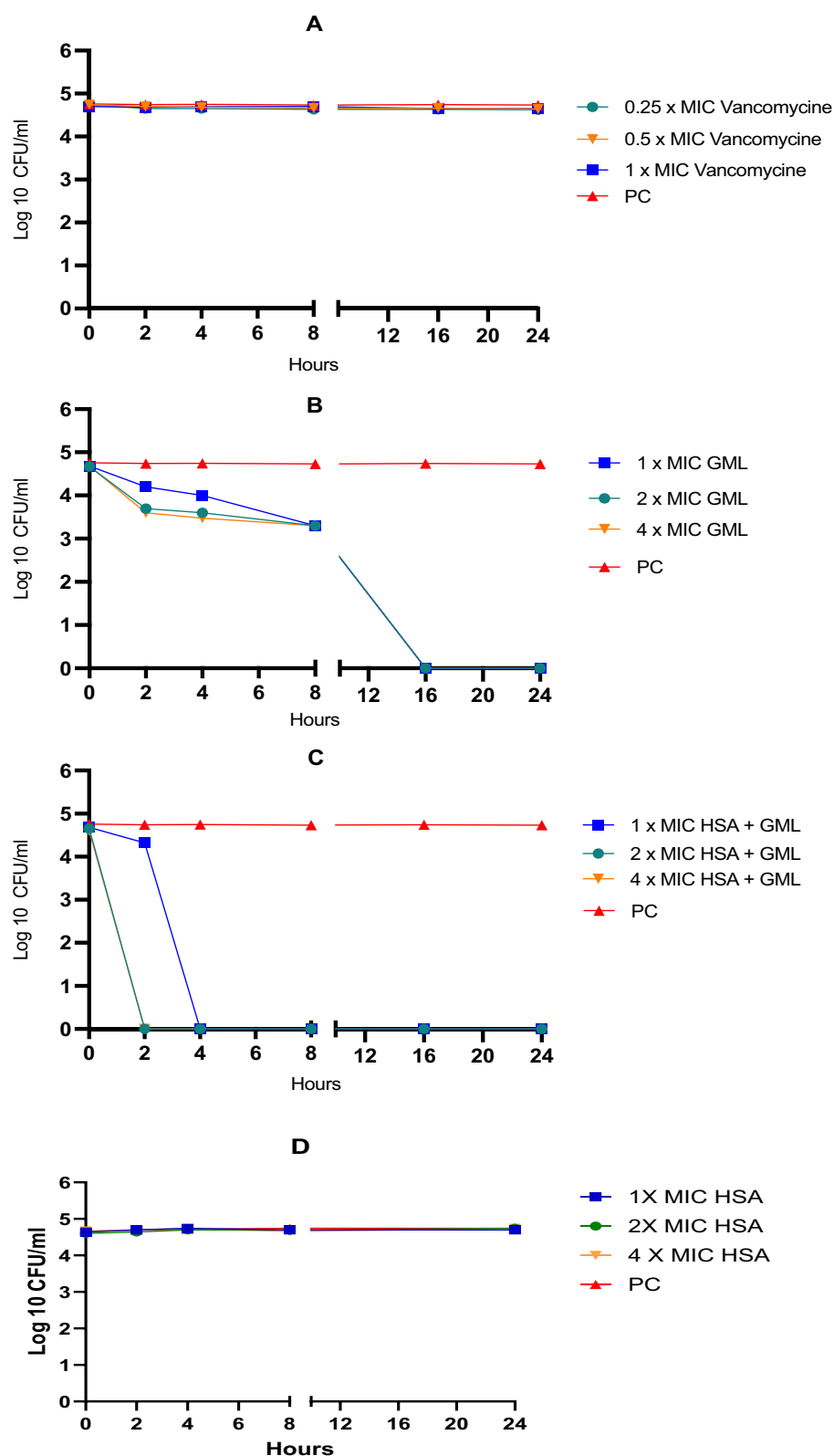


Figure 2 Time-kill assay of *E. faecalis* (VRE-19-02) isolate. **(A)** *E. faecalis* shows resistance against Vancomycin (1xMIC, 0.5xMIC, 0.25xMIC) since the log CFU/mL stayed constant over time; **(B)** GML (1x, 2x, 4xMIC) shows bactericidal activity; **(C)** HSA+GML (2x, 4xMIC) presents bactericidal activity after 2hrs, and after 4hrs with 1xMIC; **(D)** HSA had no antibacterial effect (1x, 2x, 4xMIC).

Abbreviation: PC, positive control for bacterial growth.

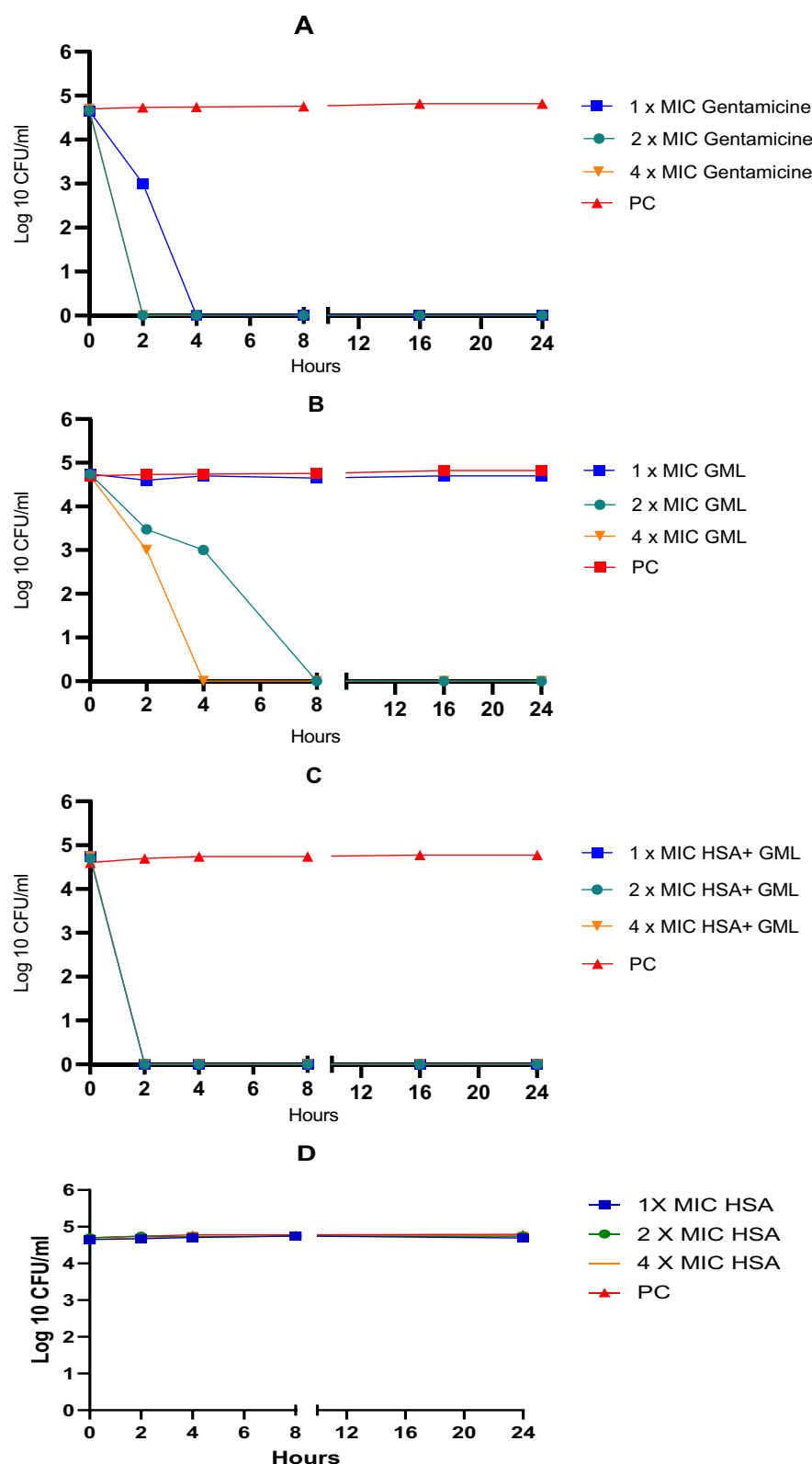


Figure 3 Time-Kill assay of *E. coli* (KPC-19-02) isolate. **(A)** Gentamicin shows bactericidal activity with (1x,2x,4xMIC); **(B)** GML (2x and 4xMIC) displays bactericidal effect; **(C)** HSA+GML at all concentrations demonstrates bactericidal effect; **(D)** HSA had no antibacterial effect (1x,2x,4xMIC).

Abbreviation: PC, positive control for bacterial growth.

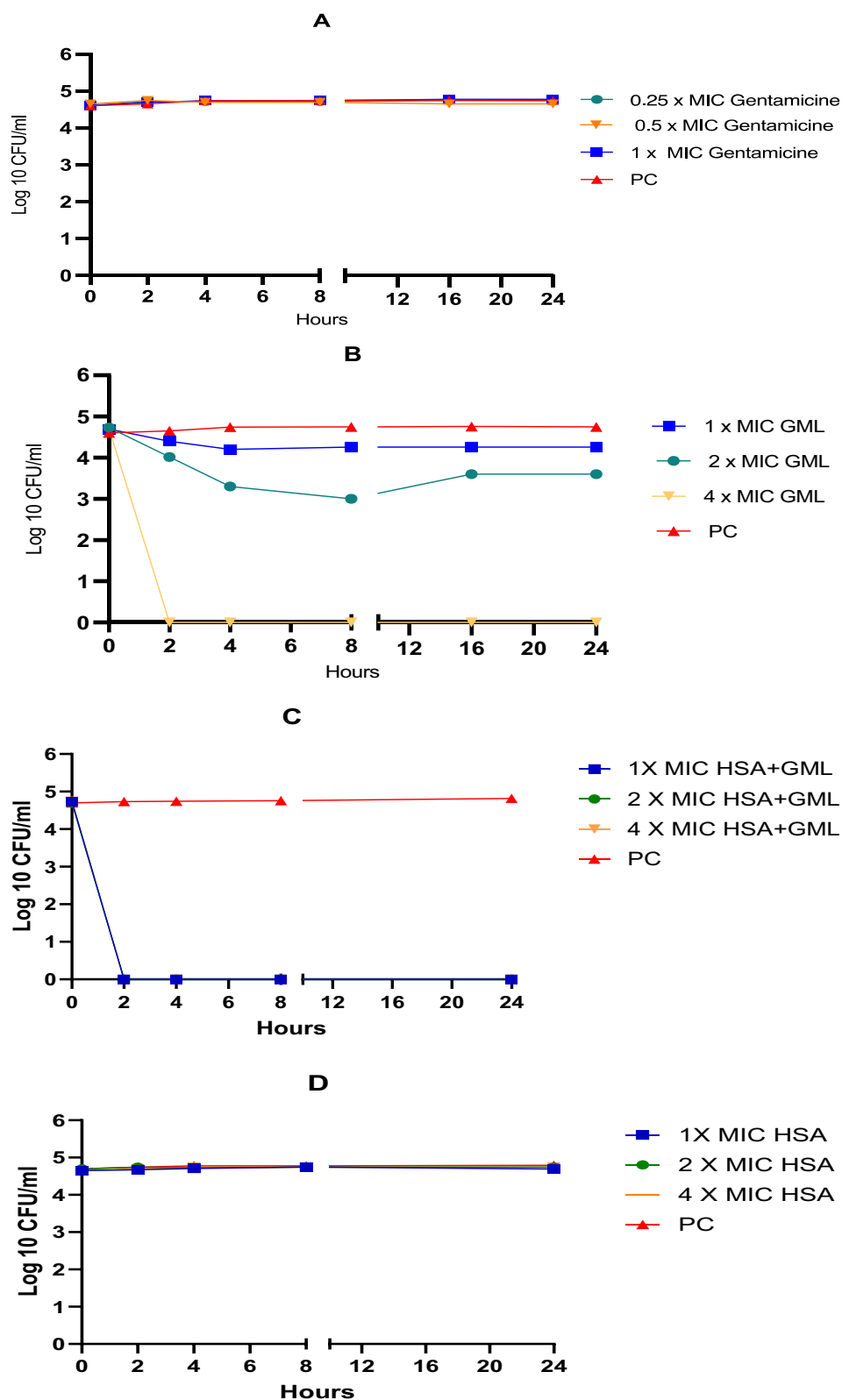


Figure 4 Time-kill assay of *K. pneumoniae* (KPC-19-07) isolate **(A)** (*K. pneumoniae* shows resistance against Gentamicin with highest MIC; **(B)** GML (1xMIC and 2xMIC) displays bacteriostatic activity while GML (4xMIC) showed bactericidal activity; **(C)** HSA+GML (1X,2X,4X MIC) presents bactericidal activity; **(D)** HSA had no effect against bacterial growth.

Abbreviation: PC, positive control for bacterial growth.

E. coli (KPC-19-02)

The isolate was killed by Gentamicin with a MIC of 2 µg/mL. The number of colonies decreased sharply after two h of incubation and showed no growth after four h with different concentrations (4xMIC, 2xMIC, and 1xMIC) (Figure 3A). However, GML killing activity varied depending on the concentration. GML (1xMIC) displayed a bacteriostatic effect, as the log CFU/mL over time remained roughly the same as the starting point. Notably, GML (2xMIC and 4xMIC) showed a bactericidal effect with the initial log CFU/mL reduction by more than three logs over time (Figure 3B). The bactericidal effect of HSA+GML was remarkable; after 2 hours of incubation with different concentrations (4xMIC, 2xMIC, and 1xMIC), HSA bound to GML was able to suppress bacterial growth to zero colonies (Figure 3C). HSA alone had no antibacterial effect against *E. coli* (Figure 3D).

K. pneumoniae (KPC-19-07)

The isolate was highly resistant to Gentamicin with an MIC of 64 µg/mL (Figure 4A). In comparison, GML at concentrations of 1xMIC and 2xMIC displayed bacteriostatic activity, as the log CFU/mL declined and remained relatively constant across time. However, the higher concentration of GML (4xMIC) showed a bactericidal effect with a significant reduction of the initial log CFU/mL by more than three logs over time (Figure 4B). Comparison to HSA alone had no antibacterial effect against *K. pneumoniae* at different concentrations (Figure 4D). Remarkably, HSA bound to GML (1xMIC, 2xMIC, and 4xMIC) was found to be bactericidal with a significant reduction of the starting log CFU/mL over time by greater than 3 logs (Figure 4C).

Discussion

Since the last few decades, an ever-increasing number of research has been conducted on antibiotic resistance, and many organizations and nations have made it a major priority in their agendas.²² Against bacteria that are resistant to many drugs, natural substances are effective in several scientific investigations as antibacterial agents.^{23,24} A recent Australian study examined the antibacterial action of twenty natural compounds and showed that vancomycin and these compounds had a synergistic effect.²⁵

To address the problem of antibiotic resistance in Gram-positive and Gram-negative bacterial strains, which is causing severe nosocomial infections in immunocompromised patients, our study has evaluated the efficacy of HSA linked to GML against clinically isolated Gram-negative and Gram-positive bacterial isolates. HSA is the most abundant protein in the blood and can bind a variety of endogenous chemical compounds. In contrast, GML is a natural chemical product that has been deemed safe, is used in foods and cosmetics, and has antibacterial activity against Gram-positive bacterial strains.

GML was found to inhibit *S. aureus* ATCC 29213 with a MIC of 25 µg/mL, while MRSA strains were also found to be inhibited by GML with a MIC of 12.5 µg/mL, but HSA+GML was shown to be equally effective with a MIC of 12.5 µg/mL. The antibacterial effects of GML alone and HSA bound to GML showed high significance with $p \leq 0.021$ when paired *t*-test was applied. In contrast to other studies, GML and MIC values for different Gram-positive bacterial strains such as *Clostridium perfringens*, *Streptococcus pneumoniae*, and *Streptococcus suis* were observed to be 300 µg/mL, ten µg/mL, and 400 µg/mL, respectively,²⁶ whereas, clinical isolate of *Staphylococcus epidermidis* required a higher concentration of GML, with MIC and MBC values of 1000 µg/mL,²⁷ In literature, GML found to inhibit the production of exotoxins in Gram-positive bacteria such as *S. aureus*, by inhibiting transcription of *S. aureus* *msrA1* genes.²⁸ Our findings indicate that vancomycin, GML alone, and HSA+GML at 1xMIC, 2xMIC, and 4xMIC were found to exhibit bactericidal effect, reducing the initial log CFU/mL of MRSA (MRSA-19-82) isolate by more than three logs over time. Correspondingly, Singh et al 2009 reported that vancomycin at a dose equal to 2xMIC suppressed MRSA after 4 and 8 hours.

Additionally, GML was found to be a potent inhibitor of *E. faecalis* (VRE-19-01) in the planktonic form (MIC 12.5 µg/mL), which was isolated from body fluids of an immunocompromised patient diagnosed with recto-sigmoid cancer metastasis to lung and liver. Moreover, the Time-kill assay of *E. faecalis* (VRE-19-02) showed resistance towards vancomycin with different concentrations. However, GML alone was found to be bactericidal, while notably, HSA bound to GML showed an earlier killing effect. *E. faecalis* (VRE-19-02) was isolated from a urine sample of a patient diagnosed

with breast cancer. As reported in the previous findings, the immunocompromised patients suffering from cancer would be given prophylactic antibiotic treatments, which would create an optimal condition for the opportunistic *Enterococci* strains to grow and *Enterococci* strains are adapted to survival on abiotic and biotic surfaces,²⁹ and also, in comparisons to earlier results, indicated efficacy of GML against the biofilm form of *E. faecalis*.³⁰

The MIC of GML for *P. aeruginosa* (ATCC 27853) was 50 µg/mL, while the MICs of GML for clinical isolates *E. coli* (KPC-19-02) and *E. coli* (KPC-19-03) were 100 µg/mL and 25 µg/mL, respectively. Furthermore, *K. pneumoniae* (KPC-19-07) and *E. cloacae* (KPC-19-16) were found to be inhibited at MIC100 µg/mL concentration with GML. In contrast to Wang et al's findings, that GML had no antibacterial effect against *E. coli* and *P. aeruginosa* with MICs greater than 10 mg/mL,³¹ however, GML in gel form was found to be effective and had a bactericidal effect against *A. baumannii*, *E. coli*, and *P. aeruginosa* with MICs greater than 10 mg/mL.^{10,32} Importantly, our findings showed successful results with lower MIC values than previous research findings. In the Time-Kill assay results, *E. coli* (KPC-19-02), isolated from a cholangiocarcinoma patient, showed bactericidal activity with Gentamicin, GML alone, and HSA +GML reducing the starting log CFU/mL over time by greater than 3 logs. Regarding *K. pneumoniae* (KPC-19-07), it showed resistance to Gentamicin, but GML exhibited bacteriostatic activity with this isolate at 1xMIC and 2xMIC and bactericidal effect at 4xMIC. Remarkably, HSA bound to GML showed a significant bactericidal effect with this Gram-negative isolate, which had been isolated from diabetic patients. The unexpected findings of GML alone and HSA bound to GML against Gram-negative isolates, which may impact their ability to resist, may be attributed to the bacterial fitness that colonizes immunocompromised and chronically ill individuals. It is worth noting that the antibacterial activity of HSA bound to GML may be attributed to HSA's composition as a single polypeptide chain of 585 amino acid residues with multiple metal-binding sites, which is known to play a key role in drug delivery.³³ Thus, the binding of GML to HSA could enhance its stability and bioavailability, thereby amplifying its antimicrobial properties. Further investigation is needed to clarify the nature of this interaction and to assess whether the effectiveness of the HSA-GML complex is primarily due to the improved delivery and stability of GML facilitated by HSA.

A key strength of this study is the exploration of HSA bound to GML as a novel therapeutic approach against antibiotic-resistant bacteria. It demonstrates broad-spectrum antibacterial activity against both Gram-positive and Gram-negative clinical isolates, including multidrug-resistant pathogens such as MRSA, VRE, and carbapenem-resistant *Enterobacterales* (CRE). The bactericidal effects of HSA+GML were confirmed through Time-Kill assays. Additionally, a comparative analysis with standard antibiotics like vancomycin and gentamicin revealed a relatively stronger efficacy of HSA+GML, with lower MIC values for GML against certain strains. This study emphasizes its relevance for treating hospital-acquired infections by focusing on clinical isolates from immunocompromised patients.

Conversely, a primary limitation of this study is the small number of clinical isolates, which may not represent the full diversity of resistant bacterial strains. A larger sample size from various healthcare settings would improve the applicability of the findings. Moreover, including a wider variety of significant multidrug-resistant isolates like *Acinetobacter baumannii* and *Pseudomonas aeruginosa* and comparing the results with other last-line antibiotics, such as daptomycin, linezolid, or colistin, would strengthen the conclusions.

Conclusion

Our findings showed that GML alone and HSA+GML had bactericidal efficacy against Gram-negative and Gram-positive isolates. GML alone, on the other hand, was found to be concentration-dependent on Gram-negative isolates. Our results demonstrate that GML alone and the HSA+GML compound could be effectively used in therapeutic medication formulations, potentially assisting in creating a new therapeutic drug target utilizing HSA connected to GML as a protein and fatty acid antibacterial agent.

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Disclosure

All authors declare that they have no competing interests.

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