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Short antimicrobial peptidomimetic SAMP-5 effective against multidrug-resistant gram-negative bacteria

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Abstract

SAMP-5 is a short histidine-derived antimicrobial peptidomimetic with pendant dialkylated tail. In this study, we evaluated the potential of SAMP-5 as an antimicrobial agent to combat multidrug-resistant gram-negative bacteria. SAMP-5 showed potent antimicrobial activity (minimum inhibitory concentration 16-64 μg/ml) comparable to melittin against multidrug-resistant *Escherichia coli* (MDREC) and multidrug-resistant (MDRPA). SAMP-5 displayed no cytotoxicity against three mammalian cells such as mouse macrophage RAW264.7, mouse embryonic fibroblast NIH-3T3, and human bone marrow SH-SY5Y cells at the concentration of 128 μg/ml. SAMP-5 showed resistance to proteolytic degradation with pepsin, trypsin, α-chymotrypsin, and proteinase K. Importantly, unlike ciprofloxacin, no antibiotic resistance against SAMP-5 arose for *Pseudomonas aeruginosa* during 7 days of serial passage at 0.5 × MIC. Moreover, SAMP-5 showed synergy or additive effects against MDRPA and MDREC, when it combined with chloramphenicol, ciprofloxacin, and oxacillin. Collectively, our results suggested that SAMP-5 is a promising alternative and adjuvant to treat infections caused by multidrug-resistant gram-negative bacteria.

Keywords: Multidrug-resistant gram-negative bacteria, Proteolytic stability, Combination therapy, Cytotoxicity, Antimicrobial resistance

Introduction

Due to the worldwide spreading of multidrug-resistant (MDR) gram-negative bacterial clones, the World Health Organization (WHO) has ranked the development of new therapeutics to treat infections caused by MDR gram-negative bacteria as a critical priority. Intrinsic antimicrobial resistance considerably limits the therapeutic options against these pathogens due to an outer membrane lipopolysaccharide permeability barrier and active multidrug efflux pumps (Olivares et al. 2013; Savage 2001). Traditional antimicrobial therapies have become ineffective in treating infections caused by MDR gram-negative pathogens (Doi et al. 2017). Thus, new therapeutic strategies are required to manage these infections.

Antimicrobial peptides (AMPs) are promising antimicrobial candidates with the potential to overcome multidrug-resistance due to their bacterial selectivity, membrane-active property, rapid killing ability, and broad antimicrobial spectrum, and uneasy induction of resistance compared with conventional antibiotics (Hancock and Chapple 1999). However, the clinical application of AMPs has been limited by (*i*) high susceptibility to proteolytic degradation by endogenous or microbial enzymes, (*ii*) moderate antimicrobial activity, (*iii*) possible toxicity due to large drug amounts required for treatment, and (*iv*) high manufacturing costs (Peters et al. 2010). Attempts to circumvent these drawbacks have been centered on the synthesis of small molecule-based antimicrobial peptidomimetics that mimic the

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structure and function of AMPs (Sgolastra et al. 2013; Scott and Tew 2017).

In previous study, our group designed a series of short histidine-derived antimicrobial peptidomimetics (SAMPs) with pendant dialkylated tail (Murugan et al. 2013). Of the designed SAMPs, SAMP-5 (Fig. 1) showed potent antimicrobial activity against gram-positive and gram-negative bacteria with minimum inhibitory concentrations (MICs) ranging from 4 to 8 μg/ml and negligible hemolytic activity until 256 μg/ml against human red blood cells (Murugan et al. 2013).

In this work, we evaluated the potential of SAMP-5 as antimicrobial agents to combat MDR gram-negative bacteria. The antimicrobial activity of SAMP-5 against multidrug-resistant *Escherichia coli* (MDREC) and multidrug-resistant Pseudomonas aeruginosa (MDRPA) was tested. SAMP-5 (MIC 16–64 μg/ml) showed potent antimicrobial activity comparable to melittin (MIC 16–32 μg/ml) is known as a powerful AMP against MDREC and MDRPA.

The cell toxicity of SAMP-5 to RAW264.7 (mouse macrophage), NIH-3T3 (mouse embryonic fibroblast), and SH-SY5Y (human bone marrow) cells was assayed by MTT viability test. Proteolytic stability of *SAMP-5* to several proteases such as pepsin, trypsin, α-chymotrypsin, and proteinase K was investigated. Furthermore, the development of resistance of bacteria to SAMP-5 as well as to ciprofloxacin as positive control was evaluated by determining the MIC using antibiotic-susceptible *Pseudomonas aeruginosa* after serial passages.

Combinational therapy is also a promising approach to overcome and prevent antibiotic resistance (Ejim et al. 2011). In recent years, several reports have revealed that AMPs combined with clinically used antibiotics could be alternatives to solve the problem of antibiotic resistance (Khara et al. 2014; Feng et al. 2015). However, the combination therapy studies using conventional antibiotics and antimicrobial peptidomimetics are rare. Therefore, the present study was planned to evaluate the synergistic effects of SAMP-5, in combination with three different antibiotics (chloramphenicol, ciprofloxacin, and oxacillin), which are conventionally used against MDREC and MDRPA.

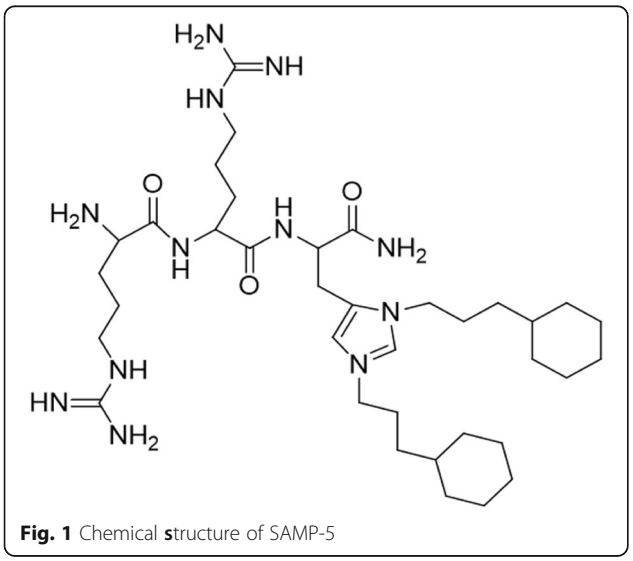

Fig. 1 Chemical **s**tructure of SAMP-5

Materials and methods

Materials and bacterial strains

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), chloramphenicol (CHL), ciprofloxacin (CIP), oxacillin (OXA), pepsin (EC 3.4.23.1, Sigma), trypsin (EC 3.4.21.4, Sigma), α-chymotrypsin (EC 3.4.21.1, Sigma), and proteinase K (EC 3.4.21.64, Sigma) were supplied from Sigma-Aldrich (St. Louis, MO, USA). HyClone Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were obtained from Seou-Lin Bioscience (Seoul, Korea). Multidrug-resistant *Pseudomonas aeruginosa* (MDRPA) (CCARM 2109) and multidrug-resistant *Escherichia coli* (MDREC) (CCARM 1229) were obtained from the Culture Collection of Antibiotic-Resistant Microbes (CCARM) of Seoul Women's University in Korea. *Pseudomonas aeruginosa* (KCTC 1637) were procured from the Korean Collection for Type Cultures (KCTC) of the Korea Research Institute of Bioscience and Biotechnology (KRIBB).

Antimicrobial activity assay (MIC determination)

The minimal inhibitory concentrations (MICs) of SAMP-5 and melittin against MDREC and MDRPA were determined via the microbroth dilution method according to Clinical and Laboratory Standards Institute guidelines (Chou et al. 2016). In brief, mid-logarithmic phase of bacteria were diluted with Mueller-Hinton broth (MHB**)** (Difco, USA) and added to a microtiter plate (2×10^6 CFU/well). A two-fold serial dilution of samples was subsequently added, and the plate was incubated for 24 h at 37 °C. The experiment was performed in triplicate using three replicates for each sample and each bacterium. The lowest peptide concentration which gave no visible growth is determined as the MIC value.

Protease resistance assay

MDRPA (CCARM 2109) was grown overnight to stationary phase at 37 °C in 10 mL of Luria-Bertani (LB) medium (Difco, USA). The overnight cultures were 10-fold diluted in fresh LB broth and incubated for additional 3 h at 37 °C to obtain mid-log phase organisms. A bacteria suspension (2×10^6 CFU/mL in LB) was mixed with 0.7% agarose, poured into a 10-cm Petri dish, and dispersed rapidly. Five microliters of an

Table 1 Minimal inhibitory concentrations (MICs; μg/ml) of SAMP-5 and melittin against multidrug-resistant Gram-negative bacteria

Strains	SAMP-5	Melittin
MDREC (CCARM 1229)	64	32
MDRPA (CCARM 2109)	16	16

MDREC: Multidrug-resistant *Escherichia coli*
MDRPA: Multidrug-resistant *Pseudomonas aeruginosa*
CCARM Culture Collection of Antibiotic-Resistant Microbes of Seoul Women's University

aqueous SAMP-5 and melittin stock solution (10 mg/mL) were added to 25 μL of trypsin (pH 7.4), α-chymotrypsin (pH 7.4), pepsin (pH 2.0), and proteinase K (pH 7.4) stock solution (0.2 mg/mL) in 50 mM Tris-HCl buffer, and incubated at 37 °C for 4 h. The reaction was stopped by freezing with liquid nitrogen, after which 30 μL of aliquots were added to each circle paper (6 mm in diameter) placed on the agarose plates, and then incubated at 37 °C overnight. The diameters of the bacterial clearance zones surrounding the circle paper were measured for the quantitation of inhibitory activities.

Cytotoxicity assay

To determine the cytotoxicity of SAMP-5, we used the MTT dye reduction assay against RAW264.7 (mouse macrophage), NIH-3T3 (mouse embryonic fibroblast), and SH-SY5Y (human bone marrow) cells as previously described (Rajasekaran et al. 2019). Briefly, the cells (2×10^4 cells/well in DMEM supplemented with 10% FBS) were placed into 96-well plates and incubated for 18–24 h in the presence of 5% CO_2 at 37 °C. The cells were treated with different concentrations (1 μg/ml to 128 μg/ml) of the peptides for 24 h. Then, 20 μl MTT (5 mg/ml) reagent in DMEM was incubated for 3 h and formed formazan crystals were dissolved in 200 μl DMSO. Cell viability was calculated by measuring absorbance at 570 nm by a microplate ELISA reader.

Resistance assay

To assess the drug resistance inducing ability of SAMP-5, *P. aeruginosa (KCTC* 1637) was chosen as the model bacteria for further research by sequential passaging method (Kim et al. 2020). Briefly, the MIC were determined according the antimicrobial activity assay, as described above. Then, the bacteria from the sub-MIC (0.5 × MIC) well were cultured overnight in fresh MHB medium and re-measured MICs. The next sub-MIC (0.5 × MIC) inoculum continued to re-measure MICs and was repeated for 7 days. Ciprofloxacin served as control in parallel cultures.

Checkerboard assay

The checkerboard titration method is performed to evaluate the combinatorial effects of SAMP-5 and conventional antibiotics (chloramphenicol, ciprofloxacin, and oxacillin) as described elsewhere (Wu et al. 2017; Qu et al. 2020). First, 2-fold serial dilutions of SAMP-5 and each antibiotic were prepared. Subsequently, 50 μl of each of different concentrations of SAMP-5 and each antibiotic was mixed and added into 100 μl of bacterial solution (containing approximately 0.5–1 × 10^6 CFU/mL) in each well of 96-well plate. The plates were then incubated in a shaking incubator at 37 °C for 24 h. Bacterial growth was assessed spectrophotometrically at A_{600nm} using microplate ELISA reader (EL800, Bio-Tek instrument). The fractional inhibitory concentration (FIC) index (FICI) was calculated as follows: FICI = [(MIC of SAMP-5 in combination)/(MIC of SAMP-5 alone)] + [(MIC of antibiotic in combination)/(MIC of antibiotic alone)]. Where FICI ≤ 0.5 is considered to indicate synergy; 0.5 < FICI ≤ 1.0 is considered additive; 1.0 < FICI ≤ 4.0 is considered indifferent; and FICI > 4.0 is considered antagonism.

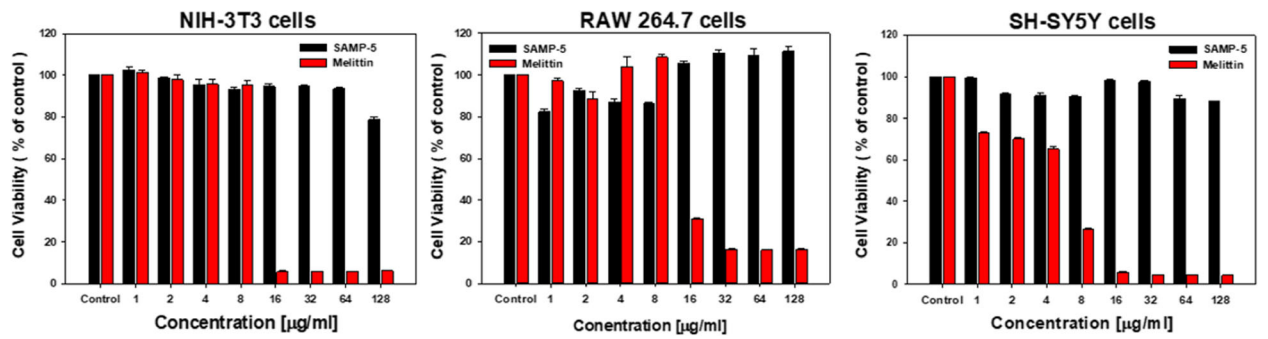

Fig. 2 Cytotoxicity of SAMP-5 against mouse embryonic fibroblast NIH-3T3 cells (**a**), mouse macrophage RAW 264.7 cells (**b**), and human SH-SY5Y bone marrow cells (**c**) determined by MTT assay

Results and discussions

Antimicrobial activity against multidrug-resistant gram-negative bacteria

The MIC (minimal inhibitory concentration) values of SMAP-5 and melittin against multidrug-resistant gram-negative bacteria such as multidrug-resistant *Escherichia coli* (MDREC) and multidrug-resistant *Pseudomonas aeruginosa* (MDRPA) were measured. Here, melittin was used as positive control AMP. The results summarized in Table 1. In multidrug-resistant *E. coli,* SMAP-5 and melittin showed potent antimicrobial activity with MIC value of 64 μg/ml and 32 μg/ml, respectively. In multidrug-resistant *P. aeruginosa,* SAMP-5 exhibited antimicrobial activity equivalent to that of melittin (MIC 16 μg/ml). Overall, SAMP-5 showed effective antimicrobial activity comparable to melittin is known as a powerful AMP against MDREC and MDRPA.

Cytotoxicity against mammalian cells

The cytotoxicity of SAMP-5 to RAW264.7 (mouse macrophage), NIH-3T3 (mouse embryonic fibroblast), and SH-SY5Y (human bone marrow) cells was evaluated by MTT assay. The results suggested that SAMP-5 showed no or less cytotoxicity against all three mammalian cells, with high cell viability up to 90% at the highest tested concentration of 128 μg/ml (Fig. 2), revealing a relatively high safety margin for SAMP-5.

Protease resistance

One of the major obstacles limiting the clinical utility of AMPs are the instability to rapid degradation by proteases which are present abundantly in biological fluids (Eckert 2011) and/or secreted by microorganisms (Wei et al. 2018). Therefore, it is also important to assess the interference effects of proteolytic enzymes, such as pepsin, trypsin, α-chymotrypsin, and proteinase K on antimicrobial activity of SAMP-5 (Low 1982). The effect of the digestion against these proteases on the bactericidal activity of SAMP-5 was investigated by the radial diffusion assay. As shown in Fig. 3, the treatment of melittin with pepsin, trypsin, α-chymotrypsin, or proteinase K completely abolished the antimicrobial activity of melittin against MDRPA. In contrast, the treatment of these digestive enzymes had no effect on the antimicrobial activity of SAMP-5. The resistance to enzymatic degradation suggests SAMP-5 is a promising candidate for therapeutic application.

Antibiotic resistance

The low possibility of emerging antibiotic resistance is one of the important factors to have as alternative therapeutic agents for conventional antibiotics (Spohn et al. 2019). Therefore, we here investigated whether a reference strain of *P. aeruginosa* would evolve resistance after multiple exposures to SAMP-5 at sub-MIC (0.5 × MIC) (Fig. 4). After 7 passages, the MICs of SAMP-5 is maintained for standard *P. aeruginosa* (KCTC 2109). In contrast, ciprofloxacin, an antibiotic acting against topoisomerase IV and DNA gyrase reached an MIC 16-fold greater than its initial MIC at 4 passage. This result indicated SAMP-5, unlike ciprofloxacin, could hardly develop antibiotic resistance.

Synergy with conventional antibiotics

One of the common approaches for the treatment of antibiotic resistant infections is the combination therapy with two antimicrobial agents that have a synergistic effect. In this study, to evaluate application of SAMP-5 in combination with conventional antibiotics, we investigated the synergistic effects of SAMP-5 in combination with three antibiotics (chloramphenicol, ciprofloxacin, and oxacillin) with different antimicrobial mechanism by checkerboard assay. Chloramphenicol and ciprofloxacin are broad-spectrum antibiotics that inhibit bacterial protein and DNA synthesis, respectively. Oxacillin is a narrow-spectrum β-lactam antibiotic that

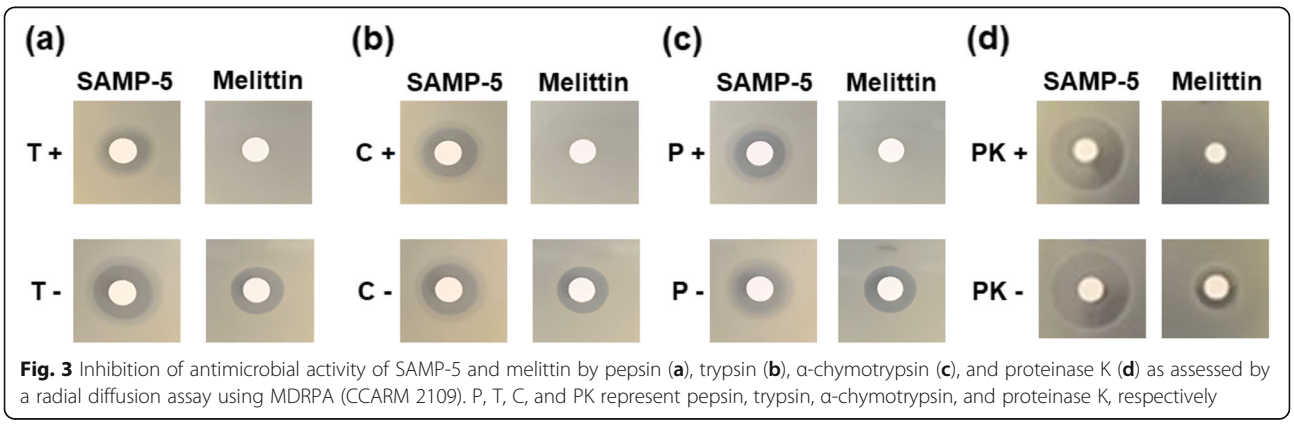

Fig. 3 Inhibition of antimicrobial activity of SAMP-5 and melittin by pepsin (**a**), trypsin (**b**), α-chymotrypsin (**c**), and proteinase K (**d**) as assessed by a radial diffusion assay using MDRPA (CCARM 2109). P, T, C, and PK represent pepsin, trypsin, α-chymotrypsin, and proteinase K, respectively

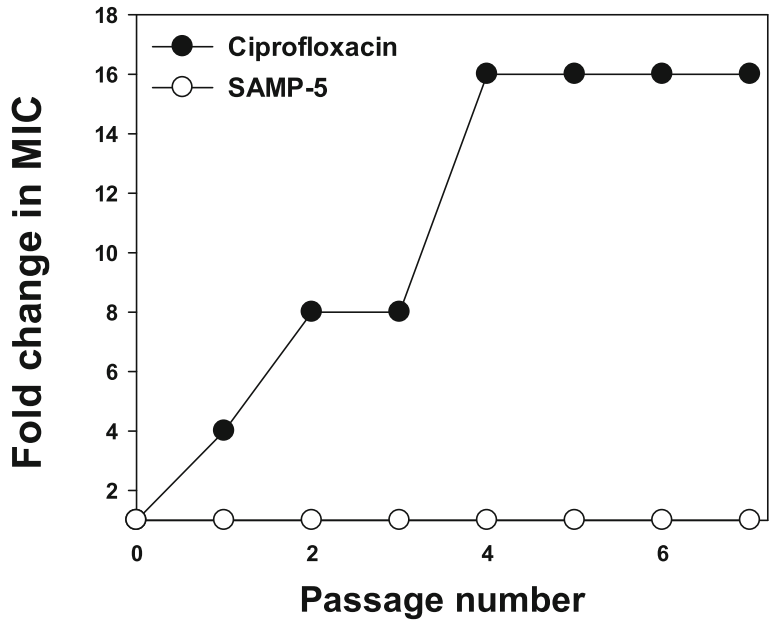

Fig. 4 Antibiotic resistance development of *Pseudomonas aeruginosa* (KCTC 1637) in the presence of sub-MIC (0.5 × MIC) concentration of SAMP-5 and ciprofloxacin

inhibits bacterial cell wall synthesis. SAMP-5 showed obvious synergist effect with all three antibiotics against MDRPA and the two antibiotics (ciprofloxacin and oxacillin) against MDREC with the FICI values ≤ 0.5 (Table 2). In particular, SAMP-5 displayed greater synergistic effect with ciprofloxacin against MDRPA and MDREC with FICI value of 0.0781 and 0.0165, respectively. Therefore, these results demonstrated that the combined application of SAMP-5 and conventional antibiotics is a potential approach to overcoming the antibiotic resistance in multidrug-resistant gram-negative bacteria in clinical practices.

Conclusions

SAMP-5 effectively prevented antibiotic resistance development and inhibited the growth of multidrug-resistant gram-negative bacteria such as MDREC and MDRPA. SAMP-5 was also demonstrated to be stable to degradation by broad spectrum proteases such as pepsin, trypsin, α-chymotrypsin, and proteinase K. Moreover, SAMP-5 showed synergistic or additive effects with the antibiotics, chloramphenicol, ciprofloxacin, and oxacillin against MDREC and MDRPA. These properties make SAMP-5 a promising candidate for the development of anti-infective agents against multidrug-resistant gram-negative bacterial infections.

Table 2 Synergy between SAMP-5 and conventional antibiotics against MDRPA and MDREC

Strains	Antibiotic	MIC_A	[A]	FIC_A	MIC_B	[B]	FIC_B	FICI [a]	Interpretation
MDRPA	CHL	256	64	0.25	16	0.25	0.0156	0.2656	synergy
	OXA	1024	256	0.25	16	0.25	0.0156	0.2656	synergy
	CIP	1024	64	0.0625	16	0.25	0.0156	0.0781	synergy
MDREC	CHL	1024	512	0.5	64	16	0.25	0.75	additive
	OXA	1024	16	0.0156	64	16	0.25	0.2656	synergy
	CIP	1	0.0009	0.0009	64	1	0.0156	0.0165	synergy

MDRPA: Multidrug-resistant *Pseudomonas aeruginosa* (CCARM 2109)
MDREC : Multidrug-resistant *Escherichia coli* (CCARM 1229)
CHL: chloramphenicol, OXA: oxacillin, CIP: ciprofloxacin
MIC_A : MIC (μg/ml) of antibiotic alone, [A]: MIC (μg/ml) of antibiotic in combination,
MIC_B : MIC (μg/ml) of SAMP-5 alone, [B]: MIC (μg/ml) of SAMP-5 in combination.
FIC_A : fractional inhibitory concentration of antibiotic
FIC_B : fractional inhibitory concentration of SAMP-5
FICI : fractional inhibitory concentration index, [a] FICI = [A] / MIC_A + [B] / MIC_B
FICI of ≤ 0.5 was interpreted as synergy, 0.5 < FICI ≤ 1.0 as additive, 1.0 < FICI ≤ 4.0 as indifferent, and an FICI > 4.0 as antagonism

Abbreviations
AMP: Antimicrobial peptide; SAMP: Short histidine-derived antimicrobial peptidomimetic; MDRPA: Multidrug-resistant *Pseudomonas aeruginosa*; MDREC: Multidrug-resistant *Escherichia coli*; MIC: Minimum inhibitory concentration; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; CHL: Chloramphenicol; CIP: Ciprofloxacin; OXA: Oxacillin

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Authors' contributions
This study was designed by SYS, SHH, and SMK. The experimental work was performed by EYK and SHH. SYS drafted the manuscript and interpreted the data. All authors read and approved the final manuscript.

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Availability of data and materials
Not applicable.

Declarations

Competing interests
The authors declare that they have no competing interests.

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