

# Synergistic antibacterial and anti-biofilm activities of resveratrol and polymyxin B against Multidrug-resistant *Pseudomonas aeruginosa*

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## Research Article

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# Abstract

Bacterial infection caused by Multidrug-resistant *Pseudomonas aeruginosa* has become a tricky problem in clinical practice. In recent years, lots of efforts have been made the treatment this type of bacterial infection. Polymyxins, which usually act as the available antibiotic for otherwise untreatable Gram-negative bacteria, can also be useful for the treatment of Multidrug-resistant *Pseudomonas aeruginosa* infection. However, current pharmacodynamic (PD) and pharmacokinetic (PK) data on polymyxins suggest that polymyxin monotherapy is unlikely to generate reliably efficacious plasma concentrations. Also, polymyxin resistance has been frequently reported, especially among Multidrug-resistant *Pseudomonas aeruginosa*, which further narrows the availability of polymyxins in clinical practice. A strategy for improving antimicrobial properties of polymyxins and preventing the development of polymyxin resistance is the use of polymyxins in combination with other agents. Indeed, it is increasingly being used clinically. In this study, we have demonstrated that resveratrol, a compound that can be well tolerated by human body, has synergistic effect with polymyxin B on antibacterial and anti-biofilm activities, which suggests that it may be an ideal partner for the combination therapy of polymyxin B against MDR *Pseudomonas aeruginosa*.

## Introduction

*Pseudomonas aeruginosa* is a type of Gram-negative bacterium that widely exists in various ecological environments, and also one of the most common opportunistic pathogens that cause clinical infection<sup>[1-2]</sup>. In 2017, it has been recognized as one of the most life-threatening bacteria and listed as a priority pathogen for research and development of new antibiotics by the World Health Organization<sup>[3]</sup>. In the past few years, with the heavy application of antibiotics in clinical practice, antibiotic resistance became more and more serious for *Pseudomonas aeruginosa*<sup>[4-5]</sup>. Especially in recent few years, with the frequent emergence of MDR strains, the treatment of *Pseudomonas aeruginosa* infection has become a big challenge facing by the clinicians<sup>[6-7]</sup>.

Polymyxin B, a type of traditional antibiotic that has been neglected for several decades due to its relatively poor clinical efficacy and high risk of nephrotoxicity, was reemployed in clinical practice in recent few years for the high prevalence of MDR bacteria<sup>[8-10]</sup>. It even be regarded as the last resorted agent for the treatment of MDR bacteria infections, even though this special antibiotic is far from being an ideal antimicrobial agent<sup>[11-12]</sup>. Unfortunately, polymyxin B resistance has emerged in various bacterial species and been frequently reported in recent few years<sup>[13-14]</sup>. Given the increasingly serious antibiotic resistance of pathogenic bacteria and the current situation of polymyxin B application, polymyxin B was suggested to be used in combination with other active antimicrobial agents when it is possible, aiming to improve its antimicrobial effect and prevent the emergence of polymyxin B resistance<sup>[15-16]</sup>.

Resveratrol is a naturally occurring polyphenolic compound that has received massive attention for its health benefits, including anti-carcinogenesis and anti-inflammation<sup>[17-18]</sup>. This compound has also shown its potential value in antibacterial therapy. For example, it has been reported to enhance the antimicrobial efficacy of aminoglycosides against *Staphylococcus aureus*, to rescue the antibacterial activity of chlorhexidine against carbapenem-resistant *Acinetobacter baumannii*, and even to increase the bactericidal effect of polymyxin B against *Klebsiella pneumoniae* and *Escherichia coli*<sup>[19-21]</sup>. However, the potential biological function of resveratrol on the antibacterial property of polymyxin B against *Pseudomonas aeruginosa* was still unknown, which deserves further investigation.

Here we investigate the synergistic effect of resveratrol and polymyxin B on anti-biofilm formation and the bactericidal effect against those cells with or without established biofilm, intending to establish an ideal partner for the combination therapy of polymyxin B for the treatment of MDR *Pseudomonas aeruginosa* infection.

## Material And Methods

### Bacterial Isolates and Chemicals

7 different isolates of MDR *Pseudomonas aeruginosa*, including 5 polymyxin B susceptible and 2 resistant isolates, were specially selected from a strain repository at the Department of Microbiology, Shiyuan Renmin Hospital. All of those isolates were obtained from different inpatients and specimens and confirmed with the MALDI-TOF mass spectrometry (MS) method. *Pseudomonas aeruginosa* ATCC 27853 was used as the control strain. Polymyxin B MICs had been interpreted accordingly to the EUCAST clinical breakpoints, version 12.0. Before each experiment, every isolate was cultured onto M-H agar plate and incubated at 37°C for 24h, and then one colony was selected for further study. Both resveratrol and polymyxin B used in this study were purchased from Solarbio (Beijing, China) and dissolved with sterile water to prepare stock solution with the original concentration of 20mg/ml for polymyxin B and 100mg/ml for resveratrol. Tiny amount of dimethyl sulfoxide (DMSO) was used to assist the dissolution of resveratrol and well-designed DMSO control groups were always included for each experiment.

### Susceptibility Testing and Genetic Analysis

MICs of resveratrol and polymyxin B were determined with the Broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) guideline. Briefly, bacterial cell suspension prepared with cation-regulated Mueller-Hinton broth (CAMHB) was dispensed into wells of 96-well microtiter plate and made the final bacterial concentration of  $5 \times 10^5$  CFU/ml for each well. Polymyxin B and resveratrol were separately added into those wells to get desired concentration, ranging from 32µg/ml to 0.125µg/ml with two-fold dilution for polymyxin B, and from 512µg/ml to 32µg/ml with two-fold dilution for resveratrol. After incubating at 37°C for 24h, the result was then read, and the MIC was defined as the lowest concentration of

drug required to visibly inhibit the growth of bacteria. PCR was performed on two polymyxin B resistant isolates with specially designed primers to detect five polymyxin B-resistance related genes including PmrA, PmrB, PhoP, PhoQ, and mcr-1, and the final products were sequenced and analyzed by Biomed (Beijing, China).

## **Checkerboard Studies**

Basing on the susceptibility testing result, checkerboard assay was further conducted to determine the interaction between polymyxin B and resveratrol on antibacterial activity. In this experiment, polymyxin B and resveratrol were prepared by two-fold serial dilutions and mixed to produce different concentration combinations. The result was read after incubated at 37°C for 24 hours and interpreted as follows: FICI  $\leq 0.5$ , synergistic effects;  $0.5 < \text{FICI} \leq 1.0$ , additive effects;  $1.0 < \text{FICI} \leq 2.0$ , no interaction; and  $\text{FICI} > 2.0$ , antagonistic effects. The result was confirmed with at least three independent experiments.

## **Time-kill assays**

Time-kill studies were performed as described previously with slight modification with all isolates<sup>[22]</sup>. Resveratrol used in this experiment was maintained at the concentration of 64 $\mu\text{g}/\text{ml}$ , while the concentration of Polymyxin B for each isolate was determined as the 1/4 MIC of polymyxin B. Bacteria suspension of  $5 \times 10^5$  CFU/ml was freshly prepared and incubated with polymyxin B and resveratrol alone or in combination at 37°C. Cell counts were determined at the time points of 0, 1, 2, 4, 6, 8, 24 hours respectively, by plating 100 $\mu\text{l}$  culture abstracted at different time points on M-H agar plates with appropriate dilution. Synergistic activity was defined as  $\geq 2\log_{10}$  decrease in CFU/mL by two-drug combination group compared with every single drug group.

## **Biofilm formation**

Bacterial cell suspension of approximately  $5 \times 10^5$  CFU/ml was freshly prepared with LB broth and dispensed into wells of 96-well microtiter plate. After statically incubated at 37°C for 24h, the liquid phase was discarded and the wells were gently washed with PBS for five times. Biofilm was fixed by adding 99% methanol to each well for 30 minutes. The fixative was then removed and the plate was allowed to air dry before the addition of crystal violet for 15 minutes to stain the adherent biofilm. The well was washed five times with PBS to remove free cells and 100 $\mu\text{l}$  of 7% acetic acid was then added to solubilize the dye combined by biofilm. Absorbance, which reflected the established biofilm, was measured at 595nm on a reader. All of those isolates were ranked according to their OD value from large to small, and the top three isolates were selected for further study.

## **Biofilm formation inhibition analysis**

Three isolates with high biofilm formation capability were selected and biofilm formation analysis was performed as described above. Bacterial cell suspension of approximately  $5 \times 10^5$  CFU/ml was dispensed into wells of 96-well microtiter plate and mixed with polymyxin B and resveratrol alone or in combination. To ensure that the inhibitory function is mainly on biofilm formation rather than on bacteria growth, the subinhibitory concentration of drug that has less than 10% influence on bacteria growth was employed in this experiment. The influence on bacteria growth was measured by incubating the bacteria suspension of  $5 \times 10^5$  CFU/ml with polymyxin B and resveratrol alone or in combination at 37°C for 24h and calculated as the decreased percentage of cell number compared with the control group. Resveratrol concentration of 8µg/ml was employed in this experiment, which has less than 10% influence on bacteria growth. While the concentration of polymyxin B for each isolate was determined, by incubating the bacterial suspension of  $5 \times 10^5$  CFU/ml with a series of diluted polymyxin B in combination with 8µg/ml resveratrol, as the highest polymyxin B concentration of those combinations which has less than 10% influence on bacteria growth. With each newly prepared bacterial suspension, two experiments were performed simultaneously, one was for inhibitory function on biofilm formation analysis, the other for influence on bacteria growth analysis. After incubating at 37°C for 24h, OD value and cell count were measured respectively, and the result was confirmed with at least three independent experiments.

### **Bactericidal activity analysis with established biofilm**

The isolate with the highest biofilm formation capability was selected for this experiment. Biofilm was cultivated as described above and the cells protected by established biofilm were obtained by discarding the content and gently washing the wells with sterilized PBS solution for five times. Change in bacteria sensitivity of this isolate to polymyxin B was confirmed by comparing the influence of polymyxin B on the growth of cells with or without established biofilm. Briefly, 24 wells with established biofilm were divided into two groups. One group was for calculating the average quantity of cells attached to the bottom of well. 100ul sterilized PBS solution was added into each well, and a sterile pipette tip was used to scrape and blow the bottom of the well to release the adherent cells and mix the suspension. The cell number was counted by plating the suspension onto M-H agar plates with appropriate dilution. The other group was for analyzing the sensitivity of those cells protected by established biofilm. 100ul cation-regulated Mueller-Hinton broth with 8µg/mL polymyxin B was added into each well and incubated at 37°C for 24h. Then total cell number in each well, including both the adherent and planktonic cells, was counted by plating onto M-H agar plates with appropriate dilution. While when the mean number of cells attached to the bottom of well with established biofilm had been obtained with the former group, 100ul freshly prepared bacterial suspension, which could provide approximately similar or small above but no more than 10% amount of cells as the calculated result, was mixed with 8µg/mL polymyxin B and added into wells of 96-well microtiter plate. After incubated at 37°C for 24h, the total cell number was counted and compared with that of the group with established biofilm.

As for bactericidal activity analysis on cells protected by biofilm, every 12 wells with established biofilm from the same batch were divided into a group. 100ul cation-regulated Mueller-Hinton broth, mixed with polymyxin

B and resveratrol alone or in combination, was added into each well. Given the decreased sensitivity of cells with established biofilm, relatively high concentration of drugs, 32µg/mL for polymyxin B and 128µg/ml for resveratrol, were employed in this experiment. After incubating at 37°C for 24h, the total cell number was counted and statistical analysis was performed. The result of this experiment was confirmed with three independent experiments.

## Statistical Analysis

Every performance was done in triplicate at least. Statistical analysis was carried out with Student's t-test and  $P < 0.01$  was considered as significant.

# Results

## Bacterial isolates

Among 7 MDR *Pseudomonas aeruginosa* isolates selected for this study, as well as one control isolate ATCC27853, 5 isolates (PA2, PA4, PA5, PA6, PA7, ATCC27853) were sensitive to polymyxin B and 2 isolates (PA1, PA3) were resistant (Table 1). *PmrA*, *PmrB*, *PhoP*, *PhoQ*, and *mcr-1* are five genes that have been reported to be closely associated with polymyxin B resistance. PCR and gene sequencing were performed with two polymyxin B resistant isolates for those five genes. *PmrA* mutation (L71R) was found in both isolates, while *PmrB* mutations (Y345H) and *PhoQ* mutation (V260G) in PA1 (Table 2). No mutation in *PhoP* was found in either isolate. Gene *mcr-1*, which has been proved to lead to polymyxin B resistance, could not be detected in either of those two isolates, suggesting that this gene may be unrelated to polymyxin B resistance in this study. Biofilm formation is another characteristic of *Pseudomonas aeruginosa*, which can lead to antibiotic resistance, but not all *Pseudomonas aeruginosa* isolates have the same biofilm formation capability. In this study, biofilm formation capability was measured as the OD value of the crystal violet adhered by established biofilm, and the top three isolates were PA2, PA1, and PA7, with the OD<sub>595</sub> of 0.958, 0.754, and 0.579 respectively, compared with the OD<sub>595</sub> of 0.107 for the negative control strain PA4.

## Antibacterial activity of resveratrol and its synergistic effect with polymyxin B

To assess the combinatorial effect of resveratrol and polymyxin B against MDR *Pseudomonas aeruginosa*, we first determined the MIC of resveratrol and polymyxin B respectively with the broth microdilution method. The MIC values of resveratrol for all isolates were >512µg/ml, indicating lack of intrinsic bactericidal activity. However, resveratrol could effectively increase the sensitivity of those isolates to polymyxin B, with the MIC of polymyxin B reduced by 4-8 fold with 64µg/ml resveratrol. The synergistic effect of resveratrol and polymyxin B could be observed with all isolates, with the FIC index keeping below 0.5 (Table 1).

## **Synergistic Activity of resveratrol and polymyxin B in Time-Kill Assays**

To further confirm the combination effect of resveratrol and polymyxin B on bactericidal activity, time-kill assay was performed with the inoculums of  $5 \times 10^5$  CFU/ml incubated with 1/4 MIC polymyxin B, alone or in combination with 64 $\mu$ g/ml resveratrol. According to the checkerboard assay results, the combination of 1/4 MIC polymyxin B and 64 $\mu$ g/ml resveratrol, with which the FICI is less than 0.5, can be expected to provide synergistic effect on bactericidal activity. That had been confirmed with all 8 isolates, as shown in Fig. 1. When compared with the control group, the single treatment group showed little or no time-dependent bactericidal activity, as bacterial load did not decrease even after 24h treatment. While in comparison, cells in all isolates except in ATCC27853 could be effectively eradicated by the combination treatment of 1/4 MIC polymyxin B and 64 $\mu$ g/ml resveratrol within 2-8h. In the isolate ATCC27853, though the combination of the chosen concentration of polymyxin B and resveratrol could not effectively eradicate bacterial cells, a decrease in cell number in the initial 8h and a wide gap in bacterial load after 24h could still be observed compared with the single treatment group. For all isolates in this study, at least 2log<sub>10</sub> decrease in CFU/mL can be obtained with the combination treatment compared with the single treatment after 24h, which supports the concept that resveratrol and polymyxin B has synergistic effect on bactericidal activity.

## **Combination effect of resveratrol and polymyxin B on inhibiting biofilm formation**

Biofilm formation is a special character for many *Pseudomonas aeruginosa* isolates, which provide bacterial cells with high protection and lead to antibiotal treatment failure. To investigate if resveratrol has combination effect with polymyxin B on inhibiting biofilm formation, three isolates (PA1, PA2, PA7) with high biofilm formation capability were selected for this experiment. The subinhibitory concentration of resveratrol and polymyxin B, defined as the concentration that has less than 10% influence on bacteria growth compared with that of the untreated group, was employed for each group. Biofilm was cultivated with the bacterial suspension mixed with a subinhibitory concentration of resveratrol and polymyxin B alone or in combination and measured as the OD value of crystal violet adhered by established biofilm. At the same time, the influence of the chosen concentration of resveratrol and polymyxin B used alone or in combination on bacteria growth was monitored with the same bacteria suspension as used for biofilm formation. After incubated at 37°C for 24h, significant decrease in OD value ( $P < 0.01$ ), but no more than 10% decrease in cell number, can be observed in all three isolates for the combination group compared with the polymyxin B or resveratrol single treatment group (Fig.2). This result demonstrates that resveratrol and polymyxin B have combination effect on inhibiting biofilm formation.

## **Resveratrol increases the bactericidal activity of polymyxin B against the cells protected by established biofilm**

Since it has been reported that biofilm can protect bacterial cells from antibiotic pressure, we intend to see if resveratrol can increase the bactericidal activity of polymyxin B against the cells with established biofilm<sup>[23-</sup>

<sup>24]</sup>. In this experiment, biofilm was cultivated with the isolate PA2, which has the highest biofilm formation capability, and biofilm influence on the sensitivity of this isolate to polymyxin B was measured by comparing the growth of bacteria with or without established biofilm.

Strictly following the operating procedures described in the section of method, we got approximately  $6.8 \times 10^5$  CFU of cells attached to the bottom of each well, which could be regarded as protected by established biofilm. While in contrast, the same number of free cells without established biofilm were obtained from freshly prepared bacteria suspension and added into wells of 96-well microtiter plate. After incubated with  $8\mu\text{g/ml}$  polymyxin B for 24h, the total number of cells for the group with established biofilm reached up to approximately  $1.8 \times 10^9$  CFU, but just  $4.3 \times 10^3$  CFU for the control group. About 5log<sub>10</sub> increase in CFU supports the concept that established biofilm leads to the decreased sensitivity of PA2 to polymyxin B. To investigate if resveratrol can increase the sensitivity of those cells protected by biofilm to polymyxin, well-prepared cells with established biofilm were treated with  $128\mu\text{g/ml}$  resveratrol and  $32\mu\text{g/ml}$  polymyxin B alone or in combination for 24h and then the total cell number in each well was counted. Significantly, cell counts of the group treated with polymyxin B in combination with resveratrol were much smaller than that of the group treated with polymyxin B alone ( $P < 0.01$ ), as well as the group treated with the same concentration of resveratrol alone ( $P < 0.01$ ). Those results demonstrate that the bactericidal activity of polymyxin B against those cells protected by established biofilm can be increased by resveratrol (Fig. 3).

## Discussion

Bacterial infection, especially MDR bacterial infection, has always been a tricky problem in clinical practice and also a big threat to people's health. Every year, people all over the world suffer a lot from bacterial infection and huge economic losses are associated with preventing and treating bacterial infection<sup>[25-27]</sup>. *Pseudomonas aeruginosa* is a ubiquitous Gram-negative bacterium that contributes a lot to clinical bacterial infection. And due to the multiple antibiotic resistance mechanisms of *Pseudomonas aeruginosa*, it is easy for this bacterium to acquire antibiotic resistance<sup>[28-29]</sup>. In recent few years, with the high prevalence of MDR *Pseudomonas aeruginosa*, and the limited number of antibacterial agents that can be available for MDR bacterial infection, the treatment of *Pseudomonas aeruginosa* infection is challenging<sup>[30]</sup>.

Polymyxin B is one of the very few antibiotic agents that can be used for the treatment of MDR Gram-negative bacterial infection. However, in recent few years, polymyxin B resistance has been frequently reported<sup>[31]</sup>. Though the polymyxin B resistance mechanism of *Pseudomonas aeruginosa* is still not completely understand, a common appreciated one is the specific modification of the lipid A component of the outer membrane lipopolysaccharide (LPS) which is the initial target of polymyxin B<sup>[32]</sup>. It has been reported that some specific mutations in PhoP/PhoQ and PmrA/PmrB two-component system (TCS) can lead to the increased LPS modification, which in turn results in polymyxin B resistance in *Pseudomonas aeruginosa*<sup>[33]</sup>. Another well-known mechanism is associated with the presence of gene *mcr-1*. Gene *mcr-1* encodes a lipid A phosphoethanolamine transferase that can catalyze the modification of lipid A moiety on bacterial lipopolysaccharide (LPS), so the plasmid carrying *mcr-1* gene can also cause polymyxin resistance

in *Pseudomonas aeruginosa*<sup>[34]</sup>. Mutations of PmrB, PmrA, and PhoQ can be detected in both polymyxin B resistant isolates, suggesting that the conclusion of this study is likely to be suitable for those types of *Pseudomonas aeruginosa*. However, gene *mcr-1* could not be found in either of those two isolates, that was one limitation of this study.

Even though having been regarded as the last resorted agent for the treatment of MDR bacteria infection, the relative poor clinical efficacy and the high risk of nephrotoxicity of polymyxin have seriously restricted its clinical application<sup>[10,35]</sup>. Undoubtedly, any drug that can improve the sensitivity of MDR *Pseudomonas aeruginosa* to this special antibiotic can greatly benefit patients. Given this consideration, resveratrol is selected as a candidate, since it can be well tolerated by human body and has been reported to have synergistic effect with different antibiotics against different bacteria species<sup>[36-37]</sup>. In this study, both checkerboard assay and time-kill assay have demonstrated that the combination of resveratrol and polymyxin B has synergistic effect against MDR *Pseudomonas aeruginosa*, which indicates that, with the assistance of resveratrol, a lower concentration of polymyxin B may be required for the treatment of MDR *Pseudomonas aeruginosa* infection. While even though in clinical practice, polymyxin B was recommended to be given with the maximum doses that could be tolerated by patients, increased sensitivity indicates that better bactericidal activity and shorter treatment course can be expected with this defined concentration of polymyxin B<sup>[38]</sup>.

For long-term consideration, the combination therapy of polymyxin B and resveratrol is likely to slow the evolution of polymyxin B resistance in *Pseudomonas aeruginosa*. According to the mutant selection window hypothesis, the development of antibiotic resistance is actually due to the accumulation of resistant mutants, and the strategy which can be expected to intervene in this enrichment step can be favorable for preserving the usefulness of antibiotics<sup>[39-40]</sup>. Though needs to be confirmed with future long-term clinic data, the hypothesis that polymyxin B used in combination with resveratrol can slow the development of polymyxin B resistance in *Pseudomonas aeruginosa* has been partly proved in this study, with the fact that polymyxin B resistant mutant isolates can still be effectively eradicated by polymyxin B with the concentration of 4ug/ml or even lower.

Biofilm can protect bacteria from surrounding environmental pressure, confer bacterial cells with high antibiotic resistance, and even impede phagocytosis<sup>[23,42]</sup>. So antibiofilm has been regarded as an important index for evaluating the antibacterial activity of antibiotics against those cells with high biofilm formation capability<sup>[42-43]</sup>. In this study, we have demonstrated that polymyxin B and resveratrol have combination effect on inhibiting the biofilm formation of *Pseudomonas aeruginosa*. This result indicates that, when used with resveratrol, a lower concentration of polymyxin B may be required for inhibiting biofilm formation. One concern of the combination antibiofilm strategy is for the respiratory *Pseudomonas aeruginosa* infection

where bactericidal cells are more likely to colonize and form biofilm and relative lower polymyxin B concentration can be achieved with routine administration.

Established biofilm is frequently observed with chronic wound *Pseudomonas aeruginosa* infection and results in poor wound healing<sup>[44-45]</sup>. Since antibiotic resistance can be greatly increased with the present of biofilm, bacterial cells, which may be initially deemed to be susceptible by the official MIC breakpoint-based criterion, with established biofilm, can still not be effectively eliminated by polymyxin B with the conventionally recommended dose strategy<sup>[46]</sup>. Even though the loading of polymyxin B for systemic administration has been greatly limited by the high risk of nephrotoxicity, there is no such restriction for topical use, so high concentration of polymyxin B can still be used topically for the treatment of bacteria infection<sup>[47-48]</sup>. In this study, polymyxin B of 32ug/ml was employed together with resveratrol against those bacterial cells protected by established biofilm, and the result has demonstrated that combination therapy has better bacterial effect compared with the polymyxin B monotherapy. This result suggests that if polymyxin B is used in combination with resveratrol in the treatment of *Pseudomonas aeruginosa* infection with established biofilm, a better clinical efficacy can be expected.

## Conclusions

Though lacking intrinsic bactericidal activity, resveratrol may still be an ideal partner for the combination therapy of polymyxin B against MDR *Pseudomonas aeruginosa*, since it can be well tolerated by human body, and also greatly increase the sensitivity of MDR *Pseudomonas aeruginosa* to polymyxin B. Besides, it can improve the antimicrobial property of polymyxin B against MDR *Pseudomonas aeruginosa* from various aspects, such as inhibiting the biofilm formation, killing the cells protected by established biofilm. It can be forecasted that resveratrol has the potential to increase the clinical efficacy of polymyxin B in the treatment of MDR *Pseudomonas aeruginosa* infection and reduce the risk of the emergence of polymyxin resistance.

## Declarations

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### Authors' contributions

LL, JJ, LQ, and RX designed the study. LQ was in charge of this study and performed the majority of those experiments. RX drafted the manuscript and revised it critically for important intellectual content. SZ, HL, YJ and MA participated in the collection and identification of MDR *Pseudomonas aeruginosa* isolates and the genetic analysis.

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## Ethics approval and consent to participate

Not applicable.

## Competing interests

The authors declare they have no competing interests.

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## Tables

Table 1 Results of checkerboard assays of polymyxin B in combination with resveratrol.

Isolates <sup>a</sup>	Source	Biofilm formation (OD595) <sup>b</sup>	MIC (mg/L)				
			Polymyxin B	resveratrol	PB +128µg/ml resveratrol	PB +64µg/ml resveratrol	PB + 32µg/ml resveratrol
PA1	Sputum	0.754	16	∞512	4*	4*	8
PA2	Urine	0.958	4	∞512	0.5*	1*	1*
PA3	Sputum	0.415	8	∞512	1*	2*	2*
PA4	Wound	0.107	2	∞512	0.5*	0.5*	1
PA5	Wound	0.176	2	∞512	0.25*	0.25*	0.5*
PA6	Wound	0.180	4	∞512	1*	1*	2
PA7	Sputum	0.579	4	∞512	1*	1*	2
PA8	ATCC	0.271	1	∞512	0.25*	0.25*	0.5

<sup>a</sup>PA1-PA7 represent seven different clinical isolated MDR Pseudomonas aeruginosa and P8 the control strain of Pseudomonas aeruginosa ATCC 27853.

<sup>b</sup>OD value of the crystal violet adhered by established biofilm measured at the spectrum of 595nm.

\*Combinations in which the selected polymyxin B and resveratrol concentration yielded a synergistic activity (FICI <0.5).

MDR was defined as resistance to at least three of those antibiotic classes, including aminoglycosides, antipseudomonal penicillins, cephalosporins, carbapenems, and fluoroquinolones.

Table 2 Mutational analysis of the PmrAB and PhoPQ regulatory pathway

Isolates <sup>a</sup>	PmrA	PmrB	PhoP	PhoQ
PA1	L71R	Y345H		V260G
PA3	L71R			

<sup>a</sup>Two polymyxin B-resistant MDR *Pseudomonas aeruginosa* isolates.

Obtained gene sequences were compared with that of the polymyxin B-susceptible *Pseudomonas aeruginosa* PAO1.

## Figures

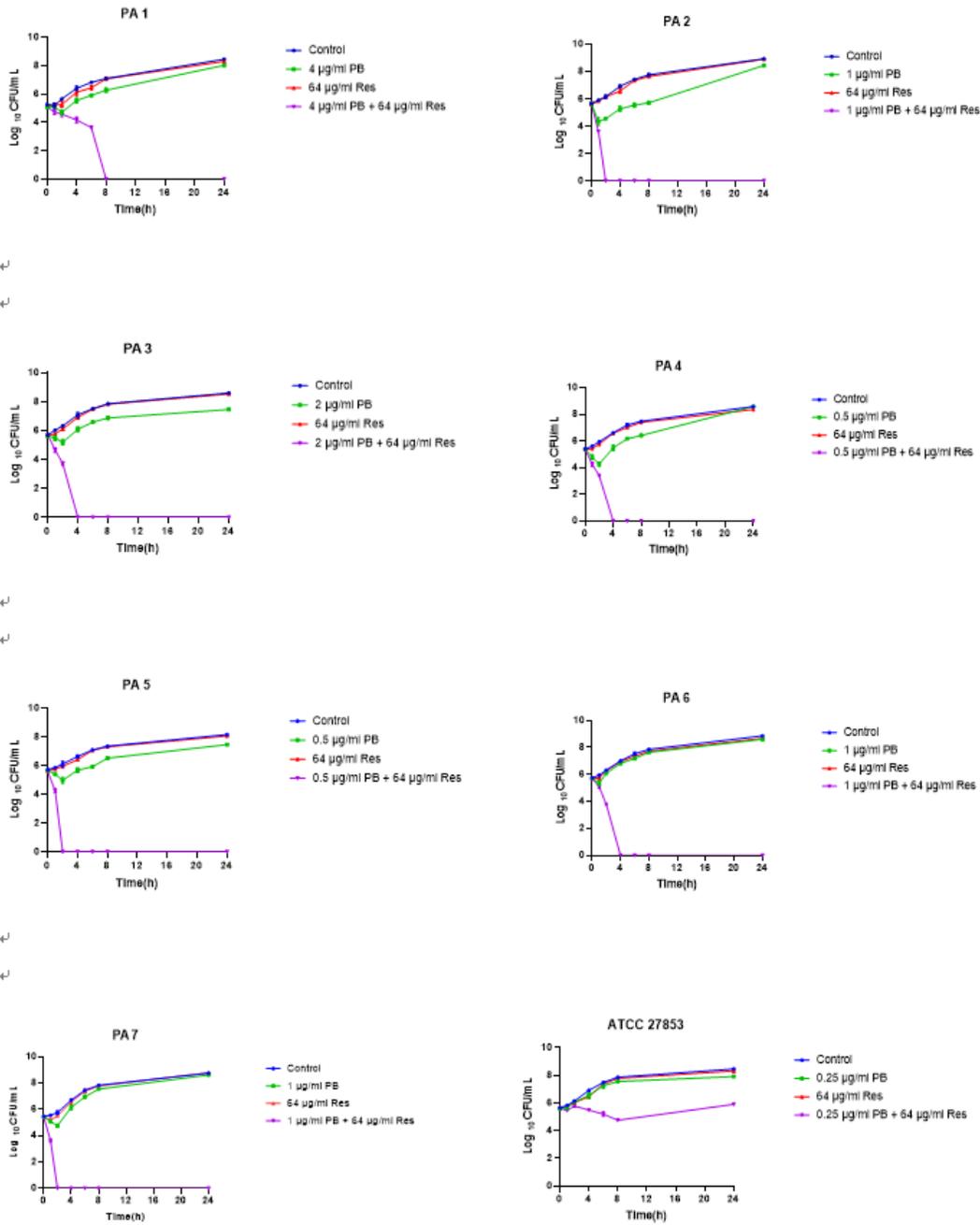


Fig. 1 Time-killing curves of polymyxin B and resveratrol alone or in combination against 8 *Pseudomonas aeruginosa* isolates.

PB = polymyxin B, Res = resveratrol.

## Figure 1

Time-killing curves of polymyxin B and resveratrol alone or in combination against 8 *Pseudomonas aeruginosa* isolates. PB = polymyxin B, Res = resveratrol.

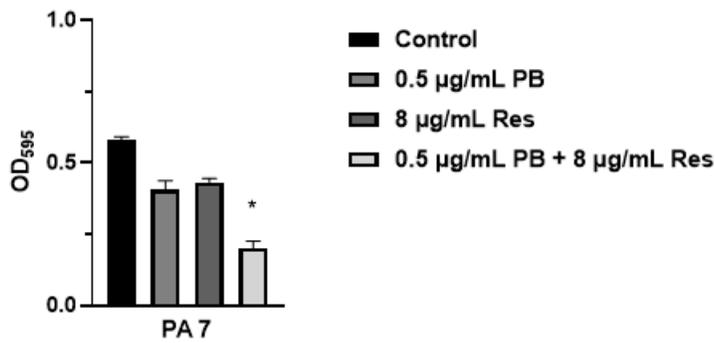
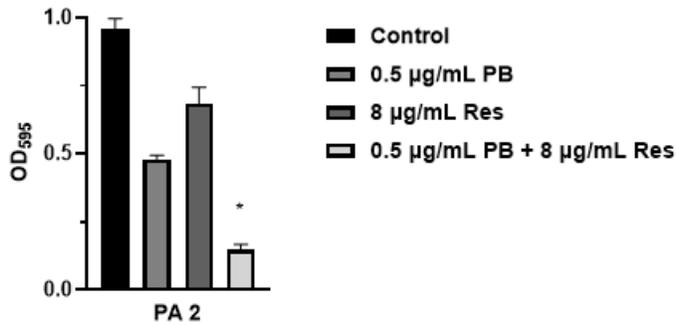
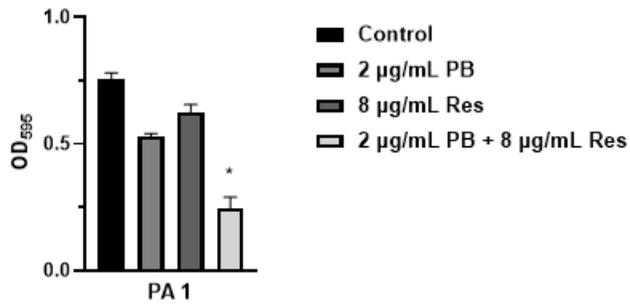


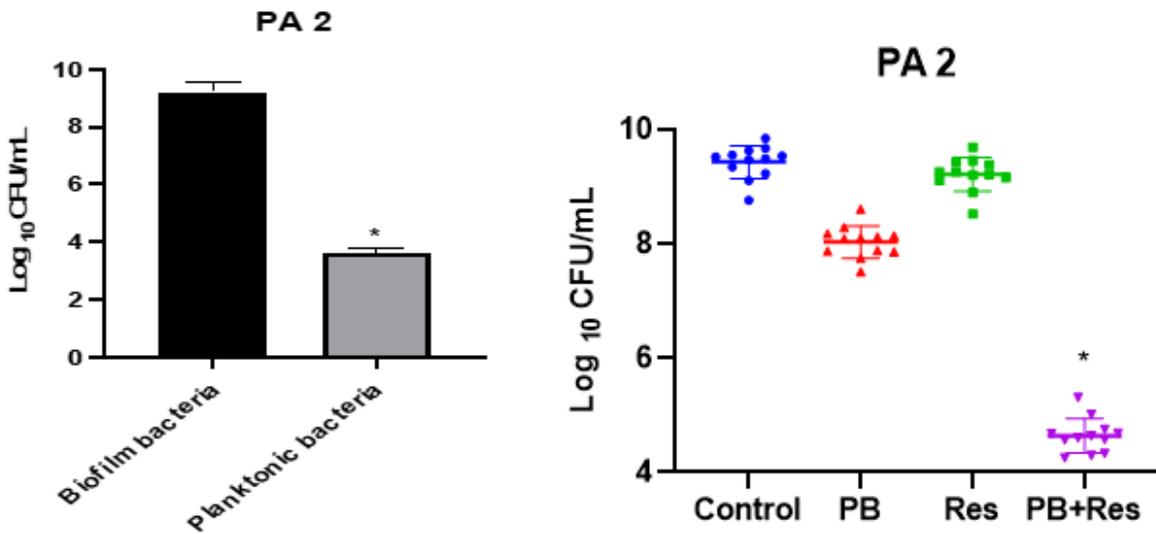
Fig. 2 Biofilm inhibitory effects of polymyxin B combined with resveratrol on 3 *Pseudomonas aeruginosa* isolates. PB = polymyxin B, Res = resveratrol.

\* There were significant differences for the combination group compared with every other group for each isolate ( $P < 0.01$ ).

## Figure 2

Fig. 2 Biofilm inhibitory effects of polymyxin B combined with resveratrol on 3 *Pseudomonas aeruginosa* isolates. PB = polymyxin B, Res = resveratrol.

\* There were significant differences for the combination group compared with every other group for each isolate ( $P < 0.01$ ).



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↵

Fig.3 (A)Growth of PA2 cells with (Biofilm bacteria group) or without (Planktonic bacteria group) established biofilm after exposing to 8µg/mL polymyxin B for 24h.↵

(B)Combination effect of polymyxin B and resveratrol against the growth of PA2 cells with established biofilm. PB, 32 µg/mL polymyxin B; Res, 128µg/ml resveratrol; PB+Res, 32 µg/mL polymyxin B + 128µg/ml resveratrol.↵

\* There were significant differences for this group compared with each other group ( $P < 0.01$ ).↵

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### Figure 3

(A)Growth of PA2 cells with (Biofilm bacteria group) or without (Planktonic bacteria group) established biofilm after exposing to 8µg/mL polymyxin B for 24h.

(B)Combination effect of polymyxin B and resveratrol against the growth of PA2 cells with established biofilm. PB, 32 µg/mL polymyxin B; Res, 128µg/ml resveratrol; PB+Res, 32 µg/mL polymyxin B + 128µg/ml resveratrol.

\* There were significant differences for this group compared with each other group ( $P < 0.01$ ).