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Research paper

Antibacterial and antibiotic synergistic activities of the extract from *Pithecellobium clypearia* against clinically important multidrug-resistant gram-negative bacteria

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ABSTRACT

Introduction: Multidrug-resistant *Acinetobacter baumannii (MDRAB)* and *Pseudomonas aeruginosa (MDRPsA)* have attracted widespread attention. Antibiotic adjuvants can give a second life to antibiotics that bacteria have developed resistance to. *Pithecellobium clypearia* is a medicinal plant used in China for treating various diseases, including infections. *Pithecellobium clypearia* may have the potential as an antibiotic adjuvant to enhance the effect of antibiotics.

Methods: The antibacterial and synergistic activities of extract from *Pithecellobium clypearia* (S20b) and antibiotics, alone and in combination with MDRAB and MDRPsA, were evaluated in vitro against 20 clinic multidrug-resistant isolates by the micro-dilution and checkerboard dilution methods. The compounds in S20b were analysed by RRLC-MS/MS. The mechanism of S20b was confirmed by transmission electron microscopy (TEM).

Results: S20b demonstrated potential inhibitory activity against MDRAB and MDRPsA and showed a synergistic effect of interaction with the antibiotics in this experiment. The TEM results confirmed that S20b could damage the bacterial cell wall. Moreover, the effects of S20b on the reversion resistance of *A. baumannii* and *P. aeruginosa* against the corresponding antibacterial agents could possibly be associated with the increase in bacterial membrane permeability.

Conclusions: S20b increased the efficiency of antibiotics against MDRAB and MDRPsA. This study is the first to demonstrate that S20b from *Pithecellobium clypearia* may have potential use as a therapeutic agent to against MDRAB and MDRPsA.

1. Introduction

The resistance of bacteria to antibiotics is an under-appreciated threat to public health in nations around the globe [1]. Originally, *Staphylococcus aureus* isolates resistant to the β-lactam group of antibiotics were identified. Subsequently, methicillin-resistant *S. aureus* (MRSA) emerged, which are even more multidrug-resistant (MDR). With clinical isolates having 95 % resistance to antibiotics today, MRSA has become the notorious MDR G + bacterium [2].

As the G- counterparts of MRSA, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* have higher survival rates and spread for extended periods of time in hospitals. The frequent causes of nosocomial infections and long-term exposure to antibiotic treatments has induced multiple drug resistances of *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. Therefore, treatment options for these pathogens are increasingly limited. The aminoglycoside class of antibiotics has become the last resort among the available drugs [3]. However, it has been clinically approved that aminoglycosides have a narrow therapeutic index due to nephrotoxic and irreversible ototoxic side effects [4]. In addition, more *Acinetobacter baumannii* and *Pseudomonas aeruginosa* isolates are now also resistant to these aminoglycosides.

Carbapenems are broad-spectrum antibiotics that show rapid and great bactericidal effects. They are commonly used to treat bacterial infections caused by various pathogens [5]. The presence of extended-spectrum beta-lactamases (ESBL) has become the predominant cause of bacterial resistance to carbapenems, which are commonly used in the

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empiric treatment of infections [6]. Due to the constant increase in antibiotic resistance, there is an increasing interest in natural products that may have antimicrobial activity.

To develop safer drugs, many studies on the antimicrobial activity of herbal extracts have been carried out. In medicinal plant research, products from natural plants are among the alternative agents examined to replace conventional antibiotics and synthetic antimicrobials [7,8]. These reports showed that herbs are less efficient than the antibiotics based on an in vitro study. However, the medicinal plant synergy with conventionally used antibiotics has become an important area of interest because many diseases possess a multi-causal aetiology and complex pathophysiology. Well-designed drug combinations are much more effective than single-drug therapy in treatments. *Pithecellobium clypearia* (*P. clypearia*) is a member of the Mimosaceae family, which is grown in Southern China as a prescribed medicine used for the treatment of upper respiratory tract infections, pharyngitis, laryngitis, acute tonsillitis, acute gastroenteritis, and bacterial dysentery. It has been reported that *P. clypearia* has antiviral activities and anti-inflammatory activities [9,10]. Two antiviral compounds, flavan 7-O-galloyltricetifavan and 7,4′-di-O-galloyltricetifavan, isolated from a methanol extract of leaves of *P. clypearia* have antiviral activity against respiratory syncytial virus, influenza A, Coxsackie B3 and herpes simplex virus type 1 [11]. 3, 3′-Neolignans and other compounds in *P. clypearia* have shown promising activity against nitric oxide (NO) and considerable antioxidant activity [12]. In conclusion, *P. clypearia* has anti-infection and anti-inflammatory effects; therefore, *P. clypearia* may be used as a drug to treat bacterial infections in the clinic.

Previous data from our lab have shown that S20b extracted from *P. clypearia* has great activity against clinically isolated MRSA strains. However, there is still no available data for its effect against multidrug-resistant *A. baumannii* and *P. aeruginosa* isolates. Therefore, the aim of this study was to investigate the antibacterial activity of *P. clypearia* alone or in combination with conventional antibacterial agents of S20b to identify a new solution to solve the drug-resistance problem. Testing was performed against a diverse strain set of MDR *A. baumannii* and *P. aeruginosa* clinical isolates.

2. Materials and methods

2.1. Plant extract preparation

The plant was collected from the herb farm of Guangzhou HuaCheng Pharmaceutical Company. The School of Life Sciences, Sun Yat-Sen University authenticated the plant material, and a voucher specimen (number #0020) was deposited.

The branches and leaf parts of *Pithecellobium clypearia* 300 g were extracted with 60 % ethanol at 60 °C for 4 h, and then the extract was successively fractionated between water and ethyl acetate. The ethyl acetate fraction was S20b.

2.2. Antibiotics

The antibiotics imipenem (Imp), cefoperazone (CFP), ceftazidime (CAZ), levofloxacin (LEV), amikacin (AMK), tetracycline (TE) and polymyxin B sulfate (PB) in powder form were obtained from Meilunebio (Dalian, China). All antibiotics were dissolved in sterile water.

2.3. Bacterial isolates

The 20 MDR *P. aeruginosa* and MDR *A. baumannii* isolates used in this study were obtained and identified by The First Affiliated Hospital of Sun Yat-sen University. The meropenem and imipenem susceptibilities of these strains were evaluated by using the E-test (AB Biodisk, Solna, Sweden) according to the CLSI standard. *P. aeruginosa* ATCC 27,853 as a control strain was used in the study.

2.4. Composition analysis by RRLC-MS/MS

Chromatographic analysis was performed on an Agilent RRLC instrument (Agilent Corp., USA). The column was an Ultimate XB-C18 column (150 × 30 mm, 0.5 μm, Welch, Shanghai, China). The column temperature was 30 °C. The mobile phases were composed of acetonitrile (A) and water with 0.1 % formic acid (B) using a gradient elution of 95–75 % A at 0–15 min and 5–25 % B at 15–40 min with a flow rate of 0.5 mL/min. The injection volume of sample was 5 μL. Mass spectrometry was performed on a Triple TOFTM5600 (AB SCIEX, Foster City, CA) hybrid triple quadrupole time-of-flight mass spectrometer equipped with an ESI source, and the mass range was set at *m/z* 100–1000. The conditions of the MS/MS detector were as follows: ion source gas, 150 psi; ion source gas, 250 psi; curtain gas, 15 psi; temperature, 550 °C; ion spray voltage, floating at 1500 V; collision gas pressure, 8 psi; entrance potential, 10 V. Acquisition and analysis of data were conducted with Peak View Software TMV. 1.1 (AB SCIEX, Foster City, CA).

2.5. Assessment of antibacterial activity against two multidrug-resistant gram-negative bacteria

The determination of the minimal inhibitory concentration (MIC) of S20b and antibiotics complied with the Clinical Laboratory and Standards Institute (CLSI) broth micro-dilution reference method [13]. Imp, CFP, CAZ, LEV and TE were used for *Pseudomonas aeruginosa* testing, and Imp, PB, CAZ, LEV and TE were used for *Acinetobacter baumannii* testing. MIC test systems were created by diluting different antibiotics in 96-well plates (JET BIOFIL, Guangzhou, China) to a volume of 50 μl, and the antibiotics were double diluted to final concentrations ranging from 0.5 to 512 μg ml^{-1}; the S20b concentrations ranged from 25 to 3200 μg/ml. A total of 50 μl of bacterial inoculum was added to each drug-containing well. Bacteria were inoculated and cultured on blood agar (bioMérieux, France) for 18 to 24 h at 37 °C, and then isolated colonies were suspended to 0.5 McFarland (1×10^8 CFU ml^{-1}) in sterile 0.9% NaCl using a DensiCHEK PLUS handheld

Table 1
Compounds identified in S20b.

No.	Retention time (min)	Formula	Compound
1	3.50	$C_7H_6O_5$	gallic acid
2	6.39	$C_{15}H_{14}O_7$	(−)-epigallocatechin
3	12.80	$C_8H_8O_5$	methyl gallate
4	25.81	$C_9H_{10}O_5$	ethyl gallate
5	27.15	$C_6H_6O_3$	pyrogallol
6	39.97	$C_{21}H_{20}O_{12}$	myricetin-3-O-α-L-rhamnopyranoside
7	50.81	$C_{21}H_{20}O_{11}$	quercetin-3-O-α-L-rhamnopyranoside
8	52.78	$C_{22}H_{18}O_{10}$	(-)-5, 3', 4', 5'-tetrahydroxy-flavan-7-gallate

Table 2
Activity S20b and antibiotics against *A. baumannii* and *P. aeruginosa* clinical isolates.

Bacterial Species (No. Isolates)	MIC (μg ml^{-1})			
	Drug	50%	90%	Range
MDRAB (20)	Imp	32	32	1–32
	TE	256	512	2–512
	LEV	8	32	0.5–32
	CAZ	512	512	64–512
	PB	4	16	0.5–64
	S20b	300	600	300–600
MDRPsA (20)	LEV	4	32	0.5–128
	Imp	32	64	4–64
	AMK	1	32	1–256
	CAZ	32	256	2–512
	CFP	64	512	8–512
	S20b	400	800	200-800

[a]Tested strains: Multidrug-resistant *Acinetobacter baumannii* (MDRAB) and *Pseudomonas aeruginosa* (MDRPsA), 20 strains each isolated from clinic patients.
[b]LEV, levofloxacin; Imp, imipenem; AMK, amikacin; CAZ, ceftazidime; CFP, cefoperazone; TE, tetracycline; PB, polymyxin B sulfate; S20b, extract from *Pithecellobium clypearia.*

Table 3
The MBC of S20b against *A. baumannii* and *P. aeruginosa* clinical isolates.

Bacterial Species (No. Isolates)	MBC (μg ml$^{-1)}$		
	50 %	90 %	Range
MDRAB (20)	600	1200	600–1200
MDRPsA (20)	1600	1600	400–1600

Tested strains: *Acinetobacter baumannii* (MDRAB) and *Pseudomonas aeruginosa* (MDRPsA), 20 strains each isolated from clinical patients.

colorimeter (bioMérieux, France). This suspension was diluted 1/150 with Mueller-Hinton Broth (OXOID LTD, England) to 1×10^6 CFU/mL to achieve a final inoculum concentration of 5×10^5 CFU ml^{-1} in the wells. MIC values were determined visually after incubation at 37 °C for 18 to 20 h. The results were considered valid if *P. aeruginosa* ATCC 27,853 (American Type Culture Collection, Manassas, VA) was tested in each experiment in accordance with the CLSI standard quality control ranges for all antibiotics tested [14,15].

Furthermore, the bacteria from wells containing S20b were cultured at 37 °C on a blood agar plate for 20 h. We observed the concentrations of the dilutions where no bacterial growth was observed on the blood agar plate, and they were noted as the minimal bactericidal concentration (MBC) of S20b.

2.6. Assessment of antibiotic activity modulation of S20b against multidrug-resistant gram-negative bacteria

The interactions between S20b and the antibiotics were tested by using the micro-broth checkerboard method [16]. The mixture of S20b and five clinically conventionally used antibiotics were combined in each well with different final concentrations ranging from 4*MIC to 1/8*MIC, and the mixture was inoculated with bacterial inoculum and incubated at 37 °C for 18 to 20 h. To calculate the fractional inhibitory concentration (FIC) index, we used the following formulas:

$$FIC_{antibiotic} = \text{MIC of antibiotic in combination} \div \text{MIC of antibiotic alone}$$

$$FIC_{S20b} = \text{MIC of S20b in combination} \div \text{MIC of S20b alone}$$

$$FIC_{index} = FIC_{antibiotic} + FIC_{S20b}$$

With this method, a FICI ≤ 0.5 indicated synergistic, FICI > 0.5–1 indicated additive, and FICI > 2 was considered to be antagonistic [17].

2.7. Morphology of S20b-exposed cells

Transmission electron microscopy (JEOL JEM-100CX-, Japan) was used to examine the morphological changes in the selected MDR *A. baumannii* and MDR *P. aeruginosa* strains, which were cultured in media containing S20b at 1/2*MIC, 1/4*MIC and 1/8* MIC and with the same volume of sterile water for 10 h. Cells were prepared for transmission

Table 4
FICI on the combination of S20b with five antibiotics against MDR *A. baumannii* (20 isolates).

FIC	S20b + Imp	S20b + TE	S20b + PB	S20b + CAZ	S20b + LVX
FIC ≤ 0.5	70 %	50 %	15 %	10 %	5 %
0.5 < FIC ≤ 1	30 %	50 %	35 %	85 %	55 %
1 < FIC ≤ 2	—	—	50 %	5 %	40 %
FIC > 2	—	—	—	—	—

LEV, levofloxacin; Imp, imipenem; CAZ, ceftazidime; TE, tetracycline; PB, polymyxin B sulfate; S20b, extract from *Pithecellobium clypearia.*

Table 5
The change in the MICs of antibiotics in combination with S20b against MDR *A. baumannii* (20 isolates).

	Imp		TE		PB		CAZ		LEV	
	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}
Alone	32	32	512	256	16	4	512	512	32	8
Combined with 75 μg ml^{-1} S20b	16	8	128	64	2	1	256	128	8	4

LEV, levofloxacin; Imp, imipenem; CAZ, ceftazidime; TE, tetracycline; PB, polymyxin B sulfate; S20b, extract from *Pithecellobium clypearia.*

electron microscopy (TEM), which were fixed by a graded series of ethanol, followed by drying and coating with gold [18].

2.8. Assessment of the effects of S20b on cell membrane function by the release of cellular K^+

The K^+ efflux was measured by a K^+ KIT (Jiancheng, Nanjing, China) according to the manufacturer's instructions. MRSA cells were grown in MHB, washed twice with buffer A, and resuspended in buffer A. Sample fluorescence (excitation, 346 nm; emission, 505 nm) background data were collected in an F-2000 fluorescence spectrophotometer (Hitachi, San Jose, Calif.) [19].

2.9. Statistical analysis

Data were expressed as the means ± SD. Statistical comparisons were performed with one-way ANOVA followed by the LSD for multiple-group comparisons, and a P-value < 0.05 was considered statistically significant.

3. Results

3.1. RRLC-MS/MS analysis of compounds in S20b

To characterize the compounds in S20b, RRLC-Q-TOF-MS/MS was conducted. Table 1 shows the MS/MS chromatograms and tentative structures of these compounds. Eight compounds were identified. Five of them, including gallic acid, (-)-epigallocatechin, methyl gallate, and ethyl gallate, belong to the catechin class. Other compounds, such as myricetin-3-O-α-L-rhamnopyranoside and quercetin-3-O-α-L-rhamnopyranoside, belong to the glycoside class.

3.2. The result of antibacterial activity

This study evaluated the antibacterial activities of S20b and 5 clinically conventionally used antibiotics such as Imp, TE, LEV, CAZ and PB against 20 *Acinetobacter baumannii* isolates and Imp, LEV, CFP, CAZ and AMK against 20 *Pseudomonas aeruginosa* isolates. All of these strains were from the clinic. The 50 % and 90% minimal inhibitory concentrations (MIC_{50}, MIC_{90}) and the MIC range for tested drugs are listed in Table 2. The 50 % and 90 % minimal bactericidal concentrations (MBC_{50}, MBC_{90}) and MBC range for S20b are listed in Table 3.

S20b, extracted from *Pithecellobium clypearia*, showed significant inhibition on the growth of all *A. baumannii* and *P. aeruginosa* clinical isolates. The MIC_{90} and MIC_{50} of S20b against *A. baumannii* were 600 μg ml^{-1} and 300 μg ml^{-1}, respectively, which showed the same antibacterial ability degree as TE with MIC_{90} and MIC_{50} values of 512 μg ml^{-1} and 256 μg ml^{-1}, respectively. The MIC_{90} and MIC_{50} of S20b were both 512 μg ml^{-1}, indicating that S20b performed better than CAZ. The MIC_{90} and MIC_{50} of S20b against *P. aeruginosa* were 400 μg ml^{-1} and 800 μg ml^{-1}, respectively. The MBC_{90}/MBC_{50} of S20b against *A. baumannii* and *P. aeruginosa* were 1200/600 μg ml^{-1} and 1600/1600 μg ml^{-1}, respectively. In conclusion, S20b shows great antibacterial activities against two kinds of G-MDR bacteria. Its activities are stronger than some antibiotics that bacteria have developed resistance to.

3.3. Determination of synergistic activity of S20b with five antibiotics

The results of the combination studies that were performed by using the microbroth checkerboard method against the 20 clinical *A. baumannii* isolates are shown in Tables 4 and 5. With FICI ≤ 0.5, synergistic interactions were observed between S20b and the various antibiotics in this test, especially with Imp and TE (14 and 10 isolates

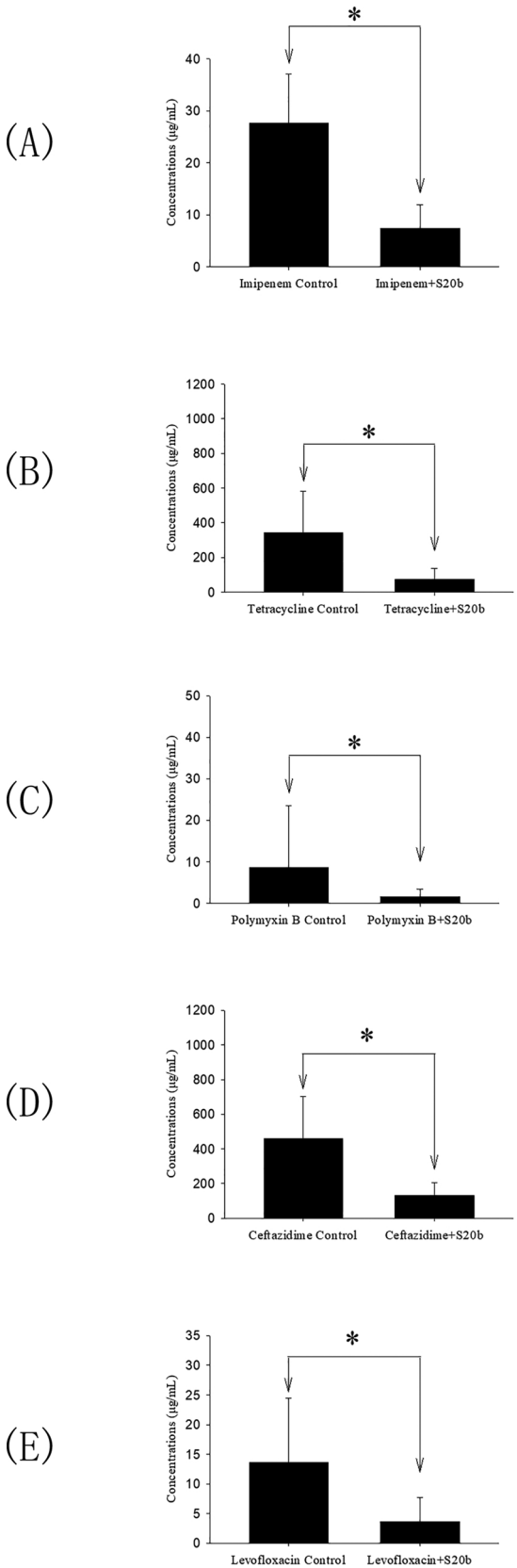

Fig. 1. Effect of S20b on the activity of antibiotics (A: imipenem; B: tetracycline; C: polymyxin B sulfate; D: ceftazidime; E: levofloxacin) against MDRAB. Values represent the geometric mean ± SD. One-way ANOVA followed by LSD. P < 0.05 antibiotic + S20b vs antibiotic control.

Table 6
FICI on the combination of S20b with five antibiotics against MDR *P. aeruginosa* (20 isolates).

FIC	S20b + CAZ	S20b + CFP	S20b + AMK	S20b + IMP	S20b + LVX
FIC ≤ 0.5	40 %	20 %	20 %	15 %	10 %
0.5 < FIC ≤ 1	35 %	55 %	45 %	55 %	35 %
1 < FIC ≤ 2	25 %	25 %	35 %	30 %	55 %
FIC > 2	—	—	—	—	—

LEV, levofloxacin; Imp, imipenem; AMK, amikacin; CAZ, ceftazidime; CFP, cefoperazone; S20b, extract from *Pithecellobium clypearia.*

Table 7
The change in the MICs of antibiotics in combination with S20b against MDR *P. aeruginosa* (20 isolates).

	LEV		Imp		AMK		CAZ		CFP	
	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}
Alone	32	4	32	64	32	1	256	32	512	64
Combined with 100 μg ml^{-1} S20b	8	1	16	2	2	0.25	64	8	256	16

LEV, levofloxacin; Imp, imipenem; AMK, amikacin; CAZ, ceftazidime; CFP, cefoperazone; S20b, extract from *Pithecellobium clypearia.*

out of 20 isolates, respectively) against *A. baumannii.* Moreover, there were no antagonist interactions with FICI > 2 between S20b and the various antibiotics. There was a significant reduction ($P < 0.05$) in the MIC values for 5 antibiotics tested against MDRAB (Fig. 1).

The results of the combination studies against the 20 clinical P. aeruginosa isolates are shown in Tables 6 and 7. With FICI ≤ 0.5, synergistic interactions were observed between S20b and the various antibiotics in the test, particularly with CAZ (8 isolates out of 20 isolates) against P. aeruginosa. In addition, there were no antagonist interactions with FICI > 2 between S20b and the various antibiotics. In this experiment, S20b in combination with all chosen antibiotics mostly showed additive effects. There was a significant reduction ($P < 0.05$) in the MIC values for 5 antibiotics tested against MDRPsA (Fig. 2).

These results confirm that S20b combined with 5 clinical antibiotics can enhance their antibacterial activity against MDR A. baumannii and P. aeruginosa. The combination of S20b and antibiotics has great potential for use in treating MDR bacterial infections.

3.4. Effects of S20b on cell wall and cell membrane integrity

The cell morphology of these bacteria changed after ten hours of exposure to S20b at 1/2*MIC, 1/4*MIC and 1/8* MIC. The cytoplasm revealed outpourings from the bacterial surface, and there were numerous protrusions on the cell surface. This showed significant damage to the bacterial cell walls of both *A. baumannii* and *P. aeruginosa* (Figs. 3 and 4).

3.5. Effects of S20b on cell membrane function

The supernatant was obtained by mixing 3 different concentrations (75, 150, and 300 μg ml^{-1}) of S20b with two *A. baumannii* isolates (a sensitive isolate and an MDR isolate) and two *P. aeruginosa* isolates (a sensitive isolate and an MDR isolate), incubating at 37 °C overnight, and centrifuging the cultures at 5000 rpm for 5 min. The concentration of K^+ in the supernatant was used to express the effect of S20b on the cell membrane. Fig. 5 shows that compared with the control group, all *A. baumannii* and *P. aeruginosa* isolates with 300 μg ml^{-1} S20b could significantly increase the amount of K^+ in the supernatant, which indicates that S20b can increase the cell membrane permeability of *A. baumannii* and *P. aeruginosa.*

4. Discussion

Currently, the emerging occurrence of MDR bacteria has led to a serious global medical crisis, which continuously challenges the scientific community [20]. The diminishing efficacy and increasing toxicity of synthetic drugs aggravate this problem. Therefore, more attention must be paid to screen new natural products for solutions. Traditional medicine has a long history that has been practised in the world, especially the application of herbal plants for therapeutic purposes. China has many sources of medicinal plants, which are sufficient to provide alternative remedies [21,22].

Since the 1970s, with the spread of multidrug-resistant (MDR) Acinetobacter strains among critically ill, hospitalized patients, and subsequent epidemics, *A. baumannii* has become an increasing cause of concern [23]. *P. aeruginosa* is a tenacious bacterium because of its ability to develop resistance to multiple antibiotics. Hence, there are a few anti-MDR *P. aeruginosa* drugs in the pipeline [24]. There is an urgent need for novel and effective infection control strategies to face this exceedingly large challenge of drug resistance [25,26].

Several traditional herbs have been reported to be used against gram-negative bacteria. For instance, BinShan et al. studied 46 extracts from dietary spices and medicinal herbs, which were investigated by the agar-well diffusion method against five foodborne bacteria (*Bacillus cereus*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella anatum*) in vitro [27]. AJ Afolayan et al. found that none of the extracts from Arctotis can inhibit *Klebsiella pneumoniae* or *Pseudomonas aeruginosa*, which are both gram-negative bacteria. However, the extracts showed significant growth inhibition against all the fungi tested [28]. Yoko Miyasaki et al. screened sixty herbal extracts against MDR *A. baumannii*; 30% of them demonstrated in vitro antibacterial activity against MDR *A. baumannii* [29].

In addition, five compounds were identified in S20b belonging to the catechin class. The catechin class of compounds have been

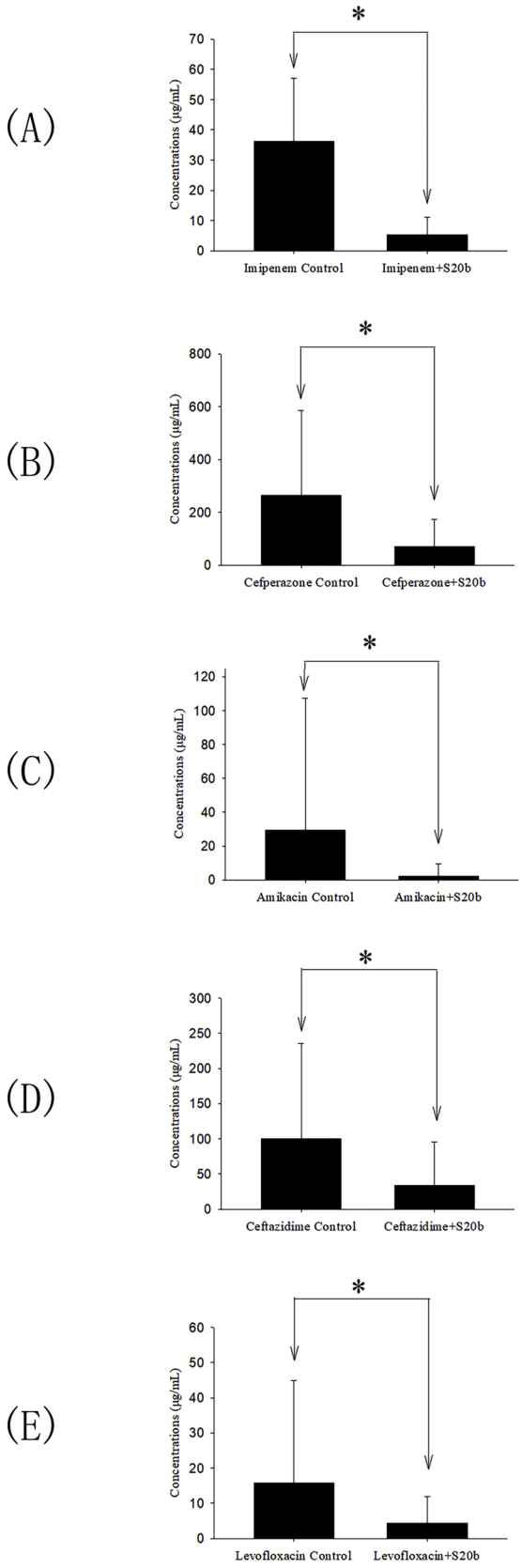

Fig. 2. Effect of S20b on the activity of antibiotics (A: imipenem; B: cefoperazone; C: amikacin; D: ceftazidime; E: levofloxacin) against MDRPsA. Values represent the geometric mean ± SD. One-way ANOVA followed by LSD. $P < 0.05$ antibiotic + S20b vs antibiotic control.

confirmed to be antibacterial. A previous report showed that the mechanism of synergy between epigallocatechin gallate (EGCG), which is a compound in S20b and b-lactams such as AMP against MRSA, was attributed to interference with the integrity of the cell wall through direct binding to peptidoglycan [30] and had been reported to have a significant effect on anti-MRSA [31,32]. Gallic acid has been reported to have antibacterial activity, with lower MICs against MRSA ranging between 3.25 and 12.5 μg/ml [33]. The other compound, ethyl gallate, has also been reported to overcome resistance in drug-resistance bacterial [34]. In addition, the glycoside class of compounds such as quercetin-3-O-α-L-rhamnopyranoside have been confirmed to have antibacterial activity against drug-resistance bacterial at 256 μg/mL [35]. Therefore, the significant antibacterial activity of S20b may ascribed to these compounds.

The morphological effect of S20b was remarkably effective on the bacterial cell wall. We also sought to confirm whether membrane depolarization by S20b caused the loss of membrane function. Bacterial cell wall structural changes and membrane permeability changes can enhance bacterial resistance [36,37]. Therefore, the possibilities of antibiotic modulation mechanisms of S20b are destroying the cell walls of bacteria and increasing cell membrane permeability.

Generally, finding a new solution involving traditional herbs to solve the MDR problem may be a feasible method. *Pithecellobium clypearia* is a Chinese herb used in traditional Chinese medicine. It is well known by its antiviral and anti-inflammation activities [10,12,38,39]. S20b is an extract from *Pithecellobium clypearia*, which was shown to have significant anti-MRSA capability by our lab. In the present report, the anti-*A. baumannii* and anti-*P. aeruginosa* potentials of S20b were demonstrated with in vitro tests. Additionally, S20b can synergistically interact with five conventionally used antibacterial agents. A broad spectrum of synergy and additive effects were revealed when S20b was mixed with clinical agents of different classes. All combinations of S20b with the 5 antibacterial agents showed positive interactions by various degrees. The morphology clearly indicated that S20b dramatically affected the *A. baumannii* and *P. aeruginosa* cell walls. It is notable that the bacterium did not grow well in the pressure of S20b at 1/2, 1/4*MIC. These two concentrations of S20b can destroy many *A. baumannii* and *P. aeruginosa* cell walls. As shown in this study, S20b at 1/2*MIC caused the bacterial membrane permeability to increase significantly.

5. Conclusion

It can be confirmed that S20b from *Pithecellobium clypearia* has anti-*A. baumannii* and anti-*P. aeruginosa* activities. The synergistic effects were observed when S20b was used in combination with antibiotics, which demonstrated that S20b may have a potential clinical use in enhancing the efficacy of current antibiotics such as imipenem (Imp), cefoperazone, ceftazidime, levofloxacin, amikacin, tetracycline and polymyxin B sulfate. The synergistic effects on *A. baumannii* and *P. aeruginosa* isolates in this study are related to the increase in membrane permeability.

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Declaration of Competing Interest

The authors declare that they have no conflict of interest.

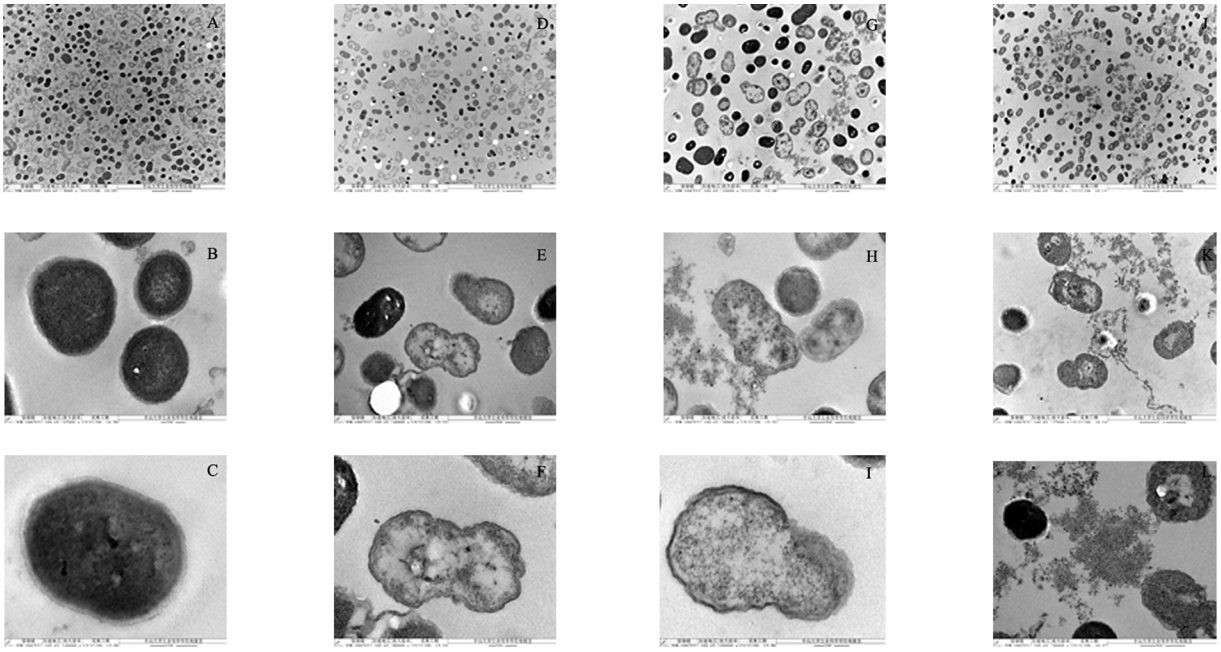

Fig. 3. Representative TEM images of MDR *A. baumannii* treated with different concentrations of drugs; A–C, sterile water; D–F, 37 μg ml^{-1} S20b; G–I, 75 μg ml^{-1} S20b; J–L, 150 μg ml^{-1} S20b.

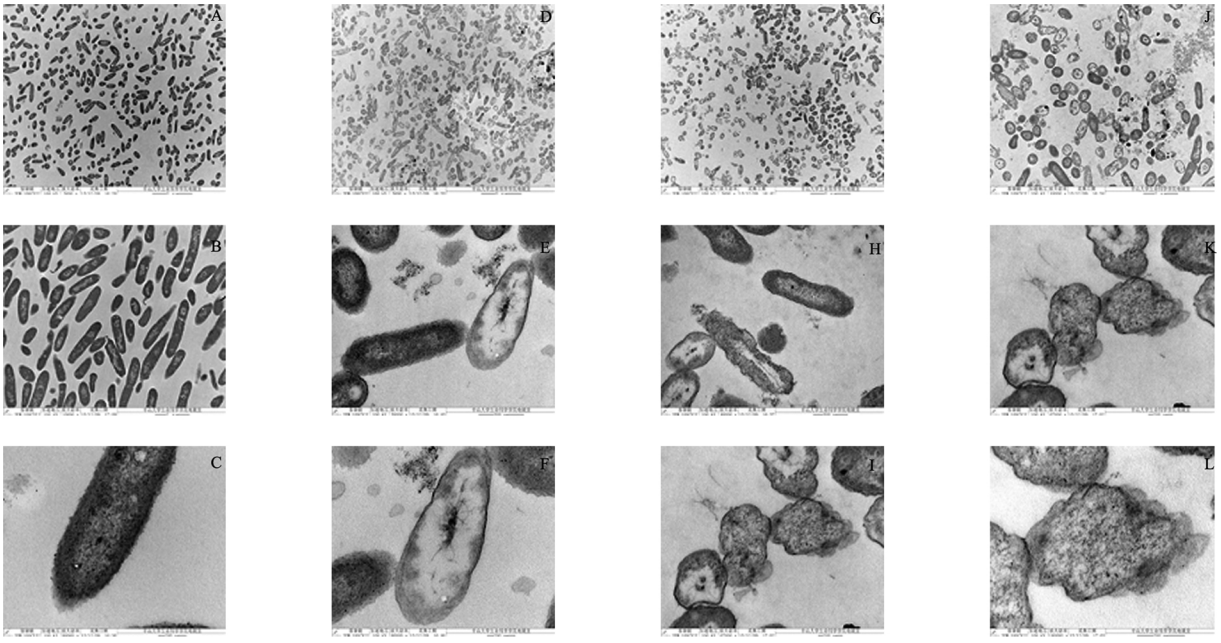

Fig. 4. Representative TEM images of MDR *P. aeruginosa* treated with different concentrations of drug; A–C, sterile water; D–F, 37 μg ml^{-1} S20b; G–I, 75 μg ml^{-1} S20b; J–L, 150 μg ml^{-1} S20b.

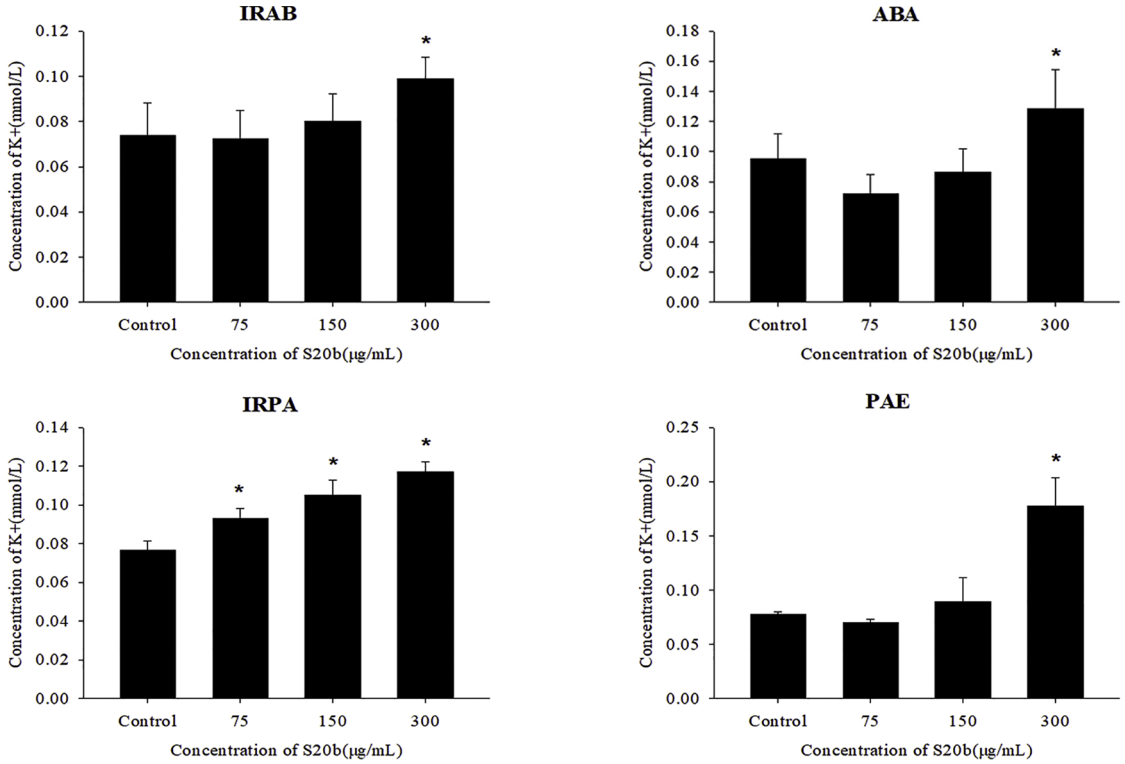

Fig. 5. The concentration of K^+ in the sensitive MDR *A. baumannii* (IRAB), *A. baumannii* (ABA), MDR *P. aeruginosa* (IRPA) and sensitive *P. aeruginosa* (PAE) cultures after treatment with different concentrations of S20b; Control: without S20b exposure, 75: 75 μg ml^{-1} S20b, 150: 150 μg ml^{-1} S20b, 300: 300 μg ml^{-1} S20b (n = 3, *p < 0.05 compared with control group.).

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