Antibacterial effects of margatoxin on ciprofloxacin resistant clinical isolates of *Acinetobacter baumannii*

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ABSTRACT

Introduction: *Acinetobacter baumannii*, a gram-negative pathogen, is recognized as a healthcare-associated opportunistic pathogens. In this study, the in vitro antibacterial properties of margatoxin (MgTX) were investigated against clinical isolates.

Method: The drug resistance profile of five antibiotics: piperacillin (100µg), imipenem (10µg), ceftazidime (30µg), amikacin (30µg), and ciprofloxacin (5µg) were evaluated with disc diffusion (Kirby-Bauer) method in 20 clinical isolates. Biofilm formation was assessed using the microtiter plate assay. Synergistic effects of MgTX in combination with ciprofloxacin was evaluated.

Results: Our results showed that the resistance to all antibiotics was ≥90%. All isolates had ability of biofilm formation (moderate to strong). Checker board method confirmed synergistic effect between MgTX and ciprofloxacin. indicated that a dose-dependent suppression of bacterial growth was achieved with the combined use of MgTX and ciprofloxacin.

discussion: It seems that MgTX, with antibacterial properties, can use in therapeutic strategies in future against antibiotic resistant isolates.

KEYWORD: *Acinetobacter baumannii*, ciprofloxacin, MgTX, Synergistic effect

1. INTRODUCTION

Acinetobacter baumannii, is a gram-negative, healthcare-associated opportunistic pathogens (Cain & Hamidian, 2023; Tacconelli et al., 2018). As one of the ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, A. baumannii, Pseudomonas aeruginosa, and Enterobacter* spp.) *A. baumannii* poses a significant global threat to human health and presents a therapeutic challenge (Kyriakidis, Vasileiou, Pana, & Tragiannidis, 2021). Its high level of antibiotic resistance and biofilm formation contributes to a variety of infections, including those affecting the bloodstream, wounds, urinary tract, and lungs (Cain & Hamidian, 2023). The antibiotic resistance mechanisms employed by *A. baumannii* include alterations to target site (Martínez-Trejo et al., 2022), reduced outer membrane permeability (Chen, Liu, Zhang, Leung, & Xia, 2021), production of beta-lactamases, and overproduction of efflux pumps (Leão et al., 2023).

Scorpions, which have existed for over 400 million years, have developed an array of venom peptides during their evolution, making them a rich resource of antimicrobial peptides (AMPs) (Li, Yuan, Li, Deng, & Wang, 2020). Scorpion venom contains a variety of low-molecular-weight peptides, including amino acids, biogenic amines, enzymes, oligopeptides, nucleotides, lipids, mucoproteins, and other substances (Pashmforoosh & Baradaran, 2023).

Previous studies have shown that AMPs interact specifically with bacterial cells membranes through electrostatic attraction, eradicating bacteria without inducing bacterial resistance (Li et al., 2020). Several AMPs have been identified from different scorpion species, including BmKn2 from *Buthus martensii Karsch* (Zeng, Wang, Zhu, Zhu, & Li, 2004), IsCTs from *Opisthacanthus madagascariensis* (Dai, Corzo, Naoki, Andriantsiferana, & Nakajima, 2002), Scorpine from Pandinus imperator (Casper, 1985), and MgTx from *Centruroides margaritatus* (Garcia-Calvo et al., 1993).

Margatoxin (MgTX), or Potassium channel toxin alpha-KTx 2.2, is a peptide derived from the venom of *Centruroides margaritatus* (de Oliveira, Soares, & Da Silva, 2023). MgTx is a 39-amino-acid peptide toxinwith a three-dimensional structure comprising an α-helix and three antiparallel β-strands; stabilized by three disulfide bonds (Naseem et al., 2021). Scorpion venom peptides have been reports to possess various therapeutic properties, including antimicrobial, antibacterial, antifungal, antimycobacterial, cancer-biomarker, and cell penetrating capabilities (Javed et al., 2022; Usmani, Agrawal, Sehgal, Patel, & Raghava, 2019). These peptides function primarily by forming pores in bacterial cell walls, leading to depolarization and subsequently antimicrobial effects (Mendoza-Tobar et al.). Given their

high biological activity,, AMPs are promising candidates for the treatment of antibiotics - resistant bacteria. This study examines the in vitro antibacterial properties of MgTx against isolates of *A. baumannii*. Additionally, we evaluated the drug resistance of the selected derivative and their synergistic effect with the antibiotic ciprofloxacin (CIP).

2. MATERIALS AND METHODS

In this study, 400 clinical samples were collected from various hospitals and laboratories in the Tehran province. The reference strain, *Acinetobacter baumannii* ATCC 19606, was purchased from the Pasteur Institute of Iran.

2.1. Antibiotic Resistance Profiles

Following the 2023 CLSI guidelines, the disc diffusion method (Kirby-Bauer) was employed to identify the antibiotic resistance profiles in 20 clinical isolates of *A. baumannii* for five antibiotics: piperacillin (100µg), imipenem (10µg), ceftazidime (30µg), amikacin (30µg), and ciprofloxacin (5µg). Additionally, six ciprofloxacin-resistant isolates and the ATCC 19606 strain were further investigated. Additional tests were performed using Mueller-Hinton Broth (MHB) and Mueller-Hinton Agar (MHA).

2.2. Biofilm Formation assay

The microtiter plate assay (MTPA) was used to evaluate biofilm formation. *A. baumannii* ATCC 19606, a known biofilm producer, served as the positive control. All isolates and the reference strain were cultivated on Tryptic Soy Agar (TSA) medium (Merck, Germany) and incubated for 24hours at 37°C. Fresh colonies were inoculated in 10 mL of Tryptic Soy Broth (TSB) (Merck, Germany) and incubated for 15–18 hours at 37°C whit shaking at 200 rpm. The optical density of each liquid culture was adjusted to 0.1 at 620 nm. The bacterial cultures were then diluted in TSB to prepare a suspension equivalent to 0.5 McFarland (0.5×10^8 CFU/mL). Each well of a 96-well polystyrene plate received 200 µL of bacterial the suspension. Plates were incubated for 72 hours at 37 °C.

Sterile broth was added to the negative control wells, and the control organism was similarly cultured and incubated. After incubation, the contents of each well were removed, and the wells were washed twice with phosphate buffer saline (PBS) (pH 7)) to eliminate free-floating bacteria. The wells were then fixed with 95% methanol for 10 minutes, stained with 1% crystal violet for 5 minutes, and excess stain was removal with sterile distilled water. After

adding 100 µl of 33% glacial acetic acid to the wells, biofilm formation was measured at 570 nm using a Biotek ELx800 ELISA auto-reader (USA) (Mohsenzadeh et al., 2021).

2.3. Minimum Inhibitory Concentrations (MIC) of MgTX and CIP

The minimum inhibitory concentrations (MIC) of Margatoxin (MgTX) and ciprofloxacin (CIP) were determined using the microdilution method, following the Clinical and Laboratory Standards Institute (CLSI) protocol (MA, 2006). A 0.5 McFarland (0.5×10^8 CFU/ml) suspension of A. baumannii ATCC 19606 and six ciprofloxacin-resistant isolates was prepared in Muller-Hinition broth. Serial dilutions of MgTX and CIP, ranging from 1 to1024 µg/mL, were prepared in tubes. After 24hours of incubation the MIC was determined.

2.4. Synergistic Effects of MgTX in combination with CIP

The synergistic effects of MgTX with CIP against *A. baumannii* isolates were evaluated in vitro using the checkerboard dilution method. Eight different concentrations of MgTX and CIP were diluted in MHB. In each longitudinal column of wells, a constant concentration of MgTX was added, while each horizontal row of wells received a constant concentration of CIP. Each well contained a total volume of 200 µL, consisting of 50 µL MgTX, 50 µL CIP, and 100 µL of a bacterial suspension (final concentration of $5\text{-}7.5\times10^5$ CFU/mL). The fractional inhibitory concentrations index (FICI) was calculated using the following formula:

Formula 1:

$$FICI = \frac{MIC_{(MgTX\ combination)}}{MIC_{(MgTX\ single)}} + \frac{MIC_{(CIP\ combination)}}{MIC_{(CIP\ single)}}$$

The results of the interaction were interpreted as follows: FICI≤0.5 shows synergy, 0.5<FICI≤0.75 shows partial synergy, 0.76<FICI≤1 shows additive interactions, 1<FICI≤4 shows lack of difference, FICI>4 shows antagonistic effects.

3. RESULTS

3.1. Antibiotic Susceptibility Profiles and Biofilm Formation of Isolates

A total of 400 clinical samples were collected, with men (65%) aged between 36 to 70 years representing the majority of participants. Biochemical testing was employed to identify samples extracted from sputum, urine, burns, wounds, blood, and other sources (Fig. 1.A). Figure 1.B summarizes the antibiotic susceptibility profiles of the 20 *A. baumannii* isolates.. Imipenem resistance was the highest among the isolates, with a rate of 100%. Ciprofloxacin and

piperacillin resistance were also notably high, at 90%, followed by amikacin and ceftazidime with resistance rates of 90%.

All clinical strains of *A. baumannii* tested positive for biofilm formation when evaluated using crystal violet staining in 96-well cell culture microtiter plates. The semiquantitative results of the biofilm formation capabilities are presented in Figure 1.C.

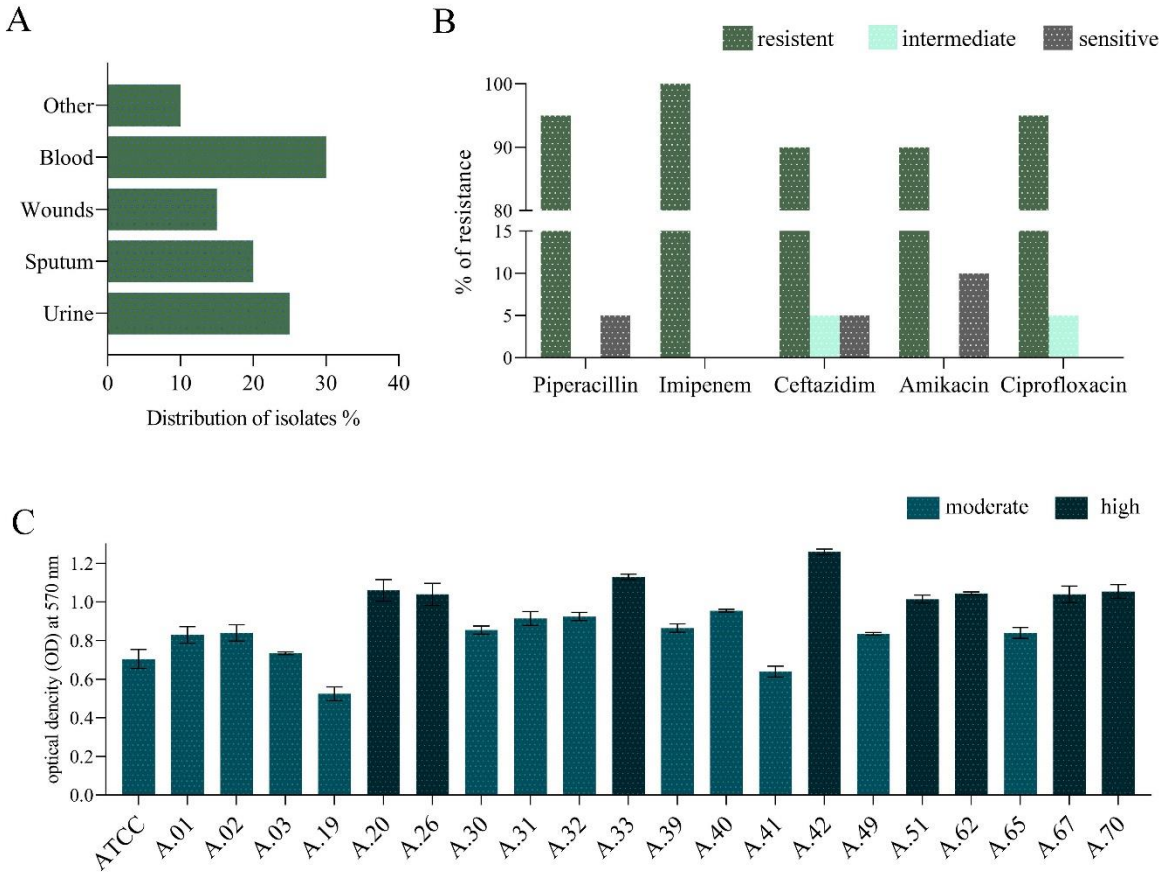

Figure.1. **A**. Distribution of *A. baumannii* isolates. **B**. profile of *A. baumannii* isolates' Antibiotic susceptibility profile of *A. baumannii* isolates as determined by the disc diffusion method. **C**. Biofilm formation ability of the isolates.

3.2. Antibacterial Properties of MgTX and CIP

The antibacterial properties of MgTX and CIP are shown in Table 1. I MgTX did not inhibit the growth of any of bacteria tested (100% resistance). In contrast, the MIC range of CIP for the tested bacteria was between 512 and 1024 µg/mL.

3.3. Synergistic Effects of MgTX in combination with CIP

The synergistic effects of MgTX and CIP were assessed using the Fractional Inhibitory Concentration Index (FICI). The results of the checkerboard assay are presented in Table 1. As indicated, *A. baumannii* isolates demonstrated synergistic interactions between MgTX and CIP.

Table 1. Interactive effects of MgTX in combination with CIP on *A. baumannii* isolates

Strains No.	**MIC $_{alone}$ (µg ml^{-1})**		**MIC $_{combined}$ (µg ml^{-1})**		**FIC(µg/ml)**
	CIP	MgTX	CIP	MgTX	
ATCC	1024	1024	16	32	0.0468*
No.19	512	1024	4	8	0.0156*
No.26	512	1024	8	8	0.0234*
No.32	512	1024	8	8	0.0234*
No.33	512	1024	4	8	0.0156*
No.62	512	1024	32	8	0.0703*
No.70	512	1024	4	8	0.0156*

*** FICI≤0.5 means synergy**

**** 0.5 <FICI≤0.75 means partial synergy**

***** 0.76 <FICI≤1 means additive**

4. DISCUSSION

It is well established from various studies that multidrug-resistant bacteria have been growing exponentially worldwide (Pfalzgraff, Brandenburg, & Weindl, 2018). In this context, new therapeutic options must be developed to treat infections of ESKAPE pathogens (Pfalzgraff et al., 2018; Vojnits et al., 2024). One such pathogen is is *Acinetobacter baumannii*, a leading sources of systemic dissemination and medical device colonization (Runci, Bonchi, Frangipani, Visaggio, & Visca, 2017).

Numerous studies have revealed a notable rise in antimicrobial resistance to many agents globally (Lari, Ardebili, & Hashemi, 2018; Leite et al., 2016). Our results align with these studies, showing that *A. baumannii* exhibits high-level resistance to all first-line antibiotics, including anti-pseudomonal cephalosporins (ceftazidime), anti-pseudomonal carbapenems (imipenem), fluoroquinolones (ciprofloxacin) and aminoglycosides (amikacin). Consequently, it is critical to determine antibiotic susceptibility profiles and implement stringent infection control measures.

Given the strong antibiofilm and antibacterial potential of antimicrobial peptides (AMPs), they may serve as promising agents for treating A. baumannii infections (Pfalzgraff et al., 2018). Several studies have investigated the antibacterial properties of MgTX, with evidence suggesting that potassium channel inhibition disrupts bacterial membrane potential(Stautz et al., 2021). Ali MK *et al.* demonstrated that *A.baumannii* exposed to ciprofloxacin stress showed increased expression of the kdpC gene, which encodes a component of the KdpD/KdpE two-component system (TCS)-regulated potassium ion transporting ATPase complex (Ali et al., 2017). The potassium transport (Kdp) system is highly conserved in *A. baumannii* strains and plays a vital role in pathogenesis (Samir et al., 2016). Disruption of ion gradientcan depolarize the bacterial cell membrane, which impairs critical biological processes, ultimately leading to bacterial cell death (Huang et al., 2020). MgTX may further interfere with ion homeostasis through its interaction with bacterial potassium channels, hindering physiological processes within bacterial cells (Stautz et al., 2021).

In a recent study, a ciprofloxacin polypeptide-based polymer (PAC-NPs) demonstrated a broad-spectrum antibacterial effect against both Gram-positive and Gram-negative bacteria, with a minimum inhibitory concentration (MIC) ranging from 1.0 to 4.0 mg/mL and a sterilization rate exceeding 91% (Zhen et al., 2023). Our findings indicated that MgTX in combination with ciprofloxacin, exhibits dose-dependent suppression of bacterial growth, making it a promising candidate for antibacterial strategies against persistent *A. baumannii* infections.

5. CONCLUSION

The growing drug resistance to conventional antibiotics underscores the urgent need to develop alternative antimicrobial agents. While few studies have explored the antibacterial properties of MgTX in recent decades, our investigation demonstrates that MgTX exerts a significant antibacterial effect against A. baumannii isolates. These findings suggest that MgTX has potential as a replacement antibiotic agent. However, further research is necessary

to comprehensively understand MgTX's mechanism of action and its full therapeutic potential.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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