Anti-*Klebsiella pneumoniae* activity of secondary metabolism of *Achromobacter* from the intestine of *Periplaneta americana*

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Abstract

Background *Klebsiella pneumoniae* is one of the main pathogens of clinical isolation and nosocomial infections, as *K. pneumoniae* show broad-spectrum resistance to β-lactam and carbapenem antibiotics. It is emerging clinical need for a safe and effective drug to anti-*K. pneumoniae*. At present, *Achromobacter* mainly focused on its degradation of petroleum hydrocarbons, polycyclic aromatic hydrocarbons, assisting insects to decompose, degrade heavy metals and utilize organic matter, but there were few reports on the antibacterial activity of the secondary metabolites of *Achromobacter*.

Results In this study, a strain WA5-4-31 from the intestinal tract of *Periplaneta americana* exhibited strong activity against *K. Pneumoniae* through preliminary screening. The strain was determined to be *Achromobacter sp.* through the morphological characteristics, genotyping and phylogenetic tree analysis, which is homologous to *Achromobacter ruhlandii* by 99%, its accession numbe in GenBank at National Center for Biotechnology Information (NCBI) is MN007235, and its deposit number was GDMCC NO.1.2520. Six compounds (Actinomycin D, Actinomycin X2, Collismycin A, Citrinin, Neoechinulin A and Cytochalasin E) were isolated and determined by activity tracking, chemical separation, nuclear magnetic resonance (NMR) and mass spectrometry (MS) analysis. Among them, Actinomycin D, Actinomycin X2, Collismycin A, Citrinin and Cytochalasin E showed a good effect on anti-*K. pneumoniae*, with MIC values of 16–64 µg/mL.

Conclusions The study reported *Achromobacter*, which was from the intestinal tract of *Periplaneta americana* with the activity against *K. Pneumoniae*, can produce antibacterial compounds for the first time. It lays the foundation for development of secondary metabolites of insect intestinal microorganisms.

Keywords *Klebsiella pneumonia*, *Periplaneta americana* intestine, *Achromobacter*, Secondary metabolites, Antibacterial activity

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Introduction

Klebsiella pneumoniae is recognized as a conditioned pathogen that colonizes the mucosal surface of patients and do not cause illness, but *K. pneumoniae* from the mucosa may spread to other tissues, leading to life-threatening infections and a variety of diseases, including pneumonia, urinary tract infections, blood infections, and sepsis [1, 2]. These infections are particularly prominent in newborns, the elderly, and individuals with weakened immune functions [3]. *K. pneumoniae* also causes a large number of community-acquired infections. The main feature of these infections is that their morbidity and mortality are related to carbapenem-resistant resistance of *K. pneumoniae* [4, 5]. Therefore, its emerging clinical need for a safe and effective drug anti-*K. pneumoniae.*

At present, *Achromobacter* is mainly isolated from the soil, plants and the digestive system of insects [6–9]. The research on *Achromobacter* at home and abroad mainly focused on its degradation of petroleum hydrocarbons, polycyclic aromatic hydrocarbons and synthetic new lipopeptide surfactants in petroleum-contaminated soil, assisting insects to decompose, degrade heavy metals and utilize organic matter [10–14], but there were few reports on the antibacterial activity of the secondary metabolites of *Achromobacter.* CHEN Zhiyu [15] isolated and identified four *Achromobacter* from the *Periplaneta american* intestinal. The four strains had inhibition ability to *B. subtilis, S. aureus, A. niger* and other pathogenic bacteria, and were both been detected the key secondary metabolite biosynthesis gene PKS I and NRPS. This revealed that the secondary metabolites of *Achromobacter* might have the ability to produce the anti-bacteria and anti fungi substances.

Periplaneta americana is a traditional Chinese medicinal material [16]. Studies had showed that the dried worms of *Periplaneta americana* had anti-inflammatory and anti-infective effects [17]. It can also promote angiogenesis, accelerate the shedding of necrotic tissues, and create conditions for tissue repair [18, 19]. In our previous studies, 159 strains of intestinal bacteria were isolated from the *Periplaneta americana* and produced a variety of antibacterial, antifungal, and anti-tumor metabolites [20–23]. In this study, a strain WA5-4-31 from the *Periplaneta americana* intestine, which exhibited strong activity against *K. Pneumoniae* through preliminary screening, was determined to be *Achromobacter* through the morphological characteristics, genotyping and phylogenetic tree analysis. The secondary metabolites of the strain WA5-4-31 were isolated and determined by activity tracking, chemical separation, nuclear magnetic resonance (NMR) and mass spectrometry (MS) analysis. This study aimed to research the material basis of the secondary metabolites of the *Achromobacter*

against *Klebsiella pneumonia*. It lays the foundation for development of secondary metabolites of insect intestinal microorganisms.

Results

Screening and identification of strain WA5-4-31

According to the preliminary activity screening of different intestinal bacteria from *Periplaneta americana*, the secondary metabolite of strain WA5-4-31 had best anti-*K. pneumonia* activity (Fig. 1A). The colony of the strain was rounded and the surface was dry and opaque on the Gauze's medium No.1 (Fig. 1B). Scanning electron microscopy shows that the strain was coryneform bacterium (Fig. 1C). Electrophoresis showed that the length of the target band was about 1500 bp, which was identical to the expected length (Fig. 1D).The strain WA5-4-31 is homologous to *Achromobacter ruhlandii* by 99% (Fig. 1E). The accession number of WA5-4-31 16 S rRNA was MN007235. The strain WA5-4-31 was finally determined to be of the genus *Achromobacter*, and its deposit number was GDMCC NO.1.2520.

Isolation and Purification of Anti-*K. Pneumoniae* Compounds

The crude extract (18.0 g) of strain WA5-4-31was loaded onto a silica gel column and eluted with an increasing polarity of ethyl acetate (dichloromethane: ethyl acetate (1-100%)), finally nine different fractions were obtained. Eight fractions showed activity against *K. pneumonia* ATCC 13,883 by Oxford Cup method, especially the fraction 2 and 3 were better, the diameters of inhibition zones of which were 25.43±0.22 and 23.61±0.34. There six pure compounds were purified from the active fractions (Table 1; Fig. 2).

Spectral analysis

Through high-resolution electrospray ionization mass spectrometry (HRESIMS) and NMR analysis and by comparison with previously reported MS and NMR data, the structures of the six pure compounds were clarified. The molecular ion peak of the mass spectrum detected by compound 1 is m/z1255.64[M+H]+, the compound 2 is m/z 1269.62[M+H]+, the compound 3 is m/z276.08[M+H]+, the compound 4 is m/z251.9[M+H]+, the compound 5 is m/z324.17[M+H]+, the compound 6 is m/z494.22[M+H]+, combined with H1 and C13 NMR spectra showed very similar to the previously reported data, so it could be determined that compounds **1**–**6** were Actinomycin D (**1**), Actinomycin X2 (**2**), Collismycin A(**3**), Citrinin (**4**), Neoechinulin A (**5**) and cytochalasin E (**6**) (Fig. 3).

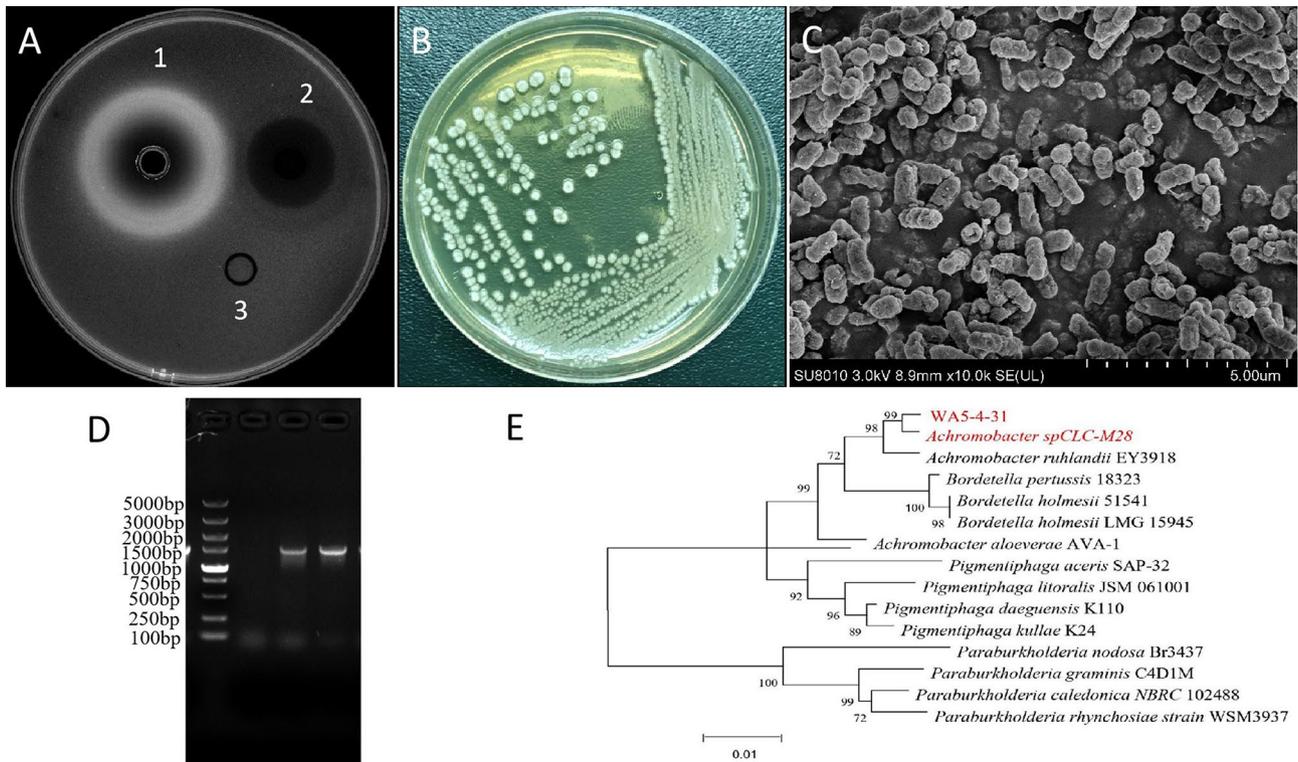Ma *et al. BMC Microbiology*   (2023) 23:162

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Fig. 1 Anti-*K. pneumonia*Activity and Identification of Strain WA5-4-31. (**A**) The Anti-*K. pneumonia* activity of WA5-4-31, 1–3 refers to sample (EtoAc 1.024 mg/mL, 150μL), positive control (Ciprofloxacin 8 μg/mL, 150 μl) and blank control (methanol solvent, 50 μl). (**B**) Culture on Gauze's medium No.1. (**C**) Observation of the culture by scanning electron microscopy (10,000×). (**D**) PCR products of strain WA5-4-31 16 S rDNA by electrophoresis. (**E**) The phylogenetic tree of strain WA5-4-31. Maximum-likelihood phylogenetic tree based on 16 S rDNA gene sequences

Table 1 Anti-*K. pneumoniae* activity and purified compounds of different fractions

| No.	Diameter of the inhibition zone (mm) (n = 3, means ± SD)	purified compound	No.	Diameter of the inhibition zone (mm) (n = 3, means ± SD)	purified compound
Fr.1	18.52 ± 0.20	compound 1 (37.52 mg)	Fr.6	8.31 ± 0.50	
Fr.2	25.43 ± 0.22	compound 2 (36.87 mg)	Fr.7	9.15 ± 0.18	
Fr.3	23.61 ± 0.34	compound 3 (71.87 mg) compound 6 (54.23 mg)	Fr.8	8.75 ± 0.23	
Fr.4	18.50 ± 0.51	compound 5 (58.72 mg)	Fr.9	Null	
Fr.5	17.12 ± 0.54	compound 4 (28.41 mg)	Positive drug	14.68 ± 0.47	
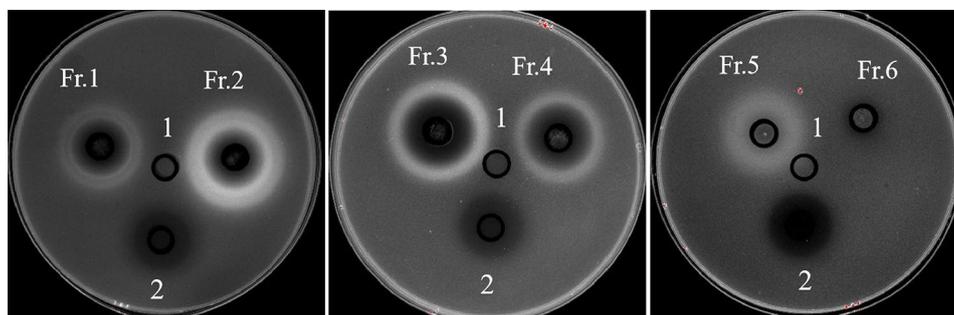
Fig. 2 The antibacterial activity of different fractions against*K. pneumoniae*by Oxford Cup method. 1: Blank control: methanol solution; 2: Positive drug: Ciprofloxacin, 4 μg/mL; Fr.1- Fr.6: Methanol solution with diferent fractions, 5 mg/mL
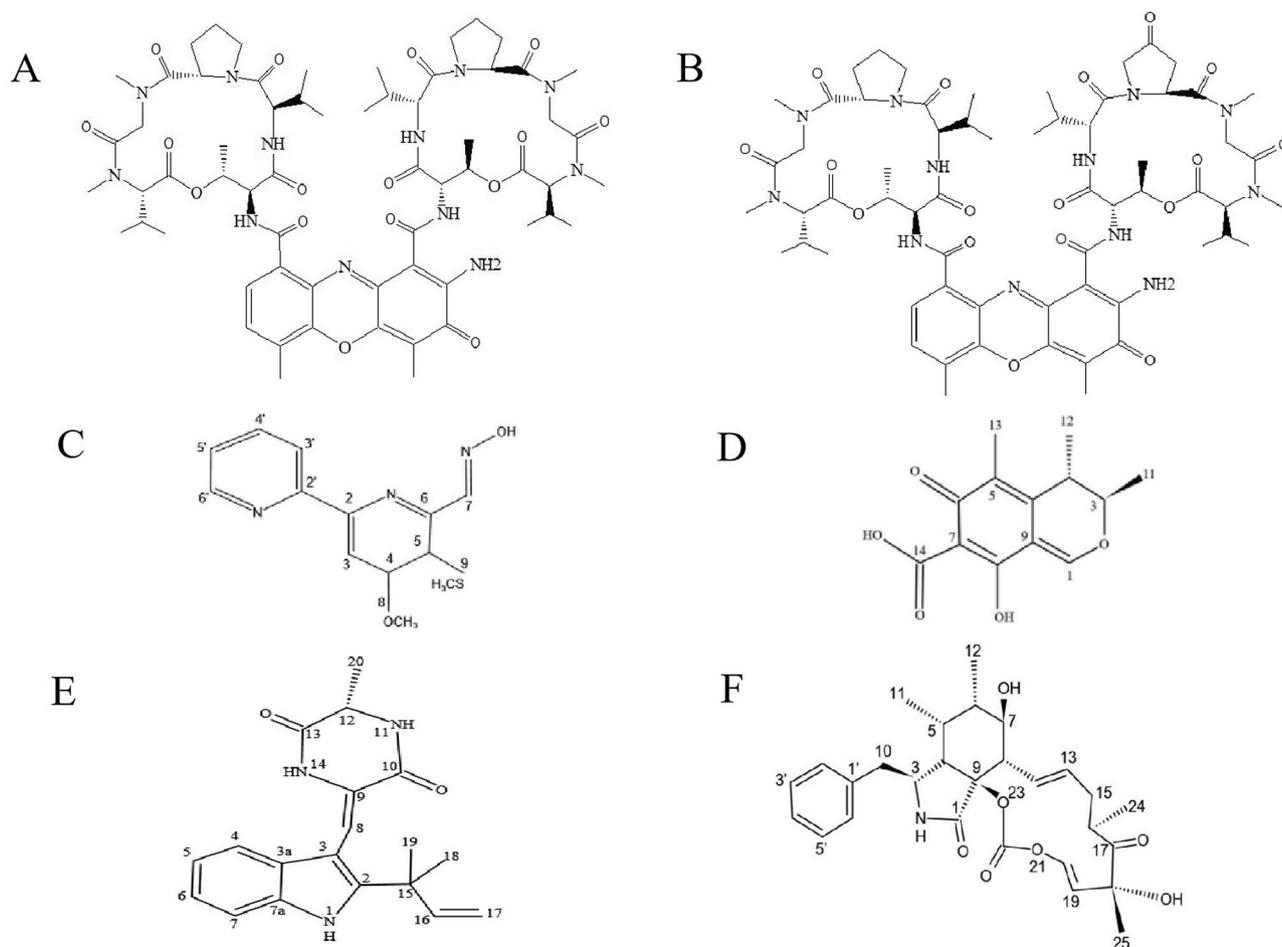
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Fig. 3 Molecular structure of the purified compound 1–6. (**A**) compound **1**: Actinomycin D. (**B**) compound **2**: Actinomycin X$_2$. (**C**) compound **3**: Collismycin A. (**D**) compound **4**: Citrinin. (**E**) compound **5**: Neoechinulin A. (**F**) compound **6**: Cytochalasin E

Table 2 The Minimal inhibitory concentration (MIC) of Compounds against *K. pneumoniae* (MIC, µg/mL, n = 3)

MIC (mL)	Compound 1 Actinomycin D	Compound 2 Actinomycin X2	Compound 3 CollismycinA	Compound 4 Citrinin	Compound 5 Neoechinulin A	Compound 6 Cytochalasin E	positive control
K. Pneumoniae (ATCC13883)	64	128	16	128	-	32	0.5
S. aureus (ATCC25923)	128	64	2	64	-	-	32

Anti-*K. pneumonia* activity of the purified compounds

As the MIC results showed in Table 2, compound 1(Actinomycin D), compound 3 (Collismycin A) and compound 6 (Cytochalasin E) showed strong activity against *K. pneumoniae* ATCC 13,883, with MIC values of 64 µg/mL, 16 µg/mL, and 32 µg/mL. The results of the Oxford cup method showed that compound 2(Actinomycin X2) and compound 4 (Citrinin) also had a strong anti-*Klebsiella pneumonia* activity with an average inhibition zone diameter of 26.83±0.52 mm, 31.00±1.12 mm (Fig. 4; Table 3). SEM results showed that the cell membrane of *K. pneumoniae* was destroyed after 24 h of treatment with compound 3 (Collismycin A) (Fig. 5) .

Discussion

With the overmining of actinomycetes for compounds acting, recent efforts to discover novel antibiotics have been focused on other groups of bacteria [24]. *Achromobacter sp.* is a short, Gram-negative rod, which occur in water, soils and the intestinal tracks of animals, can produce a variety of biologically active substances [25–27]. The insect-associated *Achromobacter sp.* is another promising system for the discovery of antibacterial and antifungal substances. Deepa I [28] found that An *Achromobacter sp.* associated with a Rhabditis entomopathogenic nematode (EPN), displayed promising antibacterial property. Three different cyclic dipeptides (CDPs), cyclo(D-Leu-D-Arg) (1), cyclo(L-Trp-L-Arg) (2), and cyclo(D-Trp-D-Arg) (3) were purified from its ethyl
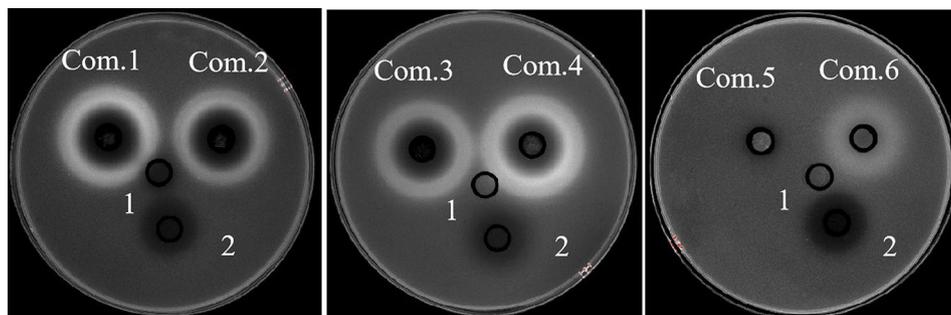
Fig. 4 The antibacterial activity of the purified compounds against *K. pneumoniae* by the Oxford Cup method. 1: Blank control: methanol solution; 2: Positive drug: Ciprofloxacin, 4 µg/mL; Com.1- Com.6: the purified compounds 1–6, 1 mg/mL

acetate extract by silica gel column chromatography and had active against wound associated bacteria. In a recent literature, *Photorhabdus*, gut symbionts of enthomopathogenic nematodes that carry up to 40 biosynthetic gene clusters coding for secondary metabolites, is attractive producers of secondary metabolites. This resulted in the isolation of a novel antimicrobial, 3'-amino 3'-deoxyguanosine (ADG), active against *E. coli* [29].

In the present study, we found that the secondary metabolites of the strain WA5-4-31 has anti-*K. pneumonia* activity, which isolated from the intestine of *Periplaneta americana*. We identify preliminarily the strain WA5-4-31 as *Achromobacter* through morphology and molecular biology. Six compounds (Actinomycin D, Actinomycin X2, Collismycin A, Citrinin, Neoechinulin A and Cytochalasin E) were isolated and determined from the secondary metabolites of the strain WA5-4-31, by activity tracking, chemical separation, nuclear magnetic resonance (NMR) and mass spectrometry (MS) analysis. Those compounds have been previously reported as natural bioactive secondary metabolites from *Streptomyces species* or fungi species [20, 30–32]. Till dated those compounds are reported here for the first time from

Table 3 Anti-*K. pneumoniae* activity of purified compounds

No.	Diameter of the inhibition zone (mm) (n = 3, means ± SD)	No.	Diameter of the inhibition zone (mm) (n = 3, means ± SD)
Compound 1 Actinomycin D	27.87 ± 0.90	Compound 5 Neoechinulin A	
Compound 2 Actinomycin X2	26.83 ± 0.52	Compound 6 Cytochalasin E	16.57.61 ± 0.46
Compound 3 CollismycinA	27.13 ± 1.69	positive control	12.67 ± 0.68
Compound 4 Citrinin	31.00 ± 1.12		

Achromobacter sp. Moreover, this is also the first report on *Achromobacter sp.*, which can produce active compounds from *Periplaneta american* intestine.

In the previous literature, Actinomycin D, Actinomycin X2, Collismycin A, Citrinin and cytochalasin E were effective biologically active substances, which can be used to treat a variety of human diseases, such as cancer, bacterial infections, et [33, 34]. Actinomycin D inhibited acute myeloid leukemia (AML) through targeting of an oncogenic mutant form of the nucleolar chaperone nucleophosmin 1 [35]. Actinomycin D, a potent anti-virulence agent, is a promising candidate to treat *Pseudomonas aeruginosa* infection by interfering with the quorum sensing (QS) systems [36]. One strain *Streptomyces globisporus* isolated from the intestinal tract of *Periplaneta americana*, produced actinomycin X2 and collismycin A which showed strong inhibition of MRSA. Citrinin is produced by fungi of the genera *Monascus* and *Penicillium*, which displayed excellent biological activities against some pathogenic bacteria with MIC of from 4 to 16 µg/ml [37]. Cytochalasin E, a common fungal metabolite, has anti-proliferative activity against human HT-29 colorectal cancer cells [38]. Our further research showed that, Actinomycin D, Collismycin A, Citrinin and Cytochalasin E, isolated from secondary metabolism of *Achromobacter* from the intestine of *Periplaneta americana*, showed strong activity against *K. pneumoniae* with MIC values of 16~64 µg/mL. *Achromobacter* has not been reported to produce any anti-*K. pneumoniae* substances previously.

Conclusions

Six antimicrobial compounds were isolated from the secondary metabolites of the *Achromobacter*, which has anti-*Klebsiella pneumonia* activity. Especially Actinomycin D, Collismycin A and Cytochalasin E showed strong activity against *Klebsiella pneumoniae*. Collismycin A had a destructive effect on the cell membrane of *Klebsiella pneumoniae*. This is the first reported *Achromobacter sp.*,
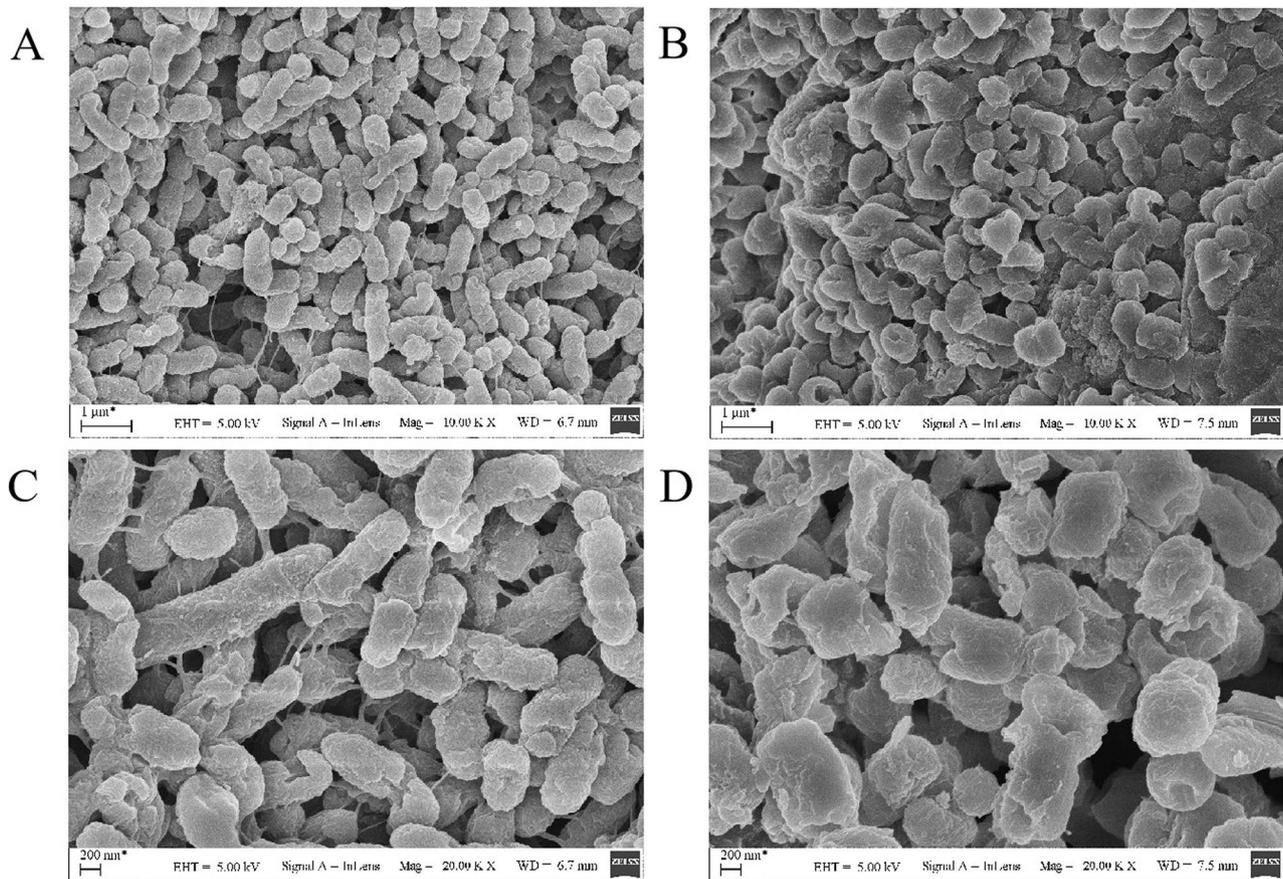
Fig. 5 **Scanning electron microscopy (SEM) images of** *K. pneumoniae* **with CollismycinA treated or untreated**. (**A**) Untreated *K. pneumoniae* (10 K×). (**B**) treated *K. pneumoniae* (10 K×). (**C**) Untreated *K. pneumoniae* (20 K×). (**D**) treated *K. pneumoniae* (20 K×)

which isolated from the intestinal tract of *P.americana*, can produce active compounds. Moreover, *Achromobacter sp.* has not been reported to produce any anti-*Klebsiella pneumoniae* substances previously, such as actinomycin D, collismycin A and Cytochalasin E. However, the activity of those compounds against some clinically relevant pathogens, such as resistant *K. pneumoniae* were still unclear. Moreover, the Whole Genome Sequencing(WGS) of the strain may need further studies.

Materials and methods
Microorganisms, Chemicals and Media
The Strain WA5-4-31 was isolated from the intestinal tract of *Periplaneta americana* and deposited in Guangdong Microbial Culture Collection Center (GDMCC NO.1.2520). *Klebsiella pneumoniae (*ATCC13883), *Staphylococcus aureus* (ATCC25923) were obtained from Guangdong Institute of Microbiology. Ezup Column Bacteria Genomic DNA Purification Kit (B518255) was from Sangon Biotech (Shanghai) Co., Ltd. Premix Taq™ (R004A), DL2000 DNA Marker (3427 A) were purchased from TakaRa Bio Co., Ltd. High performance liquid chromatography (HPLC) grade methanol was purchased from

Honeywell. Analytical pure organic reagents (methanol, ethyl acetate, dichloromethane, petroleum ether, anhydrous ethanol) were purchased from Guangdong Guanghua Technology Co., Ltd.

Identification of the *Achromobacter* WA5-4-31
159 strains of intestinal bacteria were isolated from the *Periplaneta americana* and tested for their anti-*K. pneumoniae* activity by the Oxford Cup method. The secondary metabolites of WA5-4-31 showed strong activity against *K. pneumoniae*. The WA5-4-31 was inoculated on Gauze's medium No.1 and incubate at 28 °C for 3 days. When the colony grew stably, the single colony was selected and cultivated in the ISP-1 seed medium at 160 rpm at 28 °C for 2 days. The morphology and surface characteristics of the strain WA5-4-31 were examined using a scanning electron microscope (FEI Phenom Desktop SEM, USA).

The DNA of WA5-4-31 was extracted by Ezup Column Bacteria Genomic DNA Purification Kit, and selected bacterial universal primers to amplify 16 S rDNA by PCR. The PCR was performed in a total volume of 20μL containing 10μL Premix Taq Mix, 2μL DNA template, 1μL

primer 27f, 1μL primer 1492r, and 6μL ddH$_2$O. Amplification conditions include pre-denaturation at 95 ℃ for 3 min, denaturation at 95 ℃ for 30s, annealing at 55 ℃ for 30s, and extension at 72 ℃ for 1 min, a total of 30 cycles, followed by extension at 72 ℃ for 10 min, and storage at 4 ℃. The PCR products were verified by electrophoresis on a 1% agarose gel and sequenced in BGI. Finally the sequence were uploaded to National Center for Biotechnology Information (NCBI) and compared to all sequences available in GenBank. The phylogenetic tree was constructed using the neighbor joining method in the MEGA5.0 software [39].

Fermentation, extraction and isolation

A 9 mL of seed culture was inoculated into a 500-mL Erlenmeyer flask containing 300 mL of the ISP 2 medium and incubated on rotary shakers (160 rpm) at 28℃. After 9 days, the fermentation broth was centrifuged at 5000rmp for 20 min. Then the supernatant of the fermentation broth was extracted with ethyl acetate(V:V = 1:1.5) for three times. After rotary evaporation and drying, 18 g crude ethyl acetate extract was obtained from 60 L of culture broth. Finally 9 fractions were obtained by thin layer chromatography and silica gel column gradient. Every fraction was tested for antimicrobial activity against *K. pneumoniae* [40] by Oxford Cup method. The active fractions were further purified by ODS column chromatography, Sephadex LH-20 column, and semi-prepared HPLC system(Waters e2535-2489, USA) with YMC-Pack ODS-AQ C18 column (250×10.0 mm, YMC AQ12S05-2546WT, Japan) using a UV-VIS detector and a 200μL injection loop at 25 ℃. The mobile phase was deionized water and methanol (H2O: methanol 100:0–0:100, V/V) at 1.0 mL/min over 65 min, and UV detection was recorded at λ = 280 nm.

Spectroscopic analysis

The purity of compounds was analyzed using analytical HPLC with photodiode array detector (PAD) and YMC-Pack (ODS-AQ C18) column. Compounds were dissolved in DMSO. The dissolved Compounds were aspirated with a disposable syringe and filtered with 0.22 μm organic filter membrane for impurities. The proton and carbon nuclear magnetic resonance (NMR) spectra were recorded at 600 MHz using Brucker AVANCE III 600 M spectrometer (Brucker, Germany). The compounds were dissolved in methanol to prepare the concentration of 1 mg/L. Then the dissolved compounds were aspirated using a disposable syringe and filtered with 0.22 μm organic filter membrane for impurities. Mass spectra was obtained within the range of m/z 50-2000 by TSQ Endura™ Triple-quadrupole mass spectrometer (Thermo Fisher, USA). The molecular weight of the compounds was determined using mass spectrometry

and their structure were characterized by 1 H NMR and 13 C NMR, combined with published literature.

Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) of purified compounds against the *K. pneumoniae* were determined by the tube-dilution method using individually packaged, flat bottomed, 96-well microtiter plates. The tested bacterial strain was cultivated in Mueller-Hinton Broth at 37 ℃ until the density reached approximately $1 \sim 5 \times 10^5$ CFU/mL. Each of the tested compounds and drugs were dissolved in methanol and then diluted with sterile broth by the twofold dilution method. The final concentrations of each sample in the wells were 512, 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5 and 0.25 μg/mL. Ciprofloxacin and Ampicillin were used as positive control for *K. pneumoniae and S. aureus* respectively. A serial dilution of compounds were performed in the microplates and incubated at 37 ℃ for 24 h. All the experiment was performed in triplicate.

Antibacterial activity assay

The activity of purified compounds against the *K. pneumoniae* were further determined by Oxford cup method. The tested bacterial strain was diluted to approximate 10^6 CFU/mL, and mixed into LB medium (1% agar) to reach 0.5% (v/v) concentration. The mixture (15 mL) was poured into a LB plate with a sterilized Oxford cup placed up still in the plate. After cooling, the Oxford cups were taken out carefully to form round holes. Each tested compounds (50μL) were added to the small round holes. Ciprofloxacin was used as positive control. Methanol solvent was used as the control group. Plates were incubated at 37 ℃ for 24 h and photoed by Gel Imaging System (Gel DocTMXR+, Bio-Rad). The experiment was performed in triplicate, and the mean of the diameter of the inhibition zones was calculated.

Scanning Electron Microscopy Analysis

The tested *K. pneumoniae* (1.5×10^8 CFU/mL) was treated with $4 \times$ MIC concentration of the compound at 37 ℃ for 24 h. The supernatant was removed by centrifuged and then the tested strain were placed on a cover glass and fixed overnight with 2.5% glutaraldehyde at 4 ℃. Fixed samples were washed 3 times in 1x phosphate buffered saline (PBS) for 20 min respectively and dehydrated in gradient concentrations of ethanol (20%, 40%, 60%, 80%, and 100%). Finally the morphological changes of the tested *K. pneumoniae* was observed by Scanning Electron Microscopy (SEM) (Hitachi FlexSEM 1000, Japan) at an accelerating voltage of 2–19 kV.

Abbreviations
HPLC	High Performance Liquid Chromatography
ODS	Octadecylsilyl
NMR	Nuclear Magnetic Resonance

HR-ESI-MS	High-resolution electrospray ionization mass spectroscopy
MEGA	Molecular Evolutionary Genetics Analysis
DMSO	Dimethyl Sulfoxide
CFU	Colony-forming Unit
CDCl3	Deuterated chloroform
m/z	Mass charge ratio.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12866-023-02909-7.

> **Supplementary Figures:** Mass spectrometry?Proton (1H) nuclear magnetic resonance and Carbon (13C) nuclear magnetic resonance spectrum of the compound 1-6
>
> **Supplementary Tables:** NMR data of Compound 1-6

Authors' contributions

XJ devised and supervised the experiments. YM and PG designed and performed experiments. MX and PG wrote the manuscript. X Ch and WL: analyzed data and prepared figures. All authors read and approved the manuscript.

Data Availability

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Declarations

Ethics approval and consent to participate

This article does not contain any studies with human participants or vertebrates performed by any of the authors.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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