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Antibiotic-Producing Bacteria Collected from Seawater

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Abstract: Background: Microorganisms are a known source of antibiotics. The study aimed to identify and screen antibiotic-producing microbes isolated from seawater. Method: Three of the fifty (50) bacteria isolated from seawater showed positive for antibiotic activity. The antimicrobial activity of *Pseudomonas guguanensis* (KD1) was screened against the ESKAPE pathogens using agar-well diffusion assays. *P. guguanensis* (KD1) was selected for the fermentation and extraction of antimicrobial compounds using solvent extraction assays. Results: *P. guguanensis* (KD1) produced the highest antibacterial activity after 36 h of cultivation, inhibiting *S. aureus*, *E. faecium*, *A. baumannii* and *E. cloacae*. According to sensitization assay, *K. pneumoniae* was impermeable to all the cell-free supernatants of *P. guguanensis* (KD1). Using agar-well diffusion assays, ethyl acetate extracts from the supernatant recorded zones of inhibition against *S. aureus*, *E. faecium*, and *E. cloacae*, producing zones of 20.1 ± 0.432, 17.8 ± 0.121 and 16 ± 0.162 mm, respectively. Acetonitrile extract from the supernatant inhibited *A. baumannii* and *S. aureus*, forming zones of inhibition 18.2 ± 0.323 mm and 18 ± 0.234. The minimum inhibitory concentration and minimum bactericidal concentration recorded for the ethyl acetate extract and acetonitrile extract ranged from 1.56 to 6.25 mg/mL and 12.5–25 mg/mL, respectively. Conclusions: *P. guguanensis* (KD1) offers a potential source of antibiotics for infections caused by multidrug-resistant bacteria.

Keywords: *Pseudomonas*; antibiotic; natural products; antibiotic resistance; antibacterial

Citation: Dashti, K.M.; Vali, L.; Almaraghi, N.; Ebrahim, H.; Abul, H.; Dashti, A.A. Antibiotic-Producing Bacteria Collected from Seawater. *Microbiol. Res.* **2024**, *15*, 2381–2393. https://doi.org/10.3390/microbiolres15040160

Academic Editor: Juan Ayala

Received: 4 November 2024
Revised: 21 November 2024
Accepted: 22 November 2024
Published: 23 November 2024

1. Introduction

The spread of antibiotic-resistant bacteria is one of the most alarming global issues [1]. Some of the mechanisms of antibiotic resistance have occurred due to the overuse and misuse of antibiotics in medicine and agriculture, reducing the efficacy of currently available antimicrobial agents [2]. The key mechanism of antimicrobial resistance (AMR) is the horizontal transmission of antibiotic-resistance genes (ARGs) between microorganisms [3].

Antimicrobial resistance is one of the leading causes of death, with low-income countries facing most of its effects [4]. By 2050, infections brought on by antibiotic-resistant bacteria are predicted to cause 10 million deaths annually [5]. According to recent estimates, 1.27 million fatalities are directly linked to antimicrobial resistance, out of an estimated 4.95 million deaths overall [4]. Furthermore, more than 250,000 AMR-related deaths in 2019 may have been attributed to six bacteria, including *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* [4]. Financially, AMR is expected to affect the global economy, anticipated to cause a reduction in global gross domestic product (GDP) of up to $100 trillion between 2014 and 2050 [2]. Antimicrobial resistance is therefore a global concern requiring immediate attention [6].

Nowadays, the discovery of novel antibiotics is a priority, especially to treat infections caused by multidrug-resistant Gram-negative bacteria. If the development of new antibiotics is impeded and resistance keeps rising, we may find ourselves returning to a pre-antibiotic world, where common medical treatments are considered dangerous [2]. Recent pharmaceutical industries have considered bacteria in nature with the ability to produce antimicrobials. Several soil microorganisms, including *Streptomyces* and *Actinomyces*, are known producers of metabolites with antimicrobial properties [7]. Antibiotics such as neomycin, tetracycline, erythromycin, and most recently, a novel antibiotic named picolinamycin, are all produced by the soil bacterium *Streptomyces* [8].

Some taxonomically diverse bacterial communities can withstand extreme environmental circumstances owing to specific physiological and structural traits. These groups are unique to marine environments and can produce secondary metabolites that are not present in terrestrial microorganisms [9]. Due to their exposure to a variety of environmental factors, including salinity, light, pressure, and temperature, marine microorganisms were found to produce unique natural products with antimicrobial activity [10].

Kuwait Bay is considered the most productive ecosystem in Kuwait seawater [11]. It is situated in the northwest corner of the Arabian Gulf and contains the most species diversity in the area [11]. However, numerous pollutants from local sources enter Kuwait's marine environment [12]. The primary discharges include ship trash disposal, desalination operations, sewage, and oil [12]. Furthermore, Kuwait Bay's seawater is reported to contain microplastics [13]. One strategy used by bacteria to adjust to these harsh sea environment conditions involves the production of secondary metabolites, including those with antibacterial activity [14]. It is therefore necessary to draw attention to antibiotic-producing microorganisms from Kuwait Bay, an untapped possible source of unique natural products. Therefore, the main aim of this study was to isolate, identify, and screen antibiotic-producing microbes from seawater in Kuwait Bay and assess the antimicrobial potential of those microorganisms against clinically significant bacteria.

2. Materials and Methods

2.1. Seawater Sampling Procedure

The seawater sample was collected from Kuwait Bay (Figure 1). Briefly, a seawater sample of 50 mL was collected in a sterile glass bottle from a sea depth of 10 m. The water sample was transferred to sterile plastic bottles of 150 mL capacity and was kept on ice. The sample was immediately transported to the microbiology laboratory at the Medical Laboratory Sciences at the Faculty of Allied Health Sciences, Kuwait University.

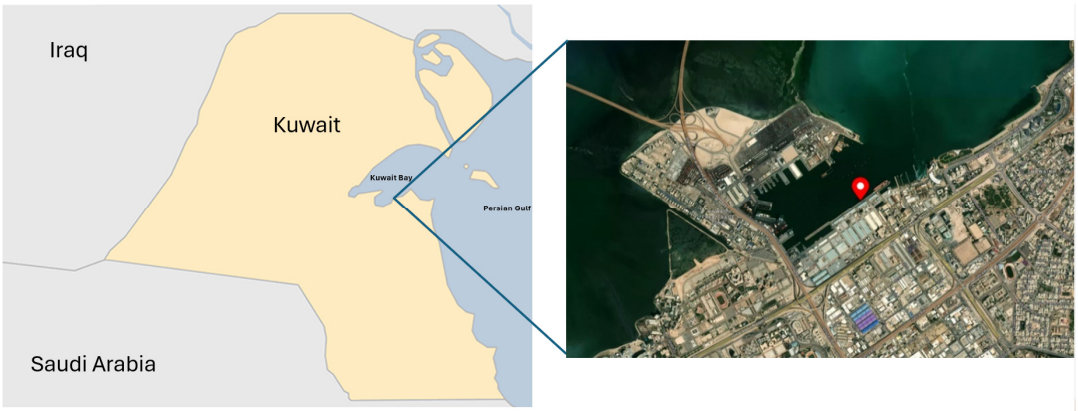

Figure 1. Location of sample collection from Kuwait Bay.

2.2. Isolation of Antibiotic-Producing Colonies from Seawater Samples

The water sample was diluted to a ten-fold dilution. The diluted samples were spread on R2A agar (Sigma, Poole, UK). After incubation at 30 °C for 4 days, 50 bacterial colonies

were isolated and tested for antimicrobial activity against methicillin-resistant *Staphylococcus aureus* (MRSA). The colonies were spotted onto nutrient agar with an overlay of 0.5% nutrient agar seeded with *S. aureus* ATCC 33592. The agar plates were incubated for 3 days at 30 °C until distinct zones of inhibition surrounding microbial colonies were observed, which served as an indicator for MRSA inhibitory activity. The obtained colonies exhibiting antibacterial activity against MRSA were identified using the Practical Handbook's "Gram-stain" flow chart [15]. Following the results of Gram staining, organisms were identified by following biochemical tests in the identification schemes [15]. Gram-negative cocci were identified using API NH (Oxoid, Basingstoke, United Kingdom), whereas Gram-positive cocci were identified using the catalase test, and using either API Staph or API Strep (Oxoid). Gram-negative bacilli were identified using the oxidase test and API 20E (Oxoid), while Gram-positive bacilli were identified using the hanging drop method of motility testing. Bacterial strains were confirmed using Vitek 2 (bioMérieux, Marcy l'Etoile, France).

2.3. Test Microorganisms

The indicator organisms in this research included the ESKAPE organisms, which is an acronym describing a collection of clinically significant multi-drug resistant Gram-positive and Gram-negative bacteria [16]. The ESKAPE organisms used in this study were *Enterococcus faecium* NCTC 12202, *Staphylococcus aureus* ATCC 33592, *Klebsiella pneumoniae* ATCC 4352, *Acinetobacter baumannii* NCTC 12156, *Pseudomonas aeruginosa* NCTC 10332, and *Enterobacter cloacae* NCTC 13405. The indicator bacteria were prepared by growing them on nutrient agar, except for *E. faecium*, which was grown on tryptic soy broth agar (Oxford, UK), followed by incubation at 37 °C for 24 h. Ten (10) mL of 24 h cultures of these pathogens were all adjusted to a starting optical density of 0.01 (OD_{600nm}), equivalent to the turbidity of a 0.5 McFarland standard.

2.4. Primary Screening of Isolated Microorganisms

The isolates were cultured in three different media (Luria broth, nutrient broth and M9 minimal medium, Oxford, UK) at two different temperatures (20 °C and 30 °C) for 24 h. Then, the cultures were centrifuged for 20 min at 1500 rpm [17]. The supernatants were collected and filtered using 0.22 µM filters and evaluated for antibacterial activity against the ESKAPE pathogens using agar-well diffusion assays. The cell-free supernatants (CFS) were stored at −20 °C for further investigation.

2.4.1. Agar-Well Diffusion Assays

The isolates were tested for their production of antimicrobial metabolites in different culture conditions using agar-well diffusion assays as previously described, with modifications [18]. A lawn of the test microorganisms was prepared by spreading inoculates of the test microorganisms, which were prepared and adjusted to a 0.5 McFarland, onto Mueller Hinton agar plates. Using a 1 mL sterile disposable pipette tip, wells of 8 mm diameters were created in each agar plate and filled with the CFS. The plates were kept at room temperature for 1 h to allow the CFS to be absorbed into the wells. The plates were subsequently incubated at 37 °C for 24 h. The diameter of zones of inhibition was measured following the incubation period. No zone of inhibition was reported as having no inhibitory activity (8 mm). The most promising isolate which showed the highest antimicrobial activity *P. guguanensis* (KD1) was chosen for further studies.

2.5. Effect of Cultivation Time on Antimicrobial Activity of Pseudomonas guguanensis KD1

The optimal timepoint for the antimicrobial potential of *Pseudomonas guguanensis* (KD1) was further investigated as previously described, with minor modifications [17]. One colony of *P. guguanensis* (KD1) was inoculated in 10 mL nutrient broth and incubated at 30 °C for 18 h. After 18 h, the inoculum was adjusted to a starting optical density of 0.01 (OD_{600nm}). Bacterial cultures were collected at timepoints of 12, 24, 36, 48, 60 and 72 h. The supernatants of these cultures were collected by centrifugation at 1500 rpm and

filtered using 0.22 µM filters. The CFS was tested against *S. aureus*, *A. baumannii*, *E. faecium* and *E. cloacae* using agar-well diffusion assays. This assay was performed using three biological replicates.

2.6. Sensitization of Gram-Negative Bacteria to the Cell-Free Supernatant

Polymyxin B nonapeptide (PMBN), a polymyxin derivative, is an outer membrane permeabilizer that can significantly sensitize antibiotic-resistant Gram-negative bacteria to certain antibiotics [19]. PMBN was used in this study to permeabilize *Klebsiella pneumoniae* ATCC 4352 to the CFS of *P. guguanensis* (KD1) as previously described [19]. A broth microdilution test was used to determine the minimum inhibitory concentration (MIC) for PMBN following the Clinical Laboratory Standards Institute (CLSI) [20]. PMBN was purchased from Sigma-Aldrich (St. Louis, MO, USA). This assay was carried out in a non-treated 96-well, microtiter plate, with the CFS serially diluted two-fold. Then, 100 µL of *K. pneumoniae* with an initial inoculum of 5×10^5 CFU/mL was pipetted into each well. The treatment of permeabilized *K. pneumoniae* was performed by adding 50 µL of 50 µg/mL PMBN. The treatment of non-permeabilized *K. pneumoniae* was performed by adding 50 µL sterile water as a control. The growth of non-permeabilized and permeabilized *K. pneumoniae* after treatment with CFS was measured for 13 h using a FLUOstar Omega™ spectrophotometer, reading the optical density (OD_{600nm}) every 6 min. This experiment was performed in triplicates.

2.7. Solvent Extraction of Antimicrobial Compounds from Isolate KD1

Crude extracts from the CFS of *P. guguanensis* (KD1) cultures were prepared by solvent extraction using solvents with different polarities (ethyl acetate, chloroform and acetonitrile, Oxford, UK) [17]. Culture supernatants were prepared by growing *P. guguanensis* (KD1) in 400 mL nutrient broth for 36 h at 30 °C on a rotary shaker set at 150 rpm. The bacterial culture was centrifuged at 19,592× *g* for 15 min at 4 °C. The supernatant was filtered using disposable 0.45 µM filter units. The CFS was concentrated to 200 mg/mL using a freeze-dryer (Fisher Scientific, Cleveland, OH, USA). The CFS was extracted for secondary metabolites using the solvents in a ratio of 1:1 for 24 h on a rolling shaker [17]. After 24 h, the mixture was allowed to stand at room temperature. The organic layer was collected and dried using nitrogen gas at 40 °C. The dried product was reconstituted using high-performance liquid chromatography (HPLC)-grade water to a concentration of 100 mg/mL.

2.8. Antimicrobial Activity of P. guguanensis (KD1) Extracts

2.8.1. Agar-Well Diffusion Assays

The extracts of *P. guguanensis* (KD1) were tested for antibacterial activity against *S. aureus*, *E. faecium*, *E. cloacae* and *A. baumannii* using agar-well diffusion assays [21]. Four concentrations (100, 50, 25 and 12.5 mg/mL) of the extract at a volume of 100 µL were prepared and tested against the test organisms using agar-well diffusion assays, as described in Section 2.4.1. Ciprofloxacin (12.5 µg/mL) was used as a positive control in this assay (Sigma-Aldrich, Poole, UK).

2.8.2. Broth Microdilution Assays

The minimum inhibitory concentration (MIC) of KD1 extract was determined using broth microdilution assay [22]. Using a 96-well microtiter plate, two-fold dilutions of the extract were prepared (100–0.78 mg/mL). In each well, 80 µL of the KD1 extract, 100 µL nutrient broth, and 20 µL of the test organism (equivalent to 10^6 CFU/mL) were dispensed into 96-well plates to make a total volume of 200 µL. The plates were incubated at 37 °C for 24 h. The minimum inhibitory concentration (MIC) was considered as the lowest concentration not showing microbial growth after the incubation period.

2.9. Chromatographic Analysis

2.9.1. Thin-Layer Chromatography

The extracts were chromatographically analyzed using thin-layer chromatography (TLC) [23]. The ethyl acetate extract of *P. guguanensis* (KD1) was profiled onto a TLC plate of the dimensions 5 × 10 cm (Fisher Scientific, Cleveland, OH, USA). The TLC plate was developed in a hexane–ethyl acetate (60:40) solvent system. The plate was subsequently viewed under an ultraviolet (UV) lamp at a wavelength of 365 nm. The spots were circled, the distance traveled by the spots and the solvent front was measured in cm, and, subsequently, the retardation factor (R_f) values were calculated [17].

2.9.2. Column Chromatography

Chromatographic separation was performed using a Sep-Pak tC18 Plus long-cartridge solid-phase extraction (SPE) column (Fisher Scientific, Cleveland, OH, USA). The column was conditioned and equilibrated prior to extraction with 5 mL methanol (Oxford, UK) followed by 6 mL HPLC-grade water (Oxford, UK). The extract (5 mL) was loaded onto the conditioned cartridge. The column was eluted using 10 mL of 70% methanol increasing in increments of 5% to 100%. Thirty-three (33) fractions were collected in a glass beaker, dried using nitrogen gas, and bulked into 6 subfractions according to their TLC profile [23]. The dried extracts were resuspended in 1 mL HPLC-grade water. The extracts were tested for antimicrobial activity against indicator bacteria using agar-well diffusion assays.

2.10. DNA Sequencing and Purification

Using the Quick-gDNA miniprep kit (Zymo Research, Irvine, CA, USA), genomic DNA was purified using an overnight culture of KD1, KD2, and KD3 grown in Luria–Bernati broth at 37 °C.

The polymerase chain reaction was performed for 16S rRNA gene-sequencing using universal primers fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and rP2 (5′-TACGGCTACCTTGTTACGACTT-3′) [24]. With 50 pmol of each primer, 200 µM of each dNTP, 0.5 units of GoTaq Flexi DNA polymerase (Promega, Madson, WI, USA), and 3 µL of DNA sample in 1× Taq polymerase buffer, the amplification reaction was carried out in a final volume of 50 µL. First, the mixture was denatured for five minutes at 94 °C. Following primer annealing at 55 °C for 45 s, primer extension at 72 °C for 90 s, and denaturation at 94 °C for 30 s, 35 PCR cycles were carried out. The mixture was incubated for 10 min at 72 °C at the end of the last cycle. Using gel electrophoresis on a 1% agarose gel, effective amplification was verified.

After the primers used in PCR reactions were removed from the PCR products using Magnesil yellow solution (Promega, Madson, WI, USA), each purified product completed a sequencing reaction in a thermocycler. A final volume of 20 µL was used for the sequencing process, which contained 2 µL of purified PCR product, 3 µL of Big Dye (version 1.1), and 20 pmol of one primer (fd1, rp2). A total of twenty-five cycles were performed: denaturation at 96 °C for 10 s, primer annealing at 55 °C for 10 s, and extension at 60 °C for 4 min. The previously mentioned Magnesil green solution was used to purify the sequencing reaction products in order to remove the excess of labeled ddNTPs.

An ABI PRISM sequencing device (ABI Prism 310 Genetic Analyser, Applied Biosystem, Foster City, CA, USA) was used to directly sequence the PCR products, and sequencing analysis software (version 1.1) was used to analyze the results.

2.11. Statistical Analysis

All experiments were conducted in triplicates and the data were recorded as ±standard error. Data were statistically analyzed using Statistical Packages for Social Sciences (SPSS) software version 24. Multiple regression analysis was conducted to determine if the difference in the sizes of zones of inhibition formed by the CFS was statistically significant. One-way analysis of variance (ANOVA) followed by Dunnett's post hoc test was used to compare the absorbance values of permeabilized and non-permeabilized *K. pneumoniae* treated with CFS. Differences between means was considered significant when the confidence interval was smaller than 5% (p value ≤ 0.05) [25].

3. Results

3.1. Identification of Antimicrobial-Producing Bacteria

Out of 50 bacterial colonies, 3 showed antibacterial action against MRSA, whereas 47 bacterial colonies from various genera, including *Streptomyces*, *Bacillus*, and *Staphylococcus*, did not. *Streptomyces* spp. was the most common microorganism identified (21 strains), followed by *Bacillus* spp. (16 strains), *Staphylococcus* spp. (10 strains), and *Pseudomonas* spp. (3 strains). Most *Bacillus* strains belonged to the species *Bacillus cereus* (15 strains) and one *Bacillus licheniformis* (1 strain). Most of the *Streptomyces* strains belonged to the species *Streptomyces griseus* (15 strains), and *Streptomyces flaveolus* (6 strain). All Staphylococci in this study were identified as *Staphylococcus aureus* (10 strains).

Despite being the least-detected microorganism in the seawater sample, the three *Pseudomonas* strains, which were tentatively named KD1, KD2, and KD3, showed antibacterial activity against MRSA and presented with different colony morphology. Using 16S rRNA sequencing, the best matches to the rRNA gene of these three isolates were *Pseudomonas guguanensis* for KD1 and *Pseudomonas aeruginosa* for both KD2 and KD3 (Table S1). These strains were referred to in this study as KD1, KD2, and KD3. The strain KD1 was identified as *P. guguanensis* and submitted to NCBI GenBank under Biosample accession SAMN44841083, whilst strains KD2 and KD3 were both identified as *P. aeruginosa* and submitted to NCBI GenBank under Biosample accession SAMN44841418 and SAMN44842870, respectively.

3.2. Antimicrobial Spectrum of Antibiotic-Producing Bacteria

The three *Pseudomonas* strains, KD1, KD2, and KD3, showed antibacterial activity against indicator bacteria, according to the results of the agar-well diffusion assays (Table S2). While *P. aeruginosa* (KD2) prevented the growth of both *S. aureus* and *E. faecium*, *P. aeruginosa* (KD3) was limited to targeting *S. aureus*. Additionally, the growth of *S. aureus*, *A. baumannii*, *E. cloacae*, and *E. faecium* was suppressed by *P. guguanensis* (KD1). The ideal conditions for *P. guguanensis* KD1 culture was nutrient broth at 30 °C for 24 h, which resulted in this broad antibacterial activity. *P. guguanensis* (KD1) was chosen for further research because it showed the broadest antibacterial action overall, suppressing both Gram-positive and Gram-negative bacteria.

3.3. Impact of Cultivation Time on Antibacterial Activity

Over a 72 h period, isolate KD1 was evaluated to determine the ideal cultivation timepoint for maximum antibacterial activity. The isolate produced the largest zones of inhibition of 28 and 25 mm, respectively, against *S. aureus* and *A. baumannii* at 36 h of incubation (Figure 2a,c), followed by 23 mm zones of inhibition against *E. faecium* and *E. cloacae* (Figure 2b,d). In contrast, the least antibacterial activity of 13 mm was recorded at 48 h incubation against *A. baumannii*.

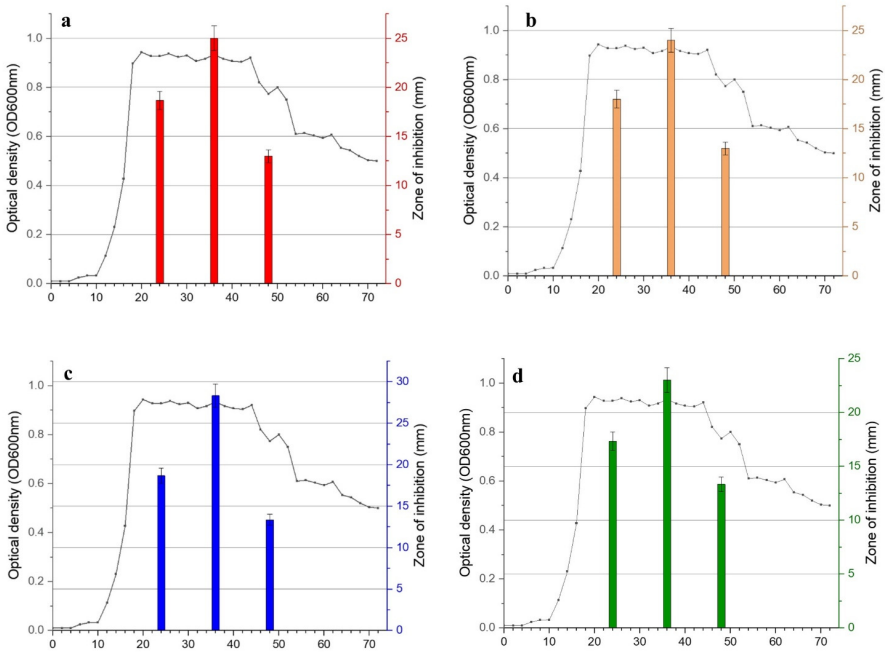

Figure 2. The growth of *P. guguanensis* (KD1) in nutrient broth and the inhibitory activity of its CFS collected at 12, 24, 36, 48, and 72 h. The bars represent zones of inhibition formed by the CFS against (**a**) *Staphylococcus aureus*, (**b**) *Enterococcus faecium*, (**c**) *Acinetobacter baumannii*, and (**d**) *Enterobacter cloacae*. Error bars represent standard deviations. The absorbance (OD_{600nm}) of *P. guguanensis* (KD1) was measured every 2 h for 72 h. Symbols represent means of three replicate measurements.

3.4. PMBN Potentiates CFS of KD1 Against K. pneumoniae

We speculated that *K. pneumoniae* was resistant to the CFS due to the impermeability of the outer membrane of the Gram-negative bacterium. As shown in Figure 3, PMBN exerted no observable impact on the growth of *K. pneumoniae*. The growth of permeabilized *K. pneumoniae* after its exposure to 100 mg/mL and 50 mg/mL CFS was significantly affected (*p*-values at 0.05 and 0.005, respectively). Treatments with CFS, respectively, inhibited the growth of *K. pneumoniae* and significantly affected the growth of the bacterium, as demonstrated by the reduced absorbance values (Figure 3).

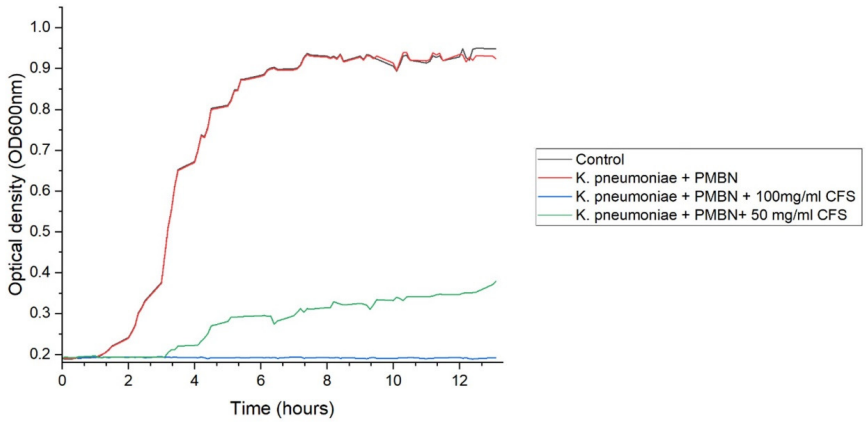

Figure 3. The growth of permeabilized and non-permeabilized *K. pneumoniae* in the presence of CFS of *P. guguanensis* (KD1). The black line (control) represents the growth of non-permeabilized *K. pneumoniae* without treatment with CFS. The red line represents the growth of *K. pneumoniae* permeabilized with PMBN. The blue line represents the growth of permeabilized *K. pneumoniae* exposed to 100 mg/mL CFS. The green line represents the growth of permeabilized *K. pneumoniae* exposed to 50 mg/mL CFS.

3.5. Antibacterial Activity of KD1 Extracts

No antimicrobial activity was observed with the chloroform extract from *P. guguanensis* (KD1). The KD1 ethyl acetate extract inhibited the growth of *E. faecium*, *S. aureus*, and *E. cloacae* (Table 1). Moreover, the KD1 acetonitrile extract exhibited antimicrobial activity against both *S. aureus* and *A. baumannii*, with respective MICs of 3.12 and 6.25 mg/mL (Table 2).

Table 1. Antibacterial activity of KD1 extracts.

Indicator Organism	Mean Zone of Inhibition (mm)		
	Ethyl Acetate Extract (100 mg/mL)	Acetonitrile Extract (100 mg/mL)	Ciprofloxacin (12.5 µg/mL)
E. faecium	17.8 ± 0.121	0	19.1 ± 0.111
S. aureus	20.1 ± 0.412	18 ± 0.234	22 ± 0.625
A. baumannii	0	18.2 ± 0.332	21 ± 0.772
E. cloacae	16 ± 0.162	0	15 ± 1.121
$n = 3$			

Table 2. MIC and MBC of KD1 extracts.

Indicator Organism	Ethyl Acetate Extract (mg/mL)		Acetonitrile Extract (mg/mL)		Ciprofloxacin (µg/mL)	
	MIC	MBC	MIC	MBC	MIC	MBC
E. faecium	1.56	3.12	N	N	6.25	12.5
S. aureus	1.56	3.12	3.12	6.25	1.56	3.12
A. baumannii	N	N	6.25	12.5	12.5	25
E. cloacae	3.12	6.25	N	N	1.56	3.12

MIC; Minimum inhibitory concentration, MBC; Minimum bactericidal concentration, N; Not explored, $n = 3$.

3.6. TLC Analysis and Antimicrobial Activity of Subfractions

Each of the active extracts revealed multiple spots according to TLC results. The KD1 ethyl acetate extract and acetonitrile extract revealed 5 and 6 components under 365 nm with different R_f values of the spots (Table S3). The six subfractions from the acetonitrile extract, labeled as F3123, F3456, F4123, F6923, F7211 and F8165, were tested for antimicrobial activity against *S. aureus* and *A. baumannii* (Figure 4a). The subfraction F4123 was inhibiting *S. aureus* and *A. baumannii*, while the subfraction F8165 was only inhibiting *A. baumannii* (Figure 4b,c).

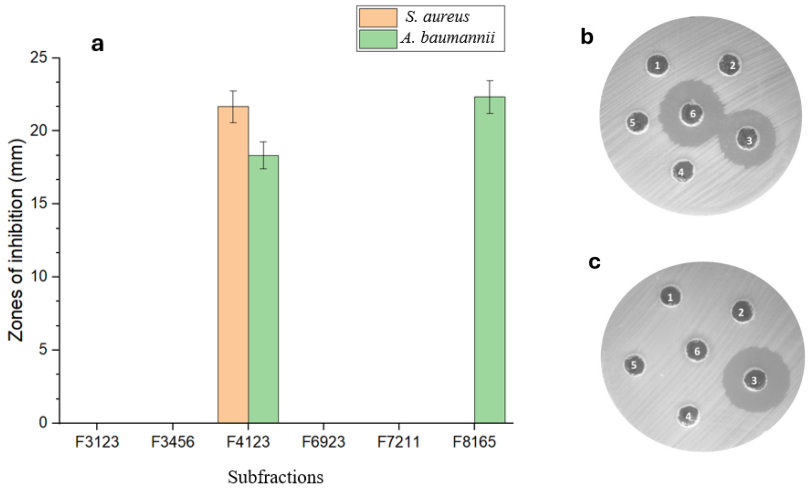

Figure 4. The antimicrobial activity of subfractions fractions collected from the extraction column. (**a**) Zones of inhibition formed by six subfractions against *S. aureus* and *A. baumannii*. $n = 3$. Figures

(**b**,**c**) are representative of the results shown in Figure 4a. (**b**) The inhibitory activity of subfractions against *A. baumannii*. (**c**) The inhibitory activity of subfractions against *S. aureus*. 1; subfraction F3123, 2; F3456, 3; F4123, 4; F6923, 5; F7211, 6; F8165. The error bars indicate the standard deviation. Statistics were conducted with unpaired, two-tailed *t*-test. p value ≤ 0.05.

4. Discussion

Healthcare environments are severely burdened by the rise and spread of antibiotic-resistant bacteria [4]. This issue has led researchers to search for novel antibiotics from a variety of sources, including microbes. This study therefore reports the isolation of antibiotic-producing bacteria from a seawater sample collected from Kuwait Bay. Out of 50 microorganisms, only 3 exhibited antibacterial activity against MRSA. The antibacterial potential of these three microorganisms was then tested against ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter cloacae*). This group of bacteria was selected as indicators in this study because they are responsible for the majority of nosocomial infections [26]. In addition, ESKAPE pathogens can "escape" the effect of antimicrobial agents and are increasingly resistant to numerous antibiotics [26]. ESKAPE pathogens display drug resistance through a variety of strategies, such as drug inactivation through irreversible enzyme cleavage, the modification of the drug-binding site, a reduction in drug permeability, and the formation of biofilms [27]. Additionally, hospital infections caused by ESKAPE pathogens are on the rise and are a leading cause of death globally [4]. It is therefore crucial to discover antibiotics that target this group of antibiotic-resistant bacteria.

Microbes can control the synthesis of secondary metabolites in response to many environmental factors, such as temperature, aeration, and the types of nutrients, particularly the carbon source [28]. *P. guguanensis* (KD1) exhibited the highest levels of antimicrobial activity in terms of the measurements of the diameters of the inhibition zones. The growth of *S. aureus*, *E. cloacae*, *E. faecium*, and *A. baumannii* was inhibited by the supernatant collected from the cultivation of *P. guguanensis* (KD1) in nutrient broth at 30 °C. This may highlight the significance of optimal conditions with a sufficient amount of nutrients in the growing medium for the production of antimicrobials [29]. Others described the maximum production of secondary metabolites by *Pseudomonas aeruginosa* when cultivated in succinate medium followed by King's B medium [30]. Additionally, *P. guguanensis* (KD1) produced the highest antibacterial activity at the stationary phase after 36 h of incubation ($p < 0.05$). This is in agreement with the previous finding, which demonstrates the ability of microbes to regulate antimicrobial production up to a specific growth phase, which is often the stationary phase [31]. Antibiotics are typically generated during a subsequent stationary phase rather than during the rapid growth phase [32]. When bacterial growth becomes limited due to the depletion of one vital nutrition source, including carbon, nitrogen, or phosphate, antibiotic synthesis begins [32]. As an example, *Penicillium chrysogenum* starts to produce penicillin when the culture media is depleted of glucose and the fungus begins to consume lactose, a sugar that is less easily absorbed [33]. At 48 h, the growth of *P. guguanensis* (KD1) declined, according to absorbance values. However, its supernatant was still able to inhibit *S. aureus*, *E. faecium*, *A. baumanii*, and *E. cloacae*. At this timepoint, *P. guguanensis* (KD1) might be experiencing the stringent response, a bacterial stress response that allows bacteria to adapt and survive in environments with limited nutrients [34]. The bacterium may start producing antibiotics as a consequence of the stringent response [34]. By 72 h, *P. guguanensis* (KD1) has reached the death phase, during which its nutritional supply is exhausted, and toxic compounds accumulate [35]. The bacterium has since lost its antibacterial activity at this point, which may suggest that the conditions in this growth phase are unfavorable for the synthesis of antibiotics by *P. guguanensis* (KD1).

Here, we investigate the resistance of *K. pneumoniae* to the CFS of *P. guguanensis* (KD1) using the permeabilizing agent PMBN. We demonstrated that PMBN can potentiate the CFS of *P. guguanensis* (KD1) in *K. pneumoniae*, without causing a lethal effect. Permeabilized

K. pneumoniae was sensitized to 100 mg/mL CFS, compared to treatment with 50 mg/mL CFS. This could be due to the leakage of periplasmic proteins essential for growth and the loss of low molecular weight (MW) substances, caused by the effect of PMBN on the outer membrane of *K. pneumoniae* [36]. Treatment with 50 mg/mL CFS reduced the growth of *K. pneumoniae*; however, growth was regained 3 h post-treatment. This can indicate the concentration-dependent inhibition of the antimicrobial compounds in the CFS. Bacteria can recognize antibiotics as extracellular chemicals at non-lethal concentrations, causing altered tolerance profiles [37].

Despite being sensitive to the CFS of *P. guguanensis* (KD1) in this study, *E. cloacae* was resistant to the acetonitrile extracts of the CFS, suggesting the impermeability of the bacteria to the antibacterial compounds in the extract. This can be due to their incapability to penetrate the lipid membrane, which is a major obstacle in the discovery and development of antibiotics [38]. This problem can be resolved by conjugating the antibiotic with a cationic cell-penetrating peptide (CPP), which has been demonstrated to improve existing antibiotics and increase their effectiveness [39]. Previous research also showed that compounds such as antimicrobial peptides were able to successfully translocate across the lipid bilayer by conjugating them with CPPs, without eliciting cytotoxicity [40]. To better understand the antibacterial compounds in the acetonitrile extracts of *P. guguanensis* (KD1), it would be useful to investigate whether the lipid membrane prevented the antibacterial compounds from inhibiting the growth of *E. cloacae* as part of a future work.

According to this study, the antibiotic-producing isolate KD1 was reported as *P. guguanensis*. This bacterium was previously reported to produce microbial surfactants [41]. Surfactants are surface-active amphiphilic compounds produced by several microorganisms, including bacteria, yeast, and fungi [42]. These surface-active substances help to inhibit the development of biofilms by reducing the surface tension and interfacial tension of fluid phases [42]. *P. guguanensis* isolated from petroleum-contaminated soils in Iraq was reported to produce a biosurfactant that is rhamnolipid in nature based on its structural characteristics [41]. Additionally, a biosurfactant produced by *P. guguanensis* was partially purified in a recent study and chemically identified as a lipopeptide [43]. According to a recent genomic study, *Pseudomonas* sp. GOM, a unique marina bacterium species, is most closely related to *P. guguanensis* in terms of taxonomy [44]. It can produce phenazines like pyocyanin, which has antibacterial activity against several Gram-negative bacteria, including *Escherichia coli* and *E. cloacae* [45]. Nevertheless, more research is needed to fully understand the antimicrobial potential of *P. guguanensis*.

In this research, secondary metabolites with potential antimicrobial activity were successfully extracted from *P. guguanensis* (KD1) using ethyl acetate and acetonitrile as extraction solvents. The ethyl acetate extract exhibited activity against *S. aureus, E. faecium, and E. cloacae*. The MIC was found to be between 1.56 and 3.12 mg/mL, whereas the MBC was found to be between 3.12 and 6.25 mg/mL. Others have reportedly extracted antibacterial compounds from marine bacteria using ethyl acetate as the extraction solvent [41]. Furthermore, acetonitrile extract exhibited antibacterial activity against *S. aureus* and *A. baumannii*, with the recorded MIC values ranging from 3.12 to 6.25 mg/mL and the MBC values between 6.25 and 12.5 mg/mL. Collectively, these results indicate the secretion of one or more antimicrobial compounds by *P. guguanensis* (KD1). The compound(s) can affect the viability of *S. aureus* and the priority Gram-negative bacterium *A. baumanii* [46]. Thus, we speculate that the *P. guguanensis* (KD1) acetonitrile extract can penetrate the cell wall of *A. baumannii*, which is a topic of interest for our future investigation. Moreover, further research using bioinformatics analysis is required to determine whether the genome of *P. guguanensis* (KD1) codes for the biosynthetic gene clusters involved in antibiotic production.

5. Conclusions

The current research concludes that seawater collected from Kuwait Bay is a good source of microorganisms with potential antibacterial properties. *Pseudomonas guguanensis* (KD1), the strain with the most potent antimicrobial activity, was isolated from the sea-

water sample. Additionally, the production of antibacterial compounds by *P. guguanensis* (KD1) was significantly impacted by the cultivation conditions. The highest antimicrobial activity produced by *P. guguanensis* (KD1) was observed when cultivated in nutrient broth at 30 °C after 36 h of incubation. The supernatant's acetonitrile extract demonstrated antibacterial activity against *S. aureus* and *A. baumannii*, while its ethyl extract demonstrated antimicrobial action against *S. aureus*, *E. faecium*, and *E. cloacae*. Our study shows that *P. guguanensis* (KD1) has the potential to be a valuable source of antibiotics and a new hope for treating infections.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/microbiolres15040160/s1, Table S1: Identification of antibiotic-producing bacteria isolated from Kuwait Bay, Table S2: Antimicrobial activity of Pseudomonas strains KD1, KD2 and KD3 using agar well diffusion assays, Table S3: Retardation factors of spots detected by TLC.

Author Contributions: Conceptualization, K.M.D. and N.A.; Formal analysis, K.M.D. and N.A.; Investigation, K.M.D. and H.E.; Methodology, K.M.D. and L.V.; Project administration, K.M.D. and H.A.; Resources, K.M.D. and A.A.D.; Software, H.E. and H.A.; Supervision, K.M.D.; Validation, L.V., N.A. and A.A.D.; Visualization, N.A. and H.A.; Writing—original draft, K.M.D., N.A. and H.E.; Writing—review and editing, K.M.D., L.V., H.E. and A.A.D. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are unavailable due to privacy restrictions.

Conflicts of Interest: The authors declare no conflicts of interest.

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