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Research Paper

Antipneumonial agent(s) from marine bacteria

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ABSTRACT

18 bacterial strains from 321 tested marine bacterial isolates showed to inhibit *Klebsiella pneumoniae* in addition to some other human pathogens. The results indicated the marine *Pseudomonas fluorescens* which isolated from El-Max bay showed activity in inhibiting the lung destructive *K. pneumoniae*. The extraction of the active agent was processed using ethyl acetate (1:1). It showed a maximum inhibition zone (30mm). The LC_{50} of the resulted crude extract was 1050ppm on using *Daphnia magana* as a toxicity biomarker. Moreover the bioactivity of the ethyl acetate crude extract against *K. pneumoniae* was compared to some common antibiotics, it showed to be more effective than amikacin and cefepime and similar in activity as imipinem.

Keywords: marine bacteria, *Pseudomonas fluorescens*, *Klebsiella pneumoniae*, bioactive agents, Biotoxicity.

INTRODUCTION

During the past two decades research on marine bacteria has highlighted the tremendous potential of these microorganisms as a source of new bioactive secondary metabolites (Jensen and Fenical, 2000) and there is a growing awareness of the need for development of new antimicrobial agents for the treatment of human, animal and plant diseases (Ahmed, et al., 2000).

Many bacterial strain isolates from marine habitats have been shown to produce secondary metabolites that display antibacterial properties (Long and Azam, 2001). In addition, it was reported that marine *Pseudomonas* sp. able to produce secondary metabolites acting against several gram negative and gram positive bacteria (Nair and Simidu, 1987). Since the mid 1980s, members of the *Pseudomonas* genus have been applied to control the growth of different pathogens (Haas and Defago, 2005).

However, in 2004, Kasanah and Hamann isolated a new species of *Pseudomonas* from a marine environment which produce novel a–pyrones. It exhibited antibacterial activity against some Gram-positive pathogens. Moreover, Lee et al., (2003) followed up the work by showing antifungal and antioomycete activities of aerugine produced by marine *Pseudomonas fluorescens* strain MM-B16. In

addition, Uzair et al., (2006a) described a marine *Pseudomonas aeruginosa* producing compounds that inhibit the growth of multi-resistant clinical bacterial isolates of *Staphylococcus, Salmonella, and Shigella* etc. Moreover, a very recent report written by Liu et al., (2007), it represented the characterization of a phenazine-producing *Pseudomonas chlororaphis* GP72 which showed broad antifungal activities.

However, *Kleibsella pneumoniae* infections are common in hospitals where they cause pneumonia (characterized by emission of bloody sputum) and urinary tract infections in catheterized patients (Schaberg, et al., 1991). *Klebsiella* infections are encountered far more often now than in the past. This is probably due to the bacterium's antibiotic resistance properties. *Klebsiella* species may contain resistance plasmids (R-plasmids) which confer resistance to several antibiotics as ampicillin and carbenicillin (Antoniadou, et al., 2006). Moreover, the mortality by *K. pneumoniae* is around 50% due to the underlying disease that tends to be present in affected persons. While normal pneumonia frequently resolves without complication, *Klebsiella pneumoniae* more frequently causes lung destruction and pockets of pus in the lung (known as abscesses). Also, the mortality rate for untreated cases is around 90% (Rashid, 2006).

As extension, the goal of this work is to isolate and identify of a potent marine bacteria able to produce bio-safe active agent(s) that inhibit the growth of *K. pneumoniae* and has a broad antimicrobial activity compared to some common antibiotics.

MATERIALS AND METHODS

Screening for the antipneumolial producer: 321 bacterial strains were isolated from different marine sites, Alexandria, Egypt according to their ability to inhibit *Klebsiella pneumoniae* in addition to some other human pathogens: *E. coli* (from spinal cord)**,** *E. coli* (from urine)*, Pseudomonas* (from urine), *Staphylococcus* (from blood) and *Staphylococcus* (from sputum). These used pathogens were kindly provided from the bacteriology department, Abo-Al-Reesh hospital, Cairo University, Egypt.

Selection of the broad spectrum producer strains: The tooth pick agar technique was carried out according to Gross and Vidaver (1990). The positive results were confirmed and also tested to inhibit some other human pathogens using tryptic soya agar medium and incubate for 24h at 30^0C (Casida, 1992).

Growth of *P. fluorescens* was performed using different medium; Glucose peptone medium (Schubert, 1989), M9 salts medium (Shanahan, et al., 1992), King's medium B (King, et al., 1954), Water peptone medium, Casamino acid medium (Aagot, et al., 2001) and water peptone medium (Gould et al., 1985). The pH was adjusted at 7 and the culture media were sterilized by autoclaving. The most active bacterial isolate was chosen for further investigation.

DNA extraction and PCR amplification: The isolation procedures of chromosomal DNA were performed according to the manufacturer's instructions using E.Z.N.A. Bacterial DNA extraction Kit (50) D3350-01(OMEGA Bio-Tek). The 16 rRNA genes were amplified according to Lee et al., (2003) using the universal primers fD1 (5 -AGAGT TTGAT CCTGG CTCAG-3) and rP2 (5 -ACGGC TACCT TGTTA CGACTT-3). The amplified DNA was carried out using 2µl of the template DNA, 2.5µl, 10x PCR Master-Mix (2.5µl of $MgCl_2$, 2.5µl of dNTPs, 0.2µl of recombinant *Taq* polymerase**)**, 2µl for each forward and reversed primers and 1µl water nuclease free. Thermal cycling was performed with the GeneAmp PCR system (model 9700

thermocycler). The thermal profile was 35 cycles consisting of 2 min of denaturation at 94°C, 45 sec. of annealing at 50°C, and 1 min of extension at 72°C. A final extension step consisting of 5 min at 72°C was included. Amplified DNA was applied on agarose gel electrophoresis systems (Taq Promega) for 30min run at 90V/cm. Trans-illuminator was used out for DNA band detection. This part was kindly carried out at Mubarak City for Scientific Research and Technology Applications, Arid Land Institute, Plant Molecular Pathology Department, New Borg El Arab City, Alexandria, Egypt.

Physiological adaptation for maximum productivity: The physiological conditions were combined using an experimental design according to Khan et al., (2006), in which three replicates for each tested factor were used. It included the following temperatures (25, 30 and 45^{o}C), pH values (6, 7 and 8) and inoculum sizes ($8x10^{6}$, $7.3x10^{7}$ and $9.8x10^{8}$ CFU/ml) and they resulted in 27 different combinations, and they were tested for estimating the most adapted culture conditions Similarly, three different concentrations (3, 5 and 7g/l) of the used culture media components: tryptone, peptone and sodium chloride were tested for maximum productivity of the active secondary metabolite substances of *P. fluorescens.* The bioactivity was expressed in mm of the obtained inhibition zone in relation to all these examined factors using the response surface plot curves as preceded in STATISTICA program (ver. 5.5, ed. 1999).

Extraction of the antipneumolial agent(s): A series of polar solvents (ethyl acetate; diethyl ether and butanol) and non-polar solvents (hexane, petroleum ether and benzene) was performed in (1:1) vol/vol for extraction process. The extracted layer was collected, dried over anhydrous sodium sulfate (Gurusiddaiah et al., 1986).The extracted layer was concentrated using a rotary evaporator at 70^{o}C (Büchi RE 111 Lab. Tech. AGCH-9230 Rota-vapor).

Bio-toxicity test: The toxicity bioassay was carried out according to Meyer et al., (1982) using the water flea *Daphnia magna*. Two hundred mg ethyl acetate crude extract were dissolved in 2 ml Dimethyl-sulfoxide (DMSO). Different concentrations of the crude (100, 200, 500, 1000, 1500, 2000 and 4000µg/ml) were made and distributed separately using clean and dry glass vials (20ml) then 10 ml of sterile dechlorinated water for *Daphnia magna* was added. Ten live *Daphnia magna* neonate were transferred to each vial. The number of the viable biomarker was counted after 24 h of application. The percentage of mortality and the half lethal concentration (LC_{50}) were determined using the probit analysis method (Reish, et al., 1987).

Comparative study of different commercial antibiotics used in human therapy in relation to the P. fluorescens ethyl acetate active product : Ten formulations of antibiotic-loaded discs including Ampicillin, Amikacin, Aztreonam, Carbenicillin, Cefoperazone, Cefepime, Doxycyclin, Imipinem, Tetracyclin and Ticarcillin (Sigma) were provided from the bacteriology department, Abu-Al-Reesh hospital, Cairo University, Egypt. They tested to inhibit *K. pneumoniae* grown on Tryptic Soy Agar (TSA). Moreover, the inhibition capacity for this pathogen was measured compared to the bioactivity of *P. fluorescens* ethyl acetate crude extract according to Streuli (2006).

RESULTS

Bioactivity against some human pathogens: From all the tested marine bacterial isolates it was found that only 18 bacterial strains were able to inhibit the *K. pneumoniae.* Moreover, these isolates showed a bioactive spectrum against the tested clinical human pathogens. The results presented in Table 1 showed that the highest activity against *K. pneumoniae* and the most potent broad spectrum was obtained by

the isolate M01 which collected from El-Max station**.** On the other hand, the other isolates showed lower activity against *K. pneumoniae* and narrow spectra.

Identification process: *Pseudomonas fluorescens* which was isolated from marine sediment sample collected from El-Max bay, Alexandria, Egypt and selected according to its high ability to inhibit the tested human pathogens. This strain was identified as an aerobic, motile, non-spore-forming, gram-negative bacterium. It produced a diffusible, fluorescent light green pigment. The identification was performed biochemically using the API 20NE identification kit (bioMérieux). However, Figure 1 illustrated the amplified PCR product of *P. fluorescens* analyzed on agarose gel; it showed a band of 600bp.

Effect of pH, temperature and inoculum size on the bioactivity: The physiological adaptation was carried out to maximize the bioactivity of *P. fluoroscens* against *K. pneumoniae* according to a statistical design. It was found that the optimum pH, temperature and inoculum size were 7, 30^{o}C and 7.3x10^{7} CFU/ml, respectively, where the inhibition zone was 24 mm against *K. pneumoniae* (Figure 2).

Effect of different medium components on the productivity: The medium constituents (tryptone, peptone and NaCl) were combined together according to a statistical design using three concentrations 3, 5 and 7g/l of each. The bioactivity of *P. fluorescens* was estimated using disc diffusion technique and the resulted zone of inhibition was expressed in (mm). The maximum bioactivity of *P. fluorescens* was obtained using a combination of 7, 7, and 3 g/l of trypton, peptone and NaCl, respectively. Where, the highest inhibition zones formed against *K. pneumoniae* was 27.7mm (Figure 3).

Determination of the appropriate solvent(s) for extraction of the active compounds: On using different extractable solvents, it was found that the highest inhibition zone (30mm) against *K. pneumoniae* was obtained using the ethyl acetate. However, the extractable products of the other solvents showed lower antimicrobial activity toward *K. pneumoniae*. Petroleum ether and diethyl ether showed inhibition zones of 15and 18mm, respectively. On the other hand, hexane and benzene showed lower inhibition zones 9 and 11 mm, respectively (Figure 4).

Biotoxicity of different concentrations of the ethyl acetate crude extracts using Daphnia magna: The biotoxicity experiment was carried out using different crude concentrations (ranging from 100 to 4000 µg/ml) and *Daphnia magna* as biomarker. The mortality percent was estimated. The results presented in Figure 5 indicated that the crude has a low toxicity effect, where LC_{50} value was 1050µg crude/ml.

Comparative study of different commercial antibiotics used in human therapy in relation to the P. fluorescens ethyl acetate crude extract: Different commercial antibiotics used in the present experiment to evaluate the zone of inhibition obtained by the ethyl acetate crude extract of *P. fluorescens* against *K. pneumoniae*. It was showed that these tested antibiotics can be categorized into four different groups according to their activity against the *K. pneumoniae.* The first group has no effect on *K. pneumoniae*, it includes A_{25}, ATM_{30}, CAR_{100}, T_{30} and TC_{75}. The second group showed a moderate inhibition zones <10mm and it includes CFP_{75} and DO_{30}. The third group which showed a relatively high activity >10mm, it includes Am_{30} and CE_{30}. While, the fourth group contains imipinem and showed a high inhibition zone 28 mm. On the other hand, the antibacterial effect of the ethyl acetate crude extract of the marine isolate showed an inhibition zone of 30mm (Table 2 and Figure 6).

DISCUSSION

Marine bacteria have been recognized as an important and untapped resource for novel bioactive compound and a great development of marine biotechnology will produce novel compounds that may contribute significantly towards drug development over the next decade (Kasanah and Hamann, 2004). However, the production of antimicrobial active agents acting against some human pathogenic bacteria was carried out through this study. 18 marine bacterial isolates from 321 tested isolates showed the ability to inhibit *K. pneumoniae* in addition to some clinical human pathogens (*E. coli* (from spinal cord)**,** *E. coli* (from urine)*, Pseudomonas* (from urine), *Staphylococcus* (from blood) and *Staphylococcus* (from sputum). The marine *Pseudomonas fluorescens* showed to be the most potent isolate in inhibiting the *K. pneumoniae* and also showed a broad spectrum in inhibiting these tested clinical pathogens. On the other hand, Isnansetyo et al., (2001) worked on *Pseudomonas* sp. which isolated from the surface of a marine red alga (*Ceratodyction spongiosum*) proved to be a producer for anti- methicillin- resistant *Staphylococcus aureus* (MSRA).

However, the present investigation showed the optimum production of the antimicrobial agent by *P. fluorescens* can be achieved at pH 7 and 30^{o}C, the highest inhibition zone was 24 mm. These results are in agreement with several authors, who showed the maximum antimicrobial activity obtained at pH ranged from 6.5 to 7.5 and temperature ranged from 25 to 30^{o}C using different *Pseudomonas* strains (Lee, et al., 2003). Moreover, on using the Plackett Burman design for the optimization of antibacterial production by *P. fluorescens* grown on a water peptone culture medium. It showed maximum inhibition (27mm) using 7, 7 and 3g/l of tryptone, peptone and NaCl, respectively. However, a recent report written by Uzair et al., (2006b) showed maximum inhibition zone (18mm) obtained from the culture filtrate of *P. fluorescens* CMG1030 grown on king B agar plates. It acts against some clinical bacteria *Staphylococcus aureus* and *Staph. epidermedis*. Similarly, many authors reported the use of Plackett Burman technique to adapt different *Pseudomonas* strains grown on different media components (He, et al., 2007, Li, et al., 2008).

The ethyl acetate showed to be the most potent organic solvent for extracting the bioactive compounds from *P. fluorescens*; 2 gm of a yellowish green crude extract was obtained from Twenty liters of *P. fluorescens* culture, it showed inhibition zone of 30mm against *K. pneumoniae*, using the disk diffusion assay. Partially similar data was reported by Uzair et al., (2006b), they showed that the dark oily brown ethyl acetate crude product (100-150mg/l) obtained from a marine *Pseudomonas aeruginosa* CMG1030 showed inhibition zones ranged from 15–33mm against some bacterial pathogens using agar well diffusion method. On the other hand, Lee et al., (2003) reported a total of 41 mg of an antibiotic was yielded from 50 liters of *Pseudomonas* cultures. It showed antifungal activity against *Colletotricum orbiculare* (31mm) using the disk-agar diffusion method.

The bio-toxicity of the produced ethyl acetate crude extract was estimated using the fresh water *Daphnia magna* biomarker. It showed low toxicity where the LC_{50} value was 1050 µg/ml. However, on the comparison to the results obtained by Yoshimura and Endoh (2005), they studied the bio-toxicity of some antiparasitic drugs (amprolium hydrochloride, bithionol, levamisole hydrochloride, pyrimethamine and trichlorfon) using *D. magna*. The LC_{50} values were 227; 0.3; 64.0; 5.2 and 0.00026µg/ml, respectively. Moreover, Kim et al., (2007) examined the biotoxicity of

some pharmaceuticals using *D. magna*. It was found that the LC_{50} of diltiazem was 8.2µg/ml. In addition, Oh et al., (2006) estimated the LC_{50} of six benzimidazole-based anthelmintics drugs (albendazole, thiabendazole, flubendazole, febantel, fenbendazole, and oxfendazole). The LC_{50} values were 0.0165, 0.0679, 0.2165, 0.0665, 0.8436 and 1.1684µg/ml, respectively.

Some recommended antibiotics were used to evaluate the inhibitory effect of the ethyl acetate crude extract obtained from the marine *P. fluorescens* against *K. pneumoniae*. It was showed that the antimicrobial activity of this extract (30mm) was more effective than A_{25}, ATM_{30}, CAR_{100}, T_{30}, TC_{75}, CFP_{75}, DO_{30}, Am_{30} and CE_{30}. While, the antibiotic imipinem showed partial similar activity (28mm) compared with the crude extract. On the other hand, Das et al., (2006) used different antibiotics: chloramphenicol, nalidixic acid, furazolidone, norfloxacin, oxytetracycline, ciprofloxacin, gentamycin and nitrofurantoin against *Aeromonas hydrophila* strain to compare the bioactive effect of the extracellular products (ECP) of *P. fluorescens*, *P. putida* and *P. aeruginosa*. They found that these tested drugs produced lower inhibition zones than that obtained with the three *Pseudomonas* strains. They showed inhibition zones ranged from 21 to 33mm, while, the inhibition zones of the ECP of *P. fluorescens* and *P. putida* were ranged from 60 to 85mm and from 52 to71mm, respectively. Moreover, the ECP of *P. aeruginosa* showed more than 52mm inhibition zone.

CONCLUSION

We can concluded that it is promising to use the ethyl acetate crude extract obtained from the marine isolate *P. fluorescens* for further investigations in order to produce antipneumolial drug acting against the lung destructive pathogen *K. pneumoniae.* It showed low bio-toxicity and more activity compared with many commercial antibiotics.

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Table 1: Bioactivity spectrum of *K. pneumoniae* inhibiting marine bacterial isolates using some clinical human pathogens and the tooth pick technique.

Sampling sites	Code of Bacterial isolates	Human pathogens					
		E.coli (spinal cord)	*E .coli* (urine)	*Klebsiella pneumoniae* (sputum)	*Pseudomonas sp.* (urine)	*Staphylococcus sp.* (blood)	*Staphylococcus sp.* (sputum)
El-Max	M01	(+)	(+)	(+++)	(++)	(+)	(-)
	M02	(++)	(++)	(++)	(+)	(-)	(-)
	M03	(++)	(+)	(+)	(++)	(-)	(-)
	M04	(-)	(-)	(+)	(-)	(-)	(+)
	M05	(+)	(+)	(-)	(-)	(-)	(+)
	M06	(+)	(-)	(+)	(+)	(-)	(-)
	M07	(-)	(-)	(+)	(-)	(-)	(-)
	M08	(-)	(-)	(+)	(-)	(-)	(-)
	M09	(+)	(+)	(+)	(-)	(-)	(-)
East harbor	EH01	(-)	(-)	(+)	(-)	(-)	(-)
	EH02	(++)	(++)	(+)	(++)	(+)	(+)
	EH03	(++)	(++)	(++)	(++)	(-)	(++)
	EH04	(-)	(+)	(-)	(-)	(-)	(-)
	EH05	(++)	(+)	(+)	(-)	(-)	(++)
	EH06	(++)	(++)	(+)	(-)	(-)	(-)
El-Shatby	SH01	(+)	(+)	(-)	(-)	(-)	(-)
	SH02	(-)	(-)	(-)	(+)	(-)	(-)
	SH03	(+)	(+)	(-)	(-)	(-)	(-)
	SH04	(-)	(-)	(+)	(+)	(+)	(-)
	SH05	(-)	(-)	(+)	(-)	(+)	(-)
Abu Qir Bay	AQ01	(+)	(-)	(-)	(-)	(-)	(-)
	AQ02	(-)	(+)	(-)	(+)	(-)	(-)
	AQ03	(-)	(-)	(+)	(-)	(+)	(-)
Suez Gulf	SC01	(+)	(-)	(-)	(-)	(+)	(+)
	SC02	(-)	(-)	(+)	(-)	(-)	(-)
	SC03	(+)	(+)	(-)	(-)	(-)	(+)
	SC04	(+)	(+)	(+)	(-)	(-)	(-)

(+++) high activity, (++) medium, (+) low, (-) no activity

Table 2: Comparison between *P. fluorescens* ethyl acetate crude extract using the disk diffusion assay and some common antibiotics used in human therapy.

Antibiotics	Concentrations (ppm)	Inhibition zone diameter (mm)
Ampicillin	25	-
Amikacin	30	10
Aztreonam	30	-
Carbenicillin	100	-
Cefoperazone	75	7
Cefepime	30	18
Doxycyclin	30	8
Tetracyclin	30	-
Ticarcillin	75	-
Imipinem	10	28
P. fluorescens crude extract (P.F.C.E)	10	30

