

Characterization of marine *Burkholderia cepacia* antibacterial agents

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ABSTRACT

A marine *Burkholderia cepacia* isolated from El-Max bay, Alexandria-Egypt. It showed a potent activity against *Aeromonas hydrophila*, *Edwardsiella tarda* and *Vibrio ordalli*. A Purification and characterization of the *B. cepacia* ethyl acetate crude extract was carried out, it resulted in five antimicrobial fractions. They were (1) Phenol, (2) Phenol-4-methyl, (3) 3-benzyl-1,4-diaza-2,5-dioxobicyclo [4.3.0]nonane, (4) Hexadecanoic acid ethyl ester and (5) 1,2-Benzene dicarboxylic acid, Bis-(2-ethylhexyl) ester. The bioactivity of these five majors against the three tested pathogens was separately estimated in comparison with the ethyl acetate crude extract itself. The results indicated the ethyl acetate crude extract had higher bioactivity against these pathogens compared to the fractionated components.

Keywords: Marine bacteria; Antimicrobial agents; TLC; GC-MS; IR analysis.

INTRODUCTION

In general, the most common bacterial diseases in aquaculture were known as Septicemia, Edwardsiellosis and Vibriosis, they caused by *Aeromonas* spp. (Gonzalez, et al., 2002), *Edwardsiella* sp. (Savan, et al., 2004) and *Vibrio* sp. (Jones, et al., 2000), respectively. However, in the last decade the biotechnological approaches to overproduce new antimicrobial agents from marine microorganisms had received widespread attention.

However, many antibiotics were intensively used worldwide to control bacterial infectious diseases, but on using these antibiotics the drug resistance patterns had been developed within the pathogenic communities (Cowen, 2001;

Lipsitch, 2001). Therefore, the production of alternatives to the ordinary used antibiotics gained big importance in many countries (Bala, 2001).

Recently, several researches had paid attention towards marine microorganisms especially aquatic *Pseudomonas* sp. as alternative resources for isolation of novel metabolites with interesting biological and pharmaceutical properties (Faulkner, 2000; Das, et al., 2006).

Burkholderia cepacia is a ubiquitous bacterium with a potential use for biological treatment against many bacterial and fungal pathogens especially the filamentous fungi, yeast, *Streptomyces*, different gram positive bacteria and some protozoa (El-Banna and Winkelmann, 1998; Cody, et al., 2000). In addition, Kang, et al., (2004) investigated the antagonistic effect of the antifungal compounds produced by *B. cepacia* using ethyl acetate crude extract. Moreover, Mao, et al., (2006) extracted four antifungal active compounds from *Burkholderia* strain designated MP-1 which undergo purification and chemical characterization using GC-MS analysis.

So, these studies aimed to investigate the physicochemical characterization of marine *Burkholderia cepacia* crude extract using: thin layer chromatography, Gas chromatography- Mass spectrum and Infra red analysis. In addition to estimate the bioactivity of these purified fractions against *Aeromonas hydrophila*, *Edwardsiella tarda* and *Vibrio ordalli* compared to the crude extractable materials.

MATERIALS AND METHODS

Bacterial strain and culture medium: *B. cepacia* was previously isolated by El-Naggar, et al., (2007) from El Max bay, Alexandria, Egypt. The water peptone culture medium was used and optimized according to El-Naggar, et al., (2007).

Screening for antimicrobial activity: *Aeromonas hydrophila*, *Edwardsiella tarda* and *Vibrio ordalli* were kindly provided from Poultry and Fish Diseases Dept., Faculty of Veterinary Medicine, Alexandria University. The cultured Tryptic Soya Agar (TSA) medium was applied and incubated at 30°C for 24-48h. The inhibition zones were estimated using the disc diffusion assay according to Kirby-Bauer bioassay technique (Bauer, et al., 1966). 2.5ml of each pre-cultured tested pathogen (OD = 1) was mixed well using 25ml of a sterile molten TSA media (45°C), then poured into three sterile Petri-dish plates (9cm) and allowed to solidify. The cultivated *B. cepacia* was under go centrifugation twice for 15 minutes at 10,000 x g then the obtained supernatant was filtered through a membrane filter (0.45mm). Then sterile discs (5mm in diameter) were immersed in the obtained free cells filtrate and placed on the prepared plates using an alcohol sterilized forceps. The plates were allowed to stand for 30min to 2hrs in a refrigerator to allow the diffusion then incubated at 30°C for 24-48h. The inhibition zone formed around each disc was measured in mm.

Extraction process: The extraction process was carried out using the ethyl acetate 1:1 according to El-Naggar, et al., (2007). Then the extracted layer was collected and concentrated using a rotary evaporator (Büchi RE 111 Lab. Tech. AGCH-9230 Rotavapor). The obtained extract undergoes dryness process using anhydrous sodium sulfate.

Characterization of the ethyl acetate crude extract:

(1) **Thin layer chromatography (TLC):** The ethyl acetate crude extract was fractionated using silica gel TLC plates. Different mobile phases were prepared separately using mixtures of solvents as follows: acetone, acetone: ethyl acetate (1:2), acetone: ethyl acetate. (2:1), methanol, methanol: ethyl acetate (1:2), methanol: ethyl acetate (2:1), chloroform, chloroform: ethyl acetate (1:2), chloroform: ethyl acetate (2:1), petroleum ether, petroleum ether: ethyl acetate

(1:2), petroleum ether: ethyl acetate (1:2) and ethyl acetate. The silica gel plates were performed in size of 20x20cm and a thickness of 0.25 mm using 60GF₂₅₄Merck. Then the crude was applied as a spot and left to complete separation using the previously prepared mobile phases. The plates stood for solvent evaporation and the R_f of resulted colored and uncolored spots were recorded using an ordinary and UV lamps, respectively. Each separated spot was scratched and its bioactivity against the three tested pathogens was estimated using the disc diffusion assay as mentioned above.

- (2) **Gas chromatography- mass spectrophotometer (GC-MS) analysis:** Analysis was conducted using an HP (Hewlett Packard, 5890 series II GC hyphenated with 5989 Mass Spectrometer). MS conditions were as follows: Detector mass spectrometer voltage 70eV and its source temperature was 300°C. The injector temperature was 240°C and the split less mode 0.5µL injection. The HP 55% dimethyl-95% diphenylpolysiloxane non-polar column was performed with length 30 cm x 0.25 mm, coating thickness film 0.25 µm. The oven was adjusted at 80°C for 1 min and initial time 1.5 min with 40°C which ended by a final temperature of 300°C and 4 min hold time where the total run time was 15 min. The components were identified by comparing their retention times with those of authentic samples, as well as by comparing their mass spectra with those of Wiley 275 Library (Wiley, 2006). Quantitative data were obtained by the peak normalization technique using integrated FID response.
- (3) **Infrared analysis (IR):** Using Perkin-ELMER1430 infrared spectrophotometer, the molecular structure of the antibacterial materials was partial identified. Separated spots of each compound were collected by scratching and dissolved in 1ml ethyl acetate and lyophilized then small discs made from the mixture of about 1mg of the lyophilized material and 300mg of pure and dry KBr were subjected to IR-spectroscopy. The measurement were carried out at infrared spectra between 800-4000nm

RESULTS

Characterization of the crude extract

(1) **Thin layer chromatography:** The results of the TLC of the crude using acetone: ethyl acetate, 1:2 as a solvent system showed to be the most potent solvent system compared to the other tested solvent systems. Five spots were separated, two yellowish brown spots (2 &3) and three colorless UV-spots (1,4 &5).The R_f values for the colored spots were 0.90 and 0.73, respectively and 0.96, 0.54 and 0.18, respectively, for the colorless UV-spots (Figure 1).



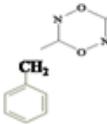
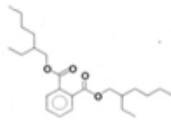
Figure-1: TLC screening of the ethyl acetate crude extract showing five major fractionated spots.

(2) **GC-MS analysis:** GC/MS analysis of the ethyl acetate crude extract showed the presence of 21 compounds. The main five detected compounds showed retention times of 2.76, 3.26, 6.29, 7.38 and 7.69 min, respectively, (Figure 2). However, based on the comparison with the standards in Wiley data base it can be recognized that these

obtained components were relatively similar to: (1) Phenol (80%), (2) Phenol-4-methyl (91%), (3) 3-benzyl-1,4-diaza-2,5-dioxobicyclo [4.3.0]nonane (89%), (4)

Hexadecanoic acid ethyl ester (93%), (5) 1,2-Benzene dicarboxylic acid, Bis- (2-ethylhexyl) ester (93%). The chemical properties of the five GC/MS obtained compounds were represented in Table 1. In addition, the mass-spectral analysis of each component was illustrated separately in Figure 3.

Table-1: The chemical properties of the five major compounds of the ethyl acetate crude extract using GC/MS analysis.

Library/ID	Amount (mg)	Molecular Formula (M.F.)	Molecular weight (M. Wt.)	Match Quality (%)	Suggested chemical Structure*
Phenol derivative	52	C ₆ H ₆ O	94.042	80	
Phenol,4-methyl-Derivative	163	C ₇ H ₈ O	108.05	91	
Hexadecanoic acid, ethyl ester	232	C ₁₈ H ₃₆ O ₂	284.272	93	
3-benzyl-1,4-diaza-2,5-dioxobicyclo[4.3.0]nonane	356	C ₁₄ H ₁₆ N ₂ O ₂	244.121	89	
1,2-Benzene dicarboxylic acid, Bis- (2-ethylhexyl) ester	97	C ₂₄ H ₃₈ O ₄	390.277	91	

* The nearest library standards.

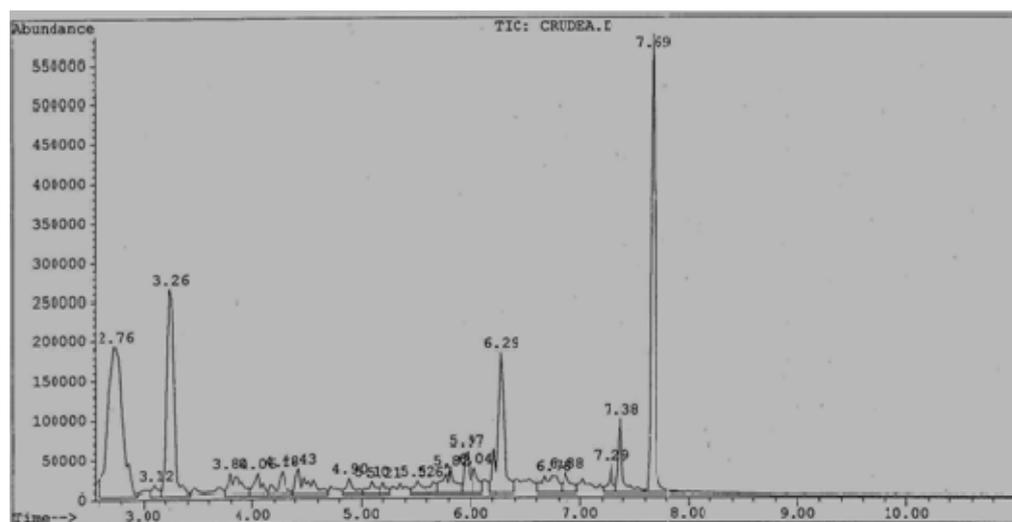


Figure 2: The gas chromatogram of *B. cepacia* ethyl acetate crude extract shows the appearance of the five major compounds at different retention times.

(3) **Infrared spectra:** In the molecular diagnosis of vibrational frequencies of absorption bands it is extremely useful to refer to the illustrated values of various functional groups and their associated characteristic group of frequencies ranges. In order to give conclusive ideas about the structure of the compound under investigation, it is necessary to have an assignment for the IR-adsorption bands corresponding to the active group in the compound (Figure 4) .

(4) **Bioactivity of majors:** The bioactivity of the elutes of the five major compounds separated by the TLC was estimated against *A. hydrophila*, *E. tarda* and *V. ordalii* using TSA agar plates. They found to be positive against these pathogens and on comparing their bioactivities with that of the crude itself, it was found that the ethyl acetate crude extract had higher activity, the inhibition zones were 30, 30 and 29mm, respectively, Table 2. On the other hand, the start spot was examined and found to be with no antibacterial activity.

Table-2: The bioactivity of the five major components compared to the crude extract using three bacterial pathogens.

Crude & TLC spots	Inhibition zone diameter (mm)		
	<i>A. hydrophila</i>	<i>E. tarda</i>	<i>V. ordalii</i>
Crude	30	30	28
Phenol derivative	15	10	12
Phenol,4-methyl- Derivative	12	8	6
Hexadecanoic acid, ethyl ester	6	10	11
3-benzyl1,4-diaza-2,5-dioxobicyclo[4.3.0]nonane	18	7	6
1,2-Benzene dicarboxylic acid, Bis- (2-ethylhexyl) ester	21	15	11

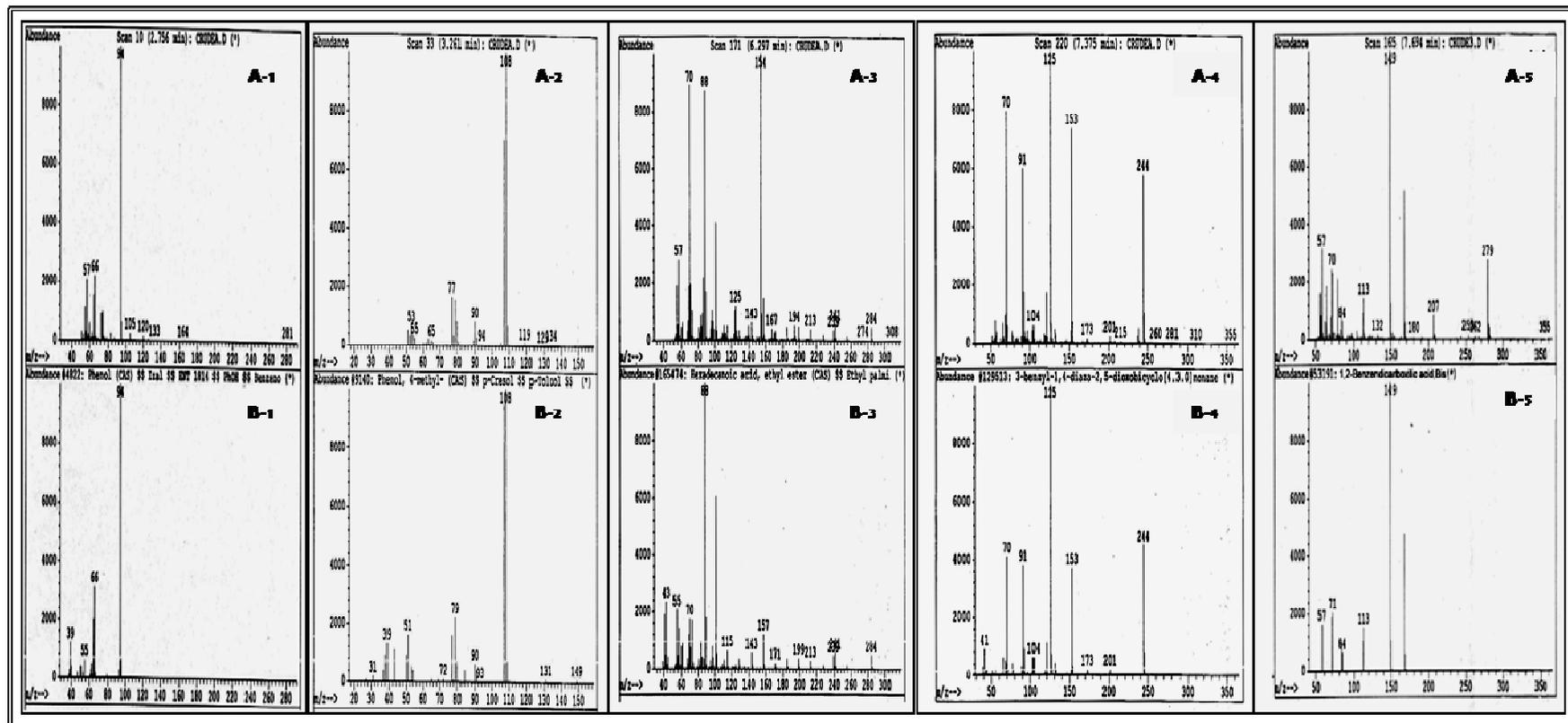


Figure 3: The mass spectra of the ethyl acetate fractions of *B. cepacia*; Phenol derivative (A-1), Phenol-4-methyl derivative (A-2) Hexadecanoic acid-ethyl ester (A-3), 3-benzyl-1,4-diaza-2,5-dioxobicyclo[4.3.0]nonane (A-4), 1,2-Benzenedicarboxylic acid, Bis(2-ethylhexyl) ester (A-5) and the mass spectra of the corresponding compounds in the authentic samples from B-1 to B-5, respectively.

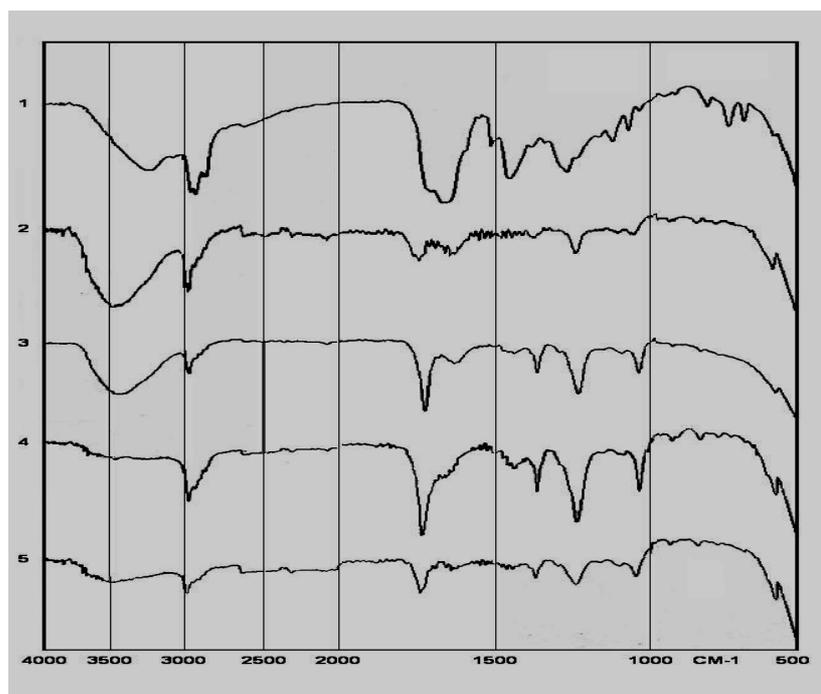


Figure-4: The IR analysis of the five major compounds of the ethyl acetate crude extract.

DISCUSSION

There is growing awareness of the need for development of new antimicrobial agents for the treatment of human, animal, and plant diseases. Marine bacteria have been recognized as an important resource for novel bioactive compounds. The development of marine biotechnology showed to be promising to produce novel compounds that may contribute significantly towards drug application (Kasanah and Hamann, 2004).

In this study several bacterial strains were isolated from different marine sites in Alexandria. They were examined for the production of antimicrobial agent(s) and the *B. cepacia* which was isolated from El-Max bay showed high activity toward *Aeromonas hydrophila*, *Edwardsiella tarda* and *Vibrio ordalli*. Similarly, several investigations were carried out using *Pseudomonas* sp. They showed antagonistic properties against different bacterial fish pathogens (Gram, et al., 2001; Das, et al., 2006).

Moreover, Upadhyay, et al., (1991) used *Burkholderia cepacia* as an important source for antimicrobial agent against several pathogens. But, the antimicrobial agents produced by *B. cepacia* showed to be influenced by different nutritional, experimental and environmental factors either *in vitro* or *in vivo* (Gatesoupe, 1999).

The physicochemical characterization of the ethyl acetate crude extract was carried out using preparative silica gel thin layer chromatography (TLC) plates. The best mobile phase was acetone: ethyl acetate (1:2 v/v) which led to five major spots with different R_f values 0.96, 0.90, 0.73, 0.54 and 0.18. Dissimilar to that Kang, et al., (2004) produced an anti-fungal metabolite from *Burkholderia* sp. MSSP, it showed inactive TLC separation using hexane: ethyl acetate (3:2, v/v), it led to a single spot with R_f value of 0.42. In addition, Lee, et al., (2003) fractionated the ethyl acetate crude extract produced by *pseudomonads* sp. using silica gel TLC plates. They found that the active band was observed with R_f of 0.75 and it exhibited strong antifungal activity against *Colletotrichum orbiculare* and *Phytophthora capsici*.

The GC-MS analysis of *B. cepacia* ethyl acetate extract showed that the extract was divided into five main compounds. They were; (1) phenol derivatives, (2) phenol, 4-methyl derivatives, (3) hexadecanoic acid, ethyl ester, (4) 3-benzyl-1,4-diaza-2,5-dioxobicyclo [4.3.0] nonane and 1,2-Benzene dicarboxylic acid. However, physical and chemical (spectral) data obtained in this study were not designed to elucidate the structure of the isolated compounds but to outline the figure of these compounds.

The bioactivity of the five components resulted from *B. cepacia* ethyl acetate crude extract was estimated and found to be positive against the three tested pathogens the inhibition zones were ranged from 6 to 21mm, while, the crude extract itself led to activity of 28-30mm. The most active fraction was, 1,2-Benzene dicarboxylic acid bis-2-ethylhexyl ester followed by the phenol derivative. On the other hand, *P. fluorescens* strain crude extract was analyzed using thin layer chromatogram on silica gel plate and a mobile phase of Ethyl acetate and hexane (1:1 v/v). it was found that thirteen bands were observed under UV trans-illuminator, but one compound only showed activity against the tested pathogenic strains *Staphylococcus aureus*, *Staphylococcus epidermids* and *Enterobacter faecalis* (Uzair, et al., 2006).

CONCLUSION

The spectral analysis of the ethyl acetate crude extract showed the majority of five active compounds acting against *Aeromonas hydrophila*, *Edwardsiella tarda* and *Vibrio ordalli*. They were (1) Phenol , (2) Phenol-4-methyl , (3) 3-benzyl-1,4-diaza-2,5-dioxobicyclo [4.3.0]nonane , (4) Hexadecanoic acid ethyl ester and (5) 1,2-Benzene dicarboxylic acid, Bis- (2-ethylhexyl) ester. However, the application of the crude extract itself exhibited more bioactivity compared to its individual components. Finally, it could be promising to use such marine *B. cepacia* extractable compounds to protect against the infection diseases caused by these pathogens.

ACHNOLEDGMENT

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REFERENCES

- Bala, R., B., (2001): Biofilm associated bacterial flora of a freshwater habitat. M. F. Sc. thesis. Central Institute of Fisheries Education, Mumbai, India, pp. 1–80.
- Bauer, A.W., Kirby, W.M., Sherris, J.C. and Turck, M., (1966): Antibiotic susceptibility testing by a standardized single disk method. *Am. J. Clin. Pathol.*, 45: 493–496.
- Cody, C.C., Henry, A.T., Robert, H., Waldo, III., Lester, J., Casida, Jr. and Joseph, O.F.III., (2000): Identification and characteristics of a novel *Burkholderia* Strain with broad-spectrum antimicrobial activity. *App. Environ. Microbiol.*, 66: 4139-4141.
- Cowen, L.E., (2001): Predicting the emergence of resistance to antifungal drugs, *FEMS Microbiol. Lett.*, 204: 1–7.
- Das, B.K., Samal, S.K., Samantaray, B.R., Sethi, S., Pattnaik, P. and Mishra, B.K., (2006): Antagonistic activity of cellular components of *Pseudomonas* species against *Aeromonas hydrophila*. *Aquacul.*, 253: 17-24.

- El-Banna, N. and Winkelmann, G., (1998): Pyrrolnitrin from *Burkholderia cepacia*: antibiotic activity against fungi and novel activities against *Streptomyces*. *J. Appl. Microbiol.*, 85: 69-76.
- El-Naggar M. M. A., Gomaa S., Gohar Y. M., Barakat K. I. and Soliman M. K., (2007): Bio-control of some fish pathogens using marine *Burkholderia cepacia* by-product. In the proceeding of the MEDCOAST 2007 International Conference, Vol-1 "Marine Biology, Chemistry & Biochemistry" pp: 345-353
- Faulkner, D. J., (2000): Marine pharmacology. *Antonie Leeuwenhoek*, 77: 135-145.
- Gatesoupe, F.J., (1999): The use of probiotics in aquaculture, *Aquacul.*, 180: 147-165.
- Gonzalez, C.J., Santos, J.A., Lopez, M.L.G. and Otero, A., (2002): Virulence markers in *Aeromonas hydrophila* and *Aeromonas veronii* biovar *sorbria* isolates from fresh water fish and from a diarrhoea case. *J. Appl. Microbiol.*, 93: 414-419.
- Gram, L., Løvold, T., Nielsen, J., Melchiorson, J. and Spanggaard, B., (2001): In vitro antagonism of the probiont *Pseudomonas fluorescens* strain AH2 against *Aeromonas salmonicida* does not confer protection of salmon against furunculosis. *Aquacul.*, 199: 1-11.
- Haas, D. and Défago, G., (2005): Biological control of soil-Born pathogens by florescent *Pseudomonas*. *Microbiol.*, 3: 307-319.
- Jones, M., Cockerill, D.J., Birkbeck, T.H. and Cox, D.I., (2000): Clinical infection in cod (*Gadus morhua* L.) in Scotland by *Vibrio anguillarum*—a case history. *Bull. Eur. Assoc. Fish Pathol.*, 20: 125-128.
- Kang, J.G., Shin, S.Y., Kim, M.J., Bajpai, V., Maheshwari, D.K. and Kang, S.C., (2004): Isolation and anti-fungal activities of 2-hydroxymethyl-chroman-4-one Produced by *Burkholderia* sp. MSSP. *J. Antibiot. (Tokyo)*, 57: 726-731.
- Kasanah, N. and Hamann, MT., (2004): Development of antibiotics and the future of marine microorganisms to stem the tide of antibiotic resistance. *Curr. Opin. Investig. Drugs*, 5: 827-837.
- Lee, J.Y., Moon, S.S. and Hwang, B.K., (2003): Isolation and antifungal and antioomycete activities of aerugine produced by *Pseudomonas fluorescens* strain MM-B16. *Appl. Environ. Microbiol.*, 69: 2023-2031.
- Lipsitch, M., (2001): The rise and fall of antimicrobial resistance. *Trends Microbiol.*, 9: 438-444.
- Mao, S., Lee, S.J., Hwangbo, H., Kim, Y.W., Park, K.H., Cha, G.S., Park, R.D. and Kim, K.Y., (2006): Isolation and characterization of antifungal substances from *Burkholderia* sp. culture broth. *Curr. Microbiol.*, 53: 358-364.
- Savan, R., Igarashi, A., Matsuoka, S. and Sakai, M., (2004): Sensitive and rapid detection of Edwardsiellosis in fish by a loop-mediated isothermal amplification method. *Appl. Environ. Microbiol.*, 70: 621-624.
- Upadhyay, R., Visintin, L. and Jayaswal, R., (1991): Environmental factors affecting antagonisms of *Pseudomonas cepacia* against *Trichoderma viride*. *Canad. J. Microbiol.*, 37: 880-884.
- Uzair, B., Ahmed, N., Ahmad, V.U. and Kousar, F., (2006): A new antibacterial compound produced by an indigenous marine bacteria--fermentation, isolation, and biological activity. *Nat. Prod. Res.*, 20: 1326-1331.
- Wiley, J., (2006): Wiley Registry of Mass Spectral Data, 8th Edition "software" Wiley's Scientific, Technical, and Medical Databases.