



## Isolation and partial characterisation of secondary metabolites from fish-borne bacterium, *Pseudomonas aeruginosa*

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### ABSTRACT

Partial characterisation of secondary metabolites of a potent antagonistic bacterium *Pseudomonas aeruginosa* FARP72 (NCBI GenBank Accession No. KC570343) isolated from the skin mucus of catfish *Clarias batrachus* was attempted in this study. The antagonistic activity of *P. aeruginosa* was confirmed by cross and parallel streaking methods against three bacterial fish pathogens (*Aeromonas caviae*, *Edwardsiella tarda* and  $\beta$ -haemolytic *Streptococcus* sp.) and seven human bacterial pathogens viz., *Klebsiella pneumoniae*, Enterohaemorrhagic *Escherichia coli*, *Escherichia coli*, *Staphylococcus aureus* ATCC 12598, *Salmonella* Typhi (serovar of *Salmonella enterica* sub sp. *enterica*), *Vibrio cholerae* and *Bacillus pumilus*. The antibacterial activities of the crude ethyl acetate extract of the whole cell of *P. aeruginosa* and the spent medium were also tested against the pathogens by well and disc diffusion assays. The ethyl acetate extract of *P. aeruginosa* cells was subjected to column chromatography packed with silica gel (60-120 mm mesh size) using petroleum ether and ethyl acetate in different concentrations as the mobile phase. The compounds of the bioactive fractions were semi purified and partially characterised using thin layer chromatography and mass spectroscopy. The secondary metabolites were identified to be pyrrols, quinoline and phenazine compounds. The results suggested that the bioactive fractions of whole *P. aeruginosa* cells have potential antibacterial activity, which can be used as an alternative to conventional antibiotics to control fish and human pathogens.

Keywords: Antagonistic bacterium, Bioactive fractions, Pathogens, Partial characterisation, *Pseudomonas aeruginosa*, Skin mucus

### Introduction

High stocking density and high feed input came in to play for increasing production in intensive aquaculture practice, which in turn, accompanies several disease problems decreasing growth rate and hence the production. The use of antimicrobials and indiscriminate use of commercial antibiotics in disease prevention results in development of antibiotic resistance (Moriarty, 1997). Alternative strategies to prevent opportunistic infections are, therefore, strongly needed. The need of the hour is to search for novel antagonistic bacteria with therapeutic potential for which the pathogens may not have resistance. The genus *Pseudomonas* is one of the most diverse Gram negative bacterial genera isolated from sources ranging from plants, soils and water. A number of disease suppressive antibiotic compounds have been chemically characterised from *pseudomonads*. The chemical compounds vary with species and strains of *Pseudomonas*. In this study,

attempts were made to study the *in vitro* antagonistic activity of a fish borne strain, *Pseudomonas aeruginosa* FARP72 against bacterial pathogens of human and fish and to characterise the bioactive secondary metabolites produced by the bacterial strain.

### Materials and methods

#### *Bacterial strains*

An antagonistic bacterium *Pseudomonas aeruginosa* FARP72 (NCBI GenBank Accession no. KC570343) isolated from the skin mucus of freshwater catfish, *Clarias batrachus* (Hoque, 2015) was used for *in vitro* antagonistic activity against three bacterial pathogens of fish viz., *Aeromonas caviae*, *Edwardsiella tarda* and  $\beta$ -haemolytic *Streptococcus* sp. as well as 7 human bacterial pathogens viz., *Bacillus pumilus*, *Escherichia coli*, Enterohaemorrhagic *E. coli*, *Klebsiella pneumoniae*, *Salmonella* Typhi (serovar of *Salmonella enterica* sub sp. *enterica*), *Staphylococcus aureus* ATCC 12598 and *Vibrio cholerae*.

### *Ethyl acetate extraction of inhibitory substances of Pseudomonas aeruginosa FARP72*

Ethyl acetate extraction as described in Wratten *et al.* (1977) was followed with minor modifications. *P. aeruginosa* FARP72 was inoculated onto six tryptic soy agar (TSA) plates and incubated for 48 h at 30±2°C. The cells were then gently scraped off and washed with sterile saline. Centrifugation was done at 10,000 rpm for 15 min at 4°C. Cell pellets were collected and suspended in 20 ml ethyl acetate for 24 h. Cells were removed by further centrifugation. The spent agar medium was cut into small pieces, homogenised with ethyl acetate (300 ml) and allowed to stand for 48 h without agitation at 30±2°C. The solvent was filtered through Whatman filter paper to remove the spent agar. Both solvents were decanted and evaporated using vacuum evaporator twice at 40-50°C at 15 psi to get a concentrated crude extract of the bacterial metabolites.

### *In vitro inhibitory activity of ethyl acetate extract of P. aeruginosa FARP72 cells and spent medium*

The antibacterial activity of the crude ethyl acetate extracts of whole cells and spent medium was tested against test organisms by agar disc diffusion assay (CLSI, 2012). Bacterial lawns of the test organisms were prepared on TSA plates separately. Sterile discs of 6.0 mm dia were placed onto the TSA plates containing bacterial lawn. Aliquots (3 µl) of the whole cell extract and spent medium extract were added separately onto the sterile discs and allowed to diffuse for 2 h. The plates were incubated at 30±2°C for 24 h and examined for a definite zone of clearance around the discs.

Well diffusion assay was performed as described by Tagg and McGiven (1971). On TSA plates, wells of 0.5 mm dia each were cut using sterile borer. The bottom of the wells was sealed with 1 µl of molten tryptic soya soft agar (tryptic soy broth+0.8% agar). Thirty microlitre of the concentrated ethyl acetate extract of spent medium of *P. aeruginosa* FARP72 were added into each well. The extract was allowed to diffuse into the medium for 2 h. The plates were then overlaid with 10 ml of soft agar seeded separately with 10 µl of overnight grown test organisms and allowed to set. The plates were then incubated at 30±2°C for 24 h. The diameter of the inhibition zone in mm formed around the well was measured. Chloramphenicol disc (30 µg disc<sup>-1</sup>) was used as positive control.

### *Isolation and partial characterisation of ethyl acetate extract of P. aeruginosa FARP72*

Crude ethyl acetate extract of *P. aeruginosa* FARP72 cells was subjected to column chromatography (CC). A glass column was packed with 100 g silica gel slurry

dissolved in petroleum ether. The crude extract was adsorbed on to dry silica gel and the solvent was allowed to evaporate. Then the dry sample adsorbed to the silica gel was applied to the column packed with silica gel (60-120 mm mesh size). Different mobile phases were prepared separately using mixtures of solvents at different concentration *viz.*, (i) petroleum ether, (ii) petroleum ether: ethyl acetate (1:2), (iii) petroleum ether: ethyl acetate (2:1) and (iv) ethyl acetate. A total of 25 fractions were collected. Solvents were removed from the fractions under reduced pressure using rotary evaporator. Thin layer chromatography (TLC) analyses of the crude material gave good separation of pigments on TLC plate in a solvent system that was composed of petroleum ether and ethyl acetate mixture. Therefore, the mixture was used in different combinations with increasing polarity to elute the column. The ethyl acetate crude extract was fractionated using silica gel TLC plates. TLC was performed on silica gel plates of 0.25 mm (60GF254 Merck) thickness as described by El-Moughith *et al.* (1986). The spots developed on TLC plates were visualised under UV light at 254 and 365 nm and then by exposure to iodine vapour. The fractions that showed the same TLC development profiles (colour and R<sub>f</sub>) were combined and concentrated to dryness under reduced pressure using rotary evaporator. Time-of-flight mass spectrometry (TOF-MS) method was used in which the ion's mass-to-charge ratio was determined *via* time measurement. Ions were accelerated by an electric field which resulted in an ion having the same kinetic energy as any other ion that has the same charge. The velocity of the ion is dependent on the mass-to-charge ratio. The molecular weights of the compounds were elucidated based on mass spectral data. The compounds were identified by comparing molecular weight with the established data from the literature (Labows *et al.*, 1980; Que *et al.*, 2011; Charyulu *et al.*, 2012).

## **Results**

### *In vitro inhibitory activity of ethyl acetate extracts of whole cells of P. aeruginosa FARP72 and spent medium*

The antibacterial substance from the whole cells and spent medium were extracted separately with ethyl acetate and both displayed inhibitory activity against the test organisms (Table 1; Fig. 1 and 2). The growth of Gram positive bacteria *S. aureus* ATCC 12598 and *B. pumilus* was inhibited by the crude extracts of *P. aeruginosa* FARP72. Crude extracts of *P. aeruginosa* FARP72 from spent medium effected larger zone of inhibition against the Gram negative bacterial pathogens of human origin such as *V. cholerae*, *S. Typhi* and *E. coli* with zone size ranging from 6 to 12 mm. *P. aeruginosa* FARP72 showed inhibitory activity against the Gram

Table 1. Susceptibility of human and fish pathogens to crude ethyl acetate extracts from *P. aeruginosa* FARP72

Test strains	Zones of inhibition (mm)		
	Disc diffusion Assay	Well diffusion Assay	
	Whole cell extract	Spent TSA medium extract	Spent TSA medium extract
Gram positive human pathogens	8.00	8.00	23.50
<i>Bacillus pumilus</i> <sup>a</sup>			
<i>Staphylococcus aureus</i> ATCC 12598 <sup>b</sup>	9.00	9.00	20.00
Gram negative human pathogens	9.00	10.00	21.00
<i>Klebsiella pneumoniae</i> <sup>c</sup>			
<i>Escherichia coli</i>	9.00	10.00	38.00
<i>Vibrio cholerae</i> <sup>b</sup>	8.50	12.00	20.00
Enterohaemorrhagic <i>Escherichia coli</i> <sup>b</sup>	8.00	7.00	24.00
<i>Salmonella</i> Typhi <sup>b</sup>	9.00	10.00	28.50
Gram positive fish pathogen	8.00	7.00	30.00
β- haemolytic <i>Streptococcus</i> sp. <sup>d</sup>			
Gram negative fish pathogens	10.00	16.00	32.00
<i>Aeromonas caviae</i> <sup>d</sup>			
<i>Edwardsiella tarda</i> <sup>e</sup>	10.00	9.00	37.00

TSA: Tryptic soy agar; a: Department of Fish Processing Technology, FFSc, Kolkata; b: Fisheries College and Research Institute, Tamil Nadu Fisheries University, Thoothukudi, c: Calcutta National Medical College and Hospital, Kolkata; d: Department of Aquatic Animal Health, FFSc, Kolkata; e: Shrimp Disease Diagnostic Laboratory, Tamil Nadu Fisheries University, Madhavaram, Chennai

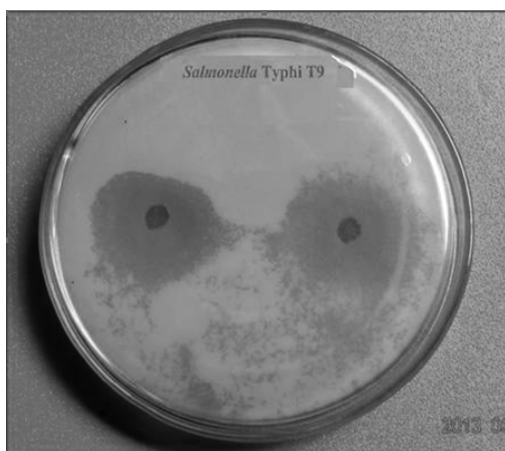


Fig. 1. *In vitro* inhibition of test strain T9, *Salmonella* Typhi by crude ethyl acetate extract of *P. aeruginosa* FARP72 spent medium by agar well diffusion assay

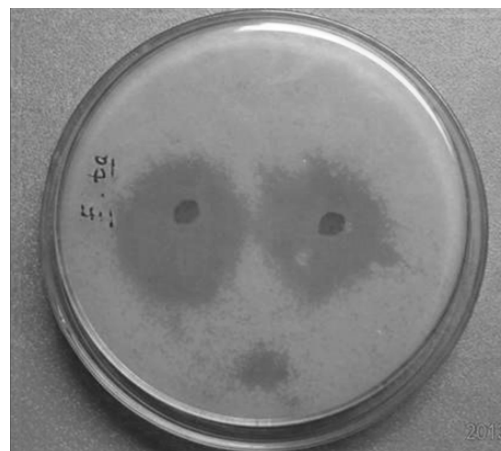


Fig. 2. *In-vitro* inhibition of test strain *Edwardsiella tarda* by crude ethyl acetate extract of *P. aeruginosa* FARP72 spent medium by agar well diffusion assay

positive fish pathogen β-haemolytic *Streptococcus* sp. isolated from diseased Nile tilapia *Oreochromis niloticus* and Gram negative fish pathogens such as *A. caviae* and *E. tarda*. Largest zone of inhibition (10-16 mm) was shown against *A. caviae*. In all the cases, the ethyl acetate extract of the spent medium produced larger zones of inhibition as compared to whole cell extract.

#### Identification and characterisation of ethyl acetate extract of *P. aeruginosa* FARP72

From TLC results, fractions were combined according to their separation and finally, four fractions were obtained. First fraction (comprising 3-5 eluents) containing upper impurities were discarded. The second and fourth fractions (comprising 7-9 and 16-25 eluents,

respectively) were considered to be partially pure. The third fraction (comprising 10-16 eluents) was considered to be mixture of second and fourth fractions. Molecular weights of the partially purified second and fourth fractions were established using the mass spectrum (MS). Due to the minute quantities of the bacterial extract obtained, analyses of the structure of the compounds were

not possible by NMR. The peaks obtained by MS analysis were, therefore, compared with the literature references and the probable compounds were ascertained by molecular weight analysis. The column chromatography and MS profiles of the partially purified second fraction of *P. aeruginosa* extract (Fig. 3. and Table 2), showed intensity of the  $m/z$  144.08 peak and hence was determined

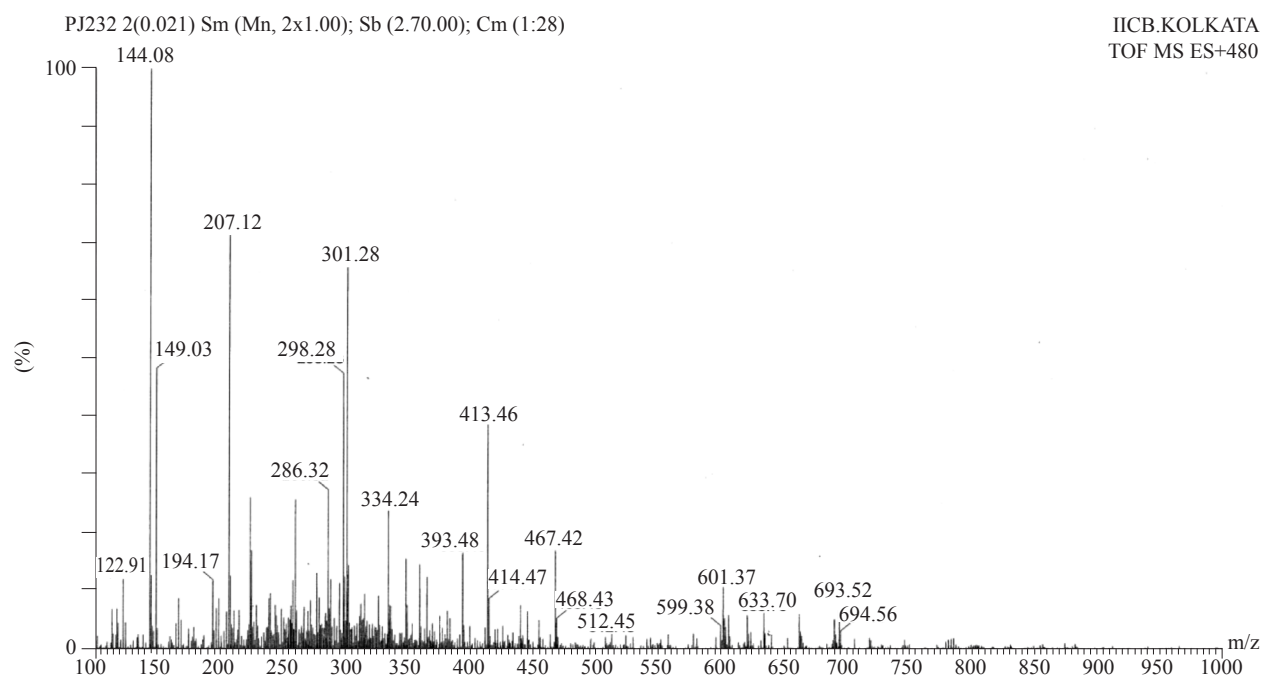


Fig. 3. Peaks obtained in Mass spectroscopy analysis of partially purified second fraction obtained by column chromatography of *P. aeruginosa* FARP72 metabolites

Table 2. Identification of secondary metabolites of *P. aeruginosa* P72 by mass spectral data Mass spectral data obtained in the fourth fraction

m/z peak obtained	Compound name	References	Antimicrobial activity
260.27	Pyrrrolnitrin (with loss of H <sup>+</sup> ions) or 3-hydroxy tetra decanoic acid ([M+NH <sub>4</sub> ] <sup>+</sup> ).	Itoh <i>et al.</i> (1971)	+
288.32	Pyoluteorin ([M+NH <sub>4</sub> ] <sup>+</sup> )	Maurhofer (1992)	+
233.21	Pyocyanin ([M+Na] <sup>+</sup> )	*	+
244.25	3- hydroxy tetra decanoic acid	*	+
315.35	4-hydroxy-2-heptyl quinoline	Que <i>et al.</i> (2011)	+

Mass spectral data obtained in the second fraction

144.08	4-methyl quinazoline	**	+
207.12	Phenazine-1-carboxylic acid	Labows <i>et al.</i> (1980)	+
149.03	3, 5, 6 –trimethyl pyrazine-2- carbaldehyde	Brown <i>et al.</i> (1955)	+
122.91	2, 3, 5-trimethyl pyrazine	Charyulu <i>et al.</i> (2012)	+
393.48	1, 1-diphenyl-2 picryl- hydrazyl radical	Charyulu <i>et al.</i> (2012)	+

\*: <http://www.chemicalbook.com/Chemical Product Property EN CB2135375.html>

\*\* : <http://www.chemsynthesis.com/base/chemical-structure-8296.html>

to be 4-methyl quinazoline. Another peak recognised at  $m/z$  207.12 supposed to be a phenazine-1-carboxylic acid with loss of one water molecule (Labows *et al.*, 1980). A peak intensified at  $m/z$  149.03 was identified as 3, 5, 6-trimethyl pyrazine-2- carbaldehyde with loss of  $H^+$  ion. The two compounds routinely detected in the mass spectral peak analysis was 2, 3, 5-trimethyl pyrazine and 1, 1- diphenyl-2 picryl- hydrazyl radical giving  $m/z$  122.91 and 393.48, with loss of  $H^+$  ion (Charyulu *et al.*, 2012).

The partially purified fourth fraction as obtained from the ethyl acetate extract of *P. aeruginosa* FARP72 (Fig. 4 and Table 2) showed intensity of the  $m/z$  260.27 peak in mass spectrum, which may be due to the presence of pyrrolnitrin (with loss of  $H^+$  ions) or 3-hydroxy tetra decanoic acid ( $[M+NH_4]^+$ ). *P. aeruginosa* produced a peak having  $m/z$  value of 288.32 ( $[M+NH_4]^+$ ) and was identified as pyoluteorin. Pyocyanin and 3-hydroxy tetra decanoic acid were found in the same fraction having the intensity of  $m/z$  233.21 ( $[M+Na]^+$ ) and 244.25, respectively. Along with other peaks, another peak was obtained at  $m/z$  315.35 due to the presence of 4-hydroxy-2-heptayl quinoline (Que *et al.*, 2011).<sup>+</sup>

## Discussion

The *in-vitro* inhibitory activity of the antagonistic bacterium *P. aeruginosa* FARP72 against 10 pathogenic bacteria belonging to the genera *Aeromonas*, *Bacillus*, *Edwardsiella*, *Escherichia*, *Klebsiella*, *Salmonella*, *Streptococcus* and *Vibrio* were evaluated. *Pseudomonas* spp. is the most common genera associated with crustaceans (Moriarty, 1997) and are common inhabitants of the aquatic environment (Otta *et al.*, 1999). They produce a wide range of secondary metabolites and used as biocontrol agents in several rhizosphere studies where their inhibitory activity has been attributed to a number of factors, such as the production of antibiotics, hydrogen cyanide or iron chelating siderophores (Raaijmakers *et al.*, 1999). Torrento and Torres (1996) reported the *in vitro* inhibition of *Vibrio harveyi* by a *Pseudomonas* species isolated from the aquatic environment. As with their terrestrial counterparts, aquatic pseudomonads are often antagonistic to other microorganisms (Gram, 1993), including fish pathogenic bacteria (Smith and Davey, 1993) and fish pathogenic fungi (Bly *et al.*, 1997). Chythanya *et al.* (2002) also reported inhibition of shrimp pathogenic vibrios by an estuarine strain of *Pseudomonas*, attributed to a low molecular weight, heat

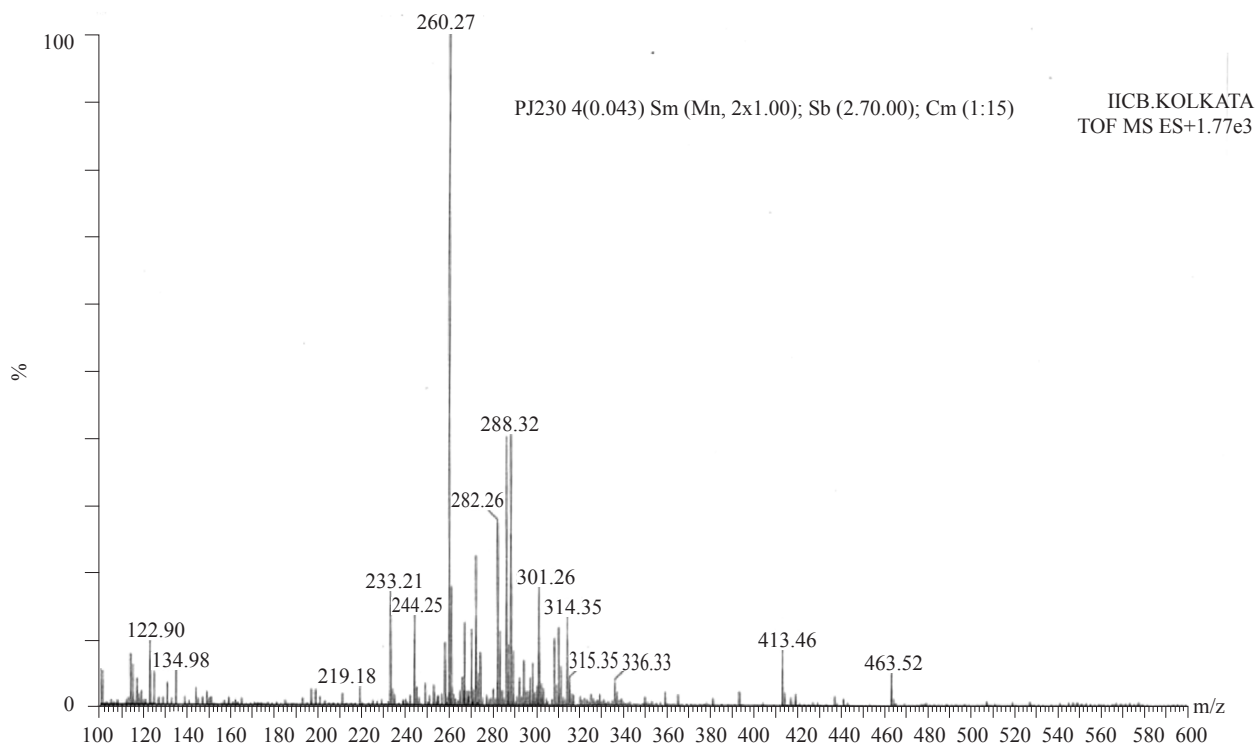


Fig. 4. Peaks obtained in Mass spectroscopy analysis of partially purified fourth fraction obtained by column chromatography of *P. aeruginosa* FARP72 metabolites

stable non-protein antimicrobial substance. The mode of action of *Pseudomonas* strains have been attributed to the production of several metabolites from different groups such as indoles (Kang *et al.*, 2006), phenazines (Cazorla *et al.*, 2006), pyocyanine, pyrrolnitrin (Ligon *et al.*, 2000), pyolu-teorin, acetyl phloroglucinols (Raaijmakers *et al.*, 1999; Guihen *et al.*, 2004), tenzin (Nielsen *et al.*, 2000), pseudotrienic acids A and B (Pohanka *et al.*, 2005) and viscosinamide (Nielsen *et al.*, 2000).

The inhibitory activity of *P. aeruginosa* FARP72 was more pronounced against Gram positive *S. aureus* ATCC 12598, followed by Gram negative *A. caviae*, *E. tarda*, Enterohaemorrhagic *E. coli*, *E. coli*, *S. Typhi* and *V. cholerae*. Results of earlier studies reported the dominance of Gram positive antagonistic bacteria in exhibiting broad spectrum action on Gram negative bacteria (Jack *et al.*, 2005; Abraham and Banerjee, 2007). However, in the present study, it has been demonstrated that both Gram positive and Gram negative human and fish pathogens were inhibited by *P. aeruginosa* FARP72. This corroborated the findings of Kariminik and Baniasadi (2010), who demonstrated the *in vitro* antagonistic properties of six Gram positive soil bacteria against Gram negative and Gram positive pathogenic bacteria. Further, Giri *et al.* (2011) demonstrated antagonistic activity of three potential probiotic strains (*Lactobacillus plantarum* VSG3, *P. aeruginosa* VSG2 and *B. subtilis* VSG1) isolated from the intestine of *Labeo rohita* using well diffusion assay against *A. hydrophila*.

The crude ethyl acetate extracts of the whole cells and spent medium exhibited inhibitory activity on all tested bacterial strains at varying levels (Table 3.1). It can be inferred from the results that the antagonistic principle of *P. aeruginosa* FARP72 was of lipid origin. Comparable results of whole cells and spent medium extracts, as presented in Table 3.1, indicate only minor variations between the antimicrobial activities of crude whole cells and spent medium extracts. Ethyl acetate extract of the spent medium, which contains lipid molecules released into the medium, has given larger zone size compared to the whole cells (lipid molecules bound to the cell wall) extract against ten pathogenic test strains. The response of bacteria in terms of susceptibility to tested extracts and isolated compounds may vary among the strains due to genetic differences between the strains (Tamokou *et al.*, 2012). However, in the present study, inhibition was noted irrespective of group, genus and species. Of the two Gram positive strains, maximum growth inhibition by the crude extracts of *P. aeruginosa* FARP72 was seen in *S. aureus* ATCC 12598 followed by *B. pumilus*. The crude extracts of *P. aeruginosa* FARP72 from spent medium effected larger zone (6-12 mm) of inhibition against *V. cholerae*,

*S. Typhi* and *E. coli*. *P. aeruginosa* FARP72 also proved its inhibitory activity against  $\beta$ -haemolytic *Streptococcus* sp., isolated from diseased *O. niloticus* as well as against *A. caviae* and *E. tarda*. It effected the largest zone of inhibition (10-16 mm) against *A. caviae*. In all the cases, the ethyl acetate extract of the spent medium has shown larger zone of inhibition as compared to the whole cells extract.

Besides the production of antimicrobial lipids, *P. aeruginosa* might employ quorum-sensing-independent extracellular products (mainly polysaccharides) to disrupt established biofilms of pathogens (Qin *et al.*, 2009). *P. aeruginosa* reportedly produces more than 55 quinolones/quinolines and these possess significant antibiotic activity against Gram positive bacteria (Deziel *et al.*, 2004). Antimicrobial quinolines can be packaged into extracellular membrane vesicles to cause direct lysis of *Staphylococcus* (Mashburn and Whiteley, 2005). It has also been reported that *P. aeruginosa* can produce an organic compound: cis-2-decenoic acid, which is capable of inducing the dispersion of established biofilms and inhibiting biofilm development of many Gram positive and Gram negative bacteria (Davies and Marques, 2009). *E. coli*, *K. pneumoniae*, *S. aureus* and *Salmonella* have emerged to be the most problematic pathogens, with alarmingly high antibiotics resistance rates. Even with the most effective antibiotic against these pathogens, the resistance rates were detected (Tamokou *et al.*, 2012). All these pathogens were found to be sensitive to the crude ethyl acetate extracts of *P. aeruginosa* FARP72. The overall results of this study can be considered as promising in the perspective of new drugs discovery from a natural source when considering the medical importance of the tested bacterial pathogens.

The genetics and biochemistry of pseudomonads, especially of *P. aeruginosa* have been explored intensively (Holloway *et al.*, 1973). Apart from the pigments, most secondary metabolites produced by *P. aeruginosa* have been detected and investigated because of their antibiotic activity. The practical use of antibiotics from pseudomonads dates back to the period before the "antibiotic era." Emmerich and Low (1899) reported that the cell free culture fluid of *P. aeruginosa*, concentrated to one-tenth of its original volume, killed several kinds of bacteria. During the antibiotic era, approximately 50 different antibiotic substances from pseudomonads have been discovered (Berdy, 1974). Only two of these *viz.*, pyocyanin and pyrrolnitrin, have been produced on a commercial basis. Most of them exhibit antibiotic or phytotoxic activity. Most antibiotics isolated from *Pseudomonas* culture filtrates, namely phenazines, pyrrolnitrin-type antibiotics, pyo compounds and indole

derivatives, fall into the class of N-containing heterocycles and have been shown to originate from intermediates or end products of the aromatic amino acid biosynthetic pathway (Leisinger and Margraff, 1977). Another substantial class of *Pseudomonas* secondary products comprises unusual amino acids and peptides (Dege, 2011). In addition to these two major groups of secondary metabolites, some glycolipids, lipids and aliphatic compounds have been isolated from *Pseudomonas* cultures (Abdel-Mawgoud *et al.*, 2010). The peaks obtained by MS analysis of the present study were matched with already available literature and the probable compounds were ascertained by molecular weight analysis. The possibility of other compounds in the same peaks obtained in the second and fourth fractions, therefore, can not be ruled out.

The partially purified fourth fraction, obtained from the ethyl acetate extract of *P. aeruginosa* FARP72, in the present study produced a peak with  $m/z$  value of 288.32 ( $[M+NH_4]^+$ ), which may be pyoluteorin as per the descriptions of Maurhofer (1992). Pyoluteorin (Plt, 4,5-dichloropyrrol-2-yl 2,6-dihydroxyphenyl), a polyketide antibiotic was first identified and separated from *P. aeruginosa* in 1958 (Takeda, 1958). Pyoluteorin is an antibiotic toxic to *Pythium ultimum* and other plant pathogenic oomycetes and has been shown to be an important factor in the biological control of such pathogens (Maurhofer, 1992). Pyoluteorin functions as an inducer of its own production when present in a culture medium at an extremely low concentration (1.47 nm). In this respect, pyoluteorin can act as signalling molecules such as the A-factor of *Streptomyces griseus* (Horinouchi, 1999) or acyl homoserine lactones of *Vibrio fischeri* and *Serratia liquefaciens* (Eberl *et al.*, 1996), which influence cell physiology and gene expression. In addition to its autoregulatory role, pyoluteorin influenced the production of at least one other secondary metabolite, 2,4-diacetylphloroglucinol, which also acts as an antimicrobial and can be completely dissolved in organic solvents (Wang *et al.*, 2008).

The fourth fraction as obtained in the present study showed a peak with an intensity of  $m/z$  260.27 in MS, which is considered to be major and the most purified compound, may be due to the presence of 3-hydroxy tetra decanoic acid ( $[M+NH_4]^+$ ) as described by Itoh *et al.* (1971). They belong to the class of rhamnolipids and are glycolipidic biosurfactants produced by various bacterial species. This fatty acid has also been identified as a constituent of the lipopolysaccharide layer of the outer membrane of certain Gram negative bacteria, including pseudomonads (Battu and Reddy, 2009). The compound is excreted into the medium during the stationary phase of growth and the optimal conditions for its formation

from various carbon sources have been defined (Hauser and Karnovsky, 1957). They were initially found as exoproducts of *P. aeruginosa* (Abdel-Mawgoud *et al.*, 2010). Biosurfactants are attracting much attention because they represent ecological alternatives to their synthetic counterparts, they exhibit lower toxicity, potentially high activities and stability at extremes of temperature, pH and salinity. Most importantly, they are biodegradable, making them environmentally friendly, “green” chemicals. Especially, they were shown to be active against a large variety of bacteria, including both Gram negative and Gram positive species, though the latter such as *B. subtilis*, are in general much more susceptible to the toxic effect of rhamnolipids (Itoh *et al.*, 1971). Growth inhibition was also observed against *Rhodococcus erythropolis* and *B. cereus* (Arino *et al.*, 1998). Various combinations of 3-hydroxy tetra decanoic acid displayed antimicrobial activity against nearly all tested Gram positive species, including *Staphylococcus*, *Mycobacterium* and *Bacillus* and significant activity against a number of Gram negative species, with *Serratia marcescens*, *Enterobacter aerogenes* and *K. pneumoniae* as specially sensitive ones. They also found an excellent inhibitory activity against a range of fungal species, including the filamentous fungi *Chaetomium globosum*, *Aureobacidium pullulans* and *Gliocladium virens* and the phytopathogens *Botrytis cinerea* and *Rhizoctonia solanii*, but no significant effect on yeasts (Benincasa *et al.*, 2004).

Another peak was obtained in the fourth fraction at  $m/z$  315.35, which may denote the presence of 4-hydroxy-2-heptyl quinoline (Que *et al.*, 2011). 4-Hydroxy-2-alkylquinolines (HAQs), especially 3,4-dihydroxy-2-heptyl quinoline (*Pseudomonas* quinolone signal) and its precursor, 4-hydroxy-2-heptyl quinoline, are attracting much attention, mainly because of their role as signalling molecules in *P. aeruginosa*. These molecules possess biological activities that endow the producer with increased fitness to compete with other species, infect various hosts and colonise diverse ecological niches. 4-Hydroxy-2-alkylquinolines (HAQs), originally named “pyo compounds,” have been long known as a class of antimicrobials produced by the opportunistic bacterial pathogen *P. aeruginosa* (Coenye *et al.*, 2003). A group of substances closely related to the pyo compounds was detected during the course of studies aimed at identifying a streptomycin antagonist excreted by *P. aeruginosa* (Sureau *et al.*, 1948). The factor responsible for this antagonism was isolated and shown to consist of a mixture of 2-n heptyl-, 2-n nonyl- and 2-n undecyl-4-hydroxy quinoline N-oxides (Cornforth *et al.*, 1956). These compounds, which probably correspond to pyo II, antagonised the inhibition by streptomycin and di-hydro streptomycin of *B. subtilis* and *S. aureus* but not of Gram negative

bacteria. They were able to antagonise the action of 1,000 times their weight of di-hydro streptomycin and at higher concentrations inhibited the growth of Gram positive bacteria. Although the mode of action of the 2-alkyl-4-hydroxyquinoline N-oxides in antagonising the inhibitory action of di- hydro streptomycin has not been elucidated, it was observed that the compounds inhibited electron transport by cytochromes. This effect was assumed to be the reason for their inhibitory action on bacterial growth (Long *et al.*, 2003).

In the fourth fraction, pyocyanin and 3-hydroxy tetra decanoic acid as per the descriptions in the literature (<http://www.chemicalbook.com/Chemical Product Property EN CB2135375.html>) were found, which produced peaks with intensities of  $m/z$  233.21 ( $[M+Na]^+$ ) and 244.25, respectively. *In vivo* studies have shown that the growth of fungus was inhibited in the presence of pyocyanin (Kerr *et al.*, 1998). The fungicidal mechanism is the activation of NAD(P)H to induce a redox active cascade to produce reactive oxygen intermediates. This allows *P. aeruginosa* to have a competitive advantage and it may dominate over other microorganisms. The synthesis of pyocyanin is primarily controlled by the quorum sensing process in *P. aeruginosa* strains (Mavrodi *et al.*, 2012).

The second fraction of ethyl acetate extract of *P. aeruginosa* FARP72 also showed a peak with an intensity of  $m/z$  144.08 peak, which may be 4-methyl quinazoline (<http://www.chemsynthesis.com/base/chemical-structure-8296.html>). It has been reported that *P. aeruginosa* has a distinct metabolism, part of which results in the production of bacteria-specific volatile organic compounds, which have proved their efficacy as antimicrobials and might be used for diagnostic purposes (Bos *et al.*, 2013). Another peak was recognised at  $m/z$  207.12 supposed to be a phenazine-1-carboxylic acid with a loss of one water molecule (Labows *et al.*, 1980). Phenazines have been implicated in the biocontrol of soil-borne plant diseases by the introduction of pseudomonads and have proved their efficacy as strong antimicrobials, especially against plant pathogens like *Gaeumannomyces graminis* var. *tritici* (Mavrodi *et al.*, 2012). The mode of action of *Pseudomonas* may be attributed to the production of phenazine compounds like phenazine-1-carboxylic acid (PCA), pyocyanin, 1-hydroxy phenazine (1-HP) and phenazine-1-carboxamide (Byng *et al.*, 1979). Formation of phenazines by *P. aureofaciens* and *P. aeruginosa* may be accommodated in the pathway by assuming phenazine-1-carboxylate as the second major branch point (Hansford *et al.*, 1972). This key intermediate would act as the precursor of pyocyanine, 1-hydroxyphenazine and its 10-monoxide, aeruginosins A and B and 2- hydroxyphenazine and its 1-carboxylate. In the past few decades it has been proved

that (i) phenazines function effectively as electron shuttles to Fe(III) minerals (Hollstein and McCamey, 1973), which may aid in Fe(II) acquisition; (ii) phenazines help modulate intracellular redox homeostasis as oxidants for NADH and/or by affecting carbon flux through central metabolic pathways; (iii) phenazines are signalling molecules, influencing the expression of a limited set of genes during the transition from exponential growth into stationary phase; (iv) phenazines are produced in biofilms, as expected, given that phenazine biosynthesis had previously been shown to be up-regulated by quorum sensing and low oxygen tension; and (v) phenazines play an important role in biofilm development, dramatically affecting the morphology of multicellular communities. Other phenazine derivatives from *P. aeruginosa* are redox-active compounds that exert many of their effects by altering oxidative metabolism and/or increasing the formation of reactive oxygen species [ROS] (Hassan and Fridovich, 1980). The ROS, in turn, can alter the function and gene expression of pathogenic bacteria (Brown, 1994).

Another peak obtained in the second fraction intensified at  $m/z$  149.03 was identified as 3, 5, 6 - trimethyl pyrazine-2- carbaldehyde with a loss of  $H^+$  ion (Brown *et al.*, 1955). In fraction "B", of the present study, the two compounds that gave  $m/z$  122.91 and 393.48 with a loss of  $H^+$  ion were detected in the mass spectral peak analysis, which corresponds to 2, 3, 5-trimethyl pyrazine and 1,1-diphenyl-2 picryl- hydrazyl radical (Charyulu *et al.*, 2012). Pyrazines are widely distributed in nature, occur naturally and exhibit wide aromas. They are widely distributed in micro-organisms and act as antimicrobials (Beck *et al.*, 2003). The pyrazine derivatives exhibiting antibacterial activity have also been synthesised. The activity of pyrazine derivative compounds towards 25 strains each of anaerobic and aerobic bacteria has been established (Gobis *et al.*, 2006).

To summarise, the present study identified several antimicrobial compounds such as 4- methyl quinazoline; 3,5,6 -trimethyl pyrazine-2- carbaldehyde; 2,3,5-trimethyl pyrazine; 1,1- diphenyl-2 picryl- hydrazyl radical, phenazine-1-carboxylic acid, pyrrolnitrin, 4-hydroxy-2-heptayl quinoline, pyocyanin, pyoluteorin and 3- hydroxy tetra decanoic acid from the two partially purified fractions of the ethyl acetate extracts of *P. aeruginosa* FARP72. All these compounds belonged to glycolipid, lipid and aliphatic derivatives, which exhibited antimicrobial activity. Nevertheless, the role of proteinaceous, carbohydrates and other antimicrobial compounds in the observed inhibition of the test bacteria in the present study could not be ruled out. However, it will be promising to separate the compounds in pure forms to explore their biological and surface activities for a variety of applications, which

may unravel the biotechnological uses of these glycolipid biosurfactants, lipids and other aliphatic compounds either in pure form or in mixtures. Moreover, characterisation of the structure-activity relationship of these compounds might be useful for prediction of the behaviour of different natural rhamnolipids mixtures comprising one major congener among other minor congeners.

Antibiotic production by fish-borne *P. aeruginosa* FARP72 is recognised as an important feature in disease suppression. The evidence that secondary microbial metabolites play a key role in the biological control of certain human and fish pathogens provides a strong incentive to develop such compounds as biocontrol agents for commercial use. The use of microbial strains producing antibiotic metabolites in controlled environments offers the best possibility for disease management. The major challenges to be resolved prior to widespread commercial exploitation of biocontrol strains lies in the ability to predict more confidently the behaviour of such strains in the field. Fortunately, as interest in these organisms grows, more information on the genetics, physiology and ecology of metabolite production become available. Such data are of immense importance for the selection of wild type strains with desirable traits from nature and to provide a more rational framework for the choice of strains for use in microbial consortia.

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