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Full Length Research Paper

Organic metabolites produced by *Vibrio parahaemolyticus* strain An3 isolated from Goan mullet inhibit bacterial fish pathogens

Anju Pandey, Milind M. Naik and S. K. Dubey*

Department of Microbiology, Laboratory of Bacterial Genetics and Environmental Biotechnology, Goa University, Goa, India.

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Identification and action of several antibacterial metabolites produced by a fish pathogen *Vibrio parahaemolyticus* strain An3 from marine ecosystem of Goa has been demonstrated. Antibacterial activity of the crude cell extract of the test bacterium has been evaluated against indicator pathogenic bacterial strains such as *Acinetobacter* sp. An2, *Aeromonas hydrophila* strain An4, *Staphylococcus arlettae* strain An1 and *Alteromonas aurentia* strain SE3 by agar well diffusion method which clearly demonstrated comparatively more significant inhibitory effect on indicator bacteria as compared to several commonly used antibiotics. Gas chromatography mass spectrometry (GC-MS) analysis of crude cell extract of the test organism interestingly revealed presence of indole, phenyl acetic acid, n-(3-methyl-1, 2, 4-oxadiazol-5-yl) - 1- pyrrolidine carboximidamide, pyrrolopyrazines, tetramethyl pyrazine and other important phenolic compounds which may be responsible for antibacterial activity against indicator microorganisms tested. It has been clearly demonstrated that *V. parahaemolyticus* strain An3 produced several medically important organic metabolites during cultivation suggesting it as a potential candidate for production of several antibacterial metabolites to control pathogenic bacterial strains causing serious fish and human diseases.

Key words: Antibacterial, gas chromatography mass spectrometry, metabolites, pathogenic bacteria, well diffusion.

INTRODUCTION

There is an increasing demand of therapeutic drugs from diverse natural resources. After many years of extensive research, the importance of terrestrial bacteria as source of valuable bioactive compounds has been very well established and exploited. As a result, the ocean and metabolites of marine organisms including associated microorganisms have now become the main focus of drug discovery research (Finical, 1993).These studies are concerned with bacteria and fungi isolated from sea water, sediments, invertebrates and fish (Kelecom, 2002). Bacteria occurring in aquatic ecosystems may have the ability to inhibit the growth of other microorganisms by producing antimicrobial substances such as antibiotics and bacteriocins. Their inhibitory mechanisms include: (i) Production of antibiotics, bacteriocins, siderophores, lysozymes, and proteases and (ii) alteration of pH through production of organic acids (Jorquera et al., 1999).

Vibrio spp. are common inhabitants of aquatic environment and are found free living as well as associated with various marine organisms such as squids, shrimps, corals, fish, molluscs, seagrasses and sponges. Some species are found as symbionts in specialized luminous organs of marine fish and invertebrates, whereas a number of other species are well-known pathogens of humans or

*Corresponding author. E-mail: santoshdubey.gu@gmail.com. Tel: 091-832-6519359, 091-832-2217770, 091-9923568466. Fax: 091-832-2225201.

Abbreviations: GC-MS, Gas chromatography mass spectrometry; **PCR ,** polymerase chain reaction; **SYEP,** sea water based yeast extract peptone agar; **TCBS,** thiosulfate citrate bile salts sucrose; **TSI,** triple sugar iron; **ONPG,** o-nitrophenyl-β-d-galactopyranoside; **VP,** Voges-Proskauer; **BLAST,** basic local alignment search tool.

marine animals (Thompson et al., 2004). *Vibrio paraheamolyticus* is one of the leading pathogens causing fish and human diseases. Recently, several biologically active substances have been isolated from marine bacteria. There are many reports about antibacterial activity shown by marine bacteria; *Pseudomonas, Yersinia, Aeromonas, Brevibacterium, Bacillus* and *Alteromonas* (Gauthier and Breittmayer, 1979; Shiozawa et al., 1997; Jorquera et al., 1999; Khalil et al., 2006; Ahmed et al., 2008; Rahman et al., 2010). There are few reports on *vibrios* producing antimicrobial substances (Sugita et al., 1997; Long and Azam, 2001; Castro et al., 2002; Hjelm et al., 2004; Norhana and Darah, 2005). In our investigation we have reported organic metabolites of a fish pathogenic strain of *V. parahaemolyticus* which inhibited the growth of other bacterial fish pathogens. We have further characterized these metabolites by gas chromatography mass spectrometry (GC-MS).

MATERIAL AND METHODS

Isolation and screening of marine bacteria

Different marine fishes with visible symptoms of hemorrhage and lesions on their body parts were selected; infected regions such as mouth, fins and gills were washed under sterile conditions with sterile deionized double distilled water and swabbed with sterile cotton wool. Suspension of this swab was prepared in saline and used for isolation of pathogenic bacteria on nutrient agar plates by serial dilutions.

Morphological characterization

The colony characteristics of the selected bacterial isolate; size, shape, colour, margin, elevation, consistency and opacity were observed and recorded. The selected bacterial isolate was gram stained and observed under the light microscope at 100 x magnification to study the cell morphology.

Identification of test bacterium using biochemical tests

Hi-Media (India) kit for biochemical tests was used to tentatively identify this bacterial isolate and results were interpreted according to Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984).

Molecular identification

Further confirmation of genus and species of the test bacterium was done by 16S rDNA sequencing and NCBI–BLAST search (Altschul et al., 1990). The genomic DNA was extracted from the bacterial isolates and used as template for polymerase chain reaction (PCR) amplification of the 16S rDNA fragment (1400 bps) according to standard procedure (Sambrook et al., 1989). The following eubacterial primers were used for PCR amplification:

27 f (5'- AGAGTTTGATCCTGGCTCAG -3')
1492 r (5'- GGTTACCTTGTTACGACTT -3')

These primers were purchased from MWG Biotech India Pvt. Ltd., Bangalore, India.

Antibiotic susceptibility test

Overnight grown bacterial suspension (0.1 mL) of indicator pathogenic bacteria *Acinetobacter sp.* strain An2 (Accession no. FJ38695), *Aeromonas hydrophila* strain An4 (accession no. FJ386959), *Staphylococcus arlettae* strain An1 (accession no. FJ386956) and *Alteromonas aurentia* strain SE3 was spread plated on Mueller Hinton agar plates; octadiscs (OD-007 and 014 from Hi-Media, India) containing multiple antibiotics were carefully placed in the center of the agar plates and incubated at room temperature (27°C) for 24 h. Sensitivity of the individual indicator bacterial isolate to a particular antibiotic was determined according to the performance standards of antibiotic disc susceptibility test approved by National Committee for Clinical Laboratory Standards (Bauer et al., 1966).

Preparation of crude cell extract

Ethyl acetate extraction procedure was followed with a slight modification to extract antimicrobial metabolites from the test bacterium (Wratten et al., 1977). Test organism was grown on sea water based yeast extract peptone agar (SYEP) agar. After 48 h, agar along with the cells was cut into pieces and suspended into ethyl acetate to extract the antibacterial metabolites. Overnight suspendsion was decanted followed by centrifugation to get cell free solvent. Solvent was subjected to evaporation at 40°C for final recovery of crude extract (Ahmed et al., 2008). Simultaneously, 48 h old culture suspension of the test bacterium was centrifuged to get cell free supernatant.

Antimicrobial bioassay (agar well diffusion test)

In order to check the antibacterial activity of the cell extract, SYEP agar (1.2%) was poured in the plates, small wells of about 6 mm diameter were made in the agar plates and bottom of the wells were sealed by 0.7% molten SYEP agar (Abraham, 2004). 100 µl crude cell extract and cell free supernatant were poured in the wells separately and allowed to diffuse in the agar media for four hours. Different indicator bacterial strains were spread plated on separate SYEP agar plates. Ethyl acetate (100 µl) was used as a control to check its inhibitory effect.

GC-MS analysis of crude cell extract

Identification of the antibacterial metabolites was done by GC-MS analysis; injecting 1 µl of sample into a RTX-5 column (7 m x 0.32 mm) of GC-MS (model GC-MS-QP-2010 plus) from Shimadzu, Japan and Helium (3 ml/min) was used as a carrier gas. The following temperature gradient program was used: 75°C for 2 min followed by an increase from 75 to 175°C at a rate of 50°C per min and finally 7 min at 175°C. The m/z peaks representing mass to charge ratio characteristic of the antimicrobial fractions were compared with those in the mass spectrum library of the corresponding organic compounds.

RESULTS

Morphological and biochemical characterization of test bacterium

This bacterial isolate appeared as spherical (2 - 3 mm diameter), dark green colonies on thiosulfate citrate bile

Table 1. Comparison of antibacterial activity of *V. parahaemolyticus* strain An3 with commonly used antibiotics on indicator bacterial isolates.

Antibiotic (µg/ml)	Zone of clearance (diameter) of indicator bacteria			
	***Acinetobacter* sp. strain An2 (mm)**	***Aeromonas hydrophila* strain An4 (mm)**	***A. aurentia* strain *SE3* (mm)**	***S. arlettae* strain An1 (mm)**
Amicacin- (Ak) 10	7	7	3	0
Carbenicillin- (Cb) 100	5	5	4.5	3
Ciprofloxacin- (Cf)10	4	4	0	0
Co-Trimazine- (Cm) 25	9	9	6	6
Kanamycine– (K) 30	6	6	10	11
Nitrofurontoin- (Nf) 300	7	7	0	0
Streptomycin- (S) 10	14	14	6	10
Tetracycline- (T) 30	14	14	2	1
Ampicillin- (A) 10	0	0	14	0
Cephalothin- (Ch) 5	0	0	13	3
Colistin methane sulphonate- (Cl) 25	1	1	7	9
Gentamycin- (G) 10	7	7	0	0
Streptomycin- (S) 10	5	5	2	3.5
Sulphatriad- (SI) 200	7	7	6	3
Tetracycline- (T) 25	9	9	3	3
Co-Trimoxazol- (Co)25	12	12	7	7
Cell free supernatant	0	0	0	0
Bacterial crude extract	11	10	12	10
Ethyl acetate	0	0	0	0

salts sucrose (TCBS) agar. Biochemical tests revealed that this motile bacteria exhibited positive activity for oxidase, gelatinase, arginine dihydrolase and lysine decarboxylase enzymes, whereas negative activity for o-nitrophenyl-β-d-galactopyranoside (ONPG), Voges-Proskauer (VP) and urease enzyme. On triple sugar iron (TSI) slant, it showed alkaline slant and acidic butt. Based on specific biochemical and morphological characters and as per Bergey's manual of systematic bacteriology, this fish pathogenic bacterial strain was tentatively identified as *Vibrio* sp.

PCR amplification of ribosomal gene encoding 16S rDNA followed by DNA sequencing and Basic Local Alignment Search Tool (BLAST) search clearly confirmed the identity of this bacterial pathogen as *V. parahaemolyticus*. Subsequently, we designated this pathogenic isolate as *V. parahaemolyticus* strain AN3 (Accession No. FJ386958). We have also confirmed presence of tdh gene (amplicon size: 264 bp), characteristically encoding thermostable direct hemolysin in *V. parahaemolyticus* (Data not shown).

Antibiotic susceptibility test for the indicator bacteria

All indicator bacterial strains exhibited significant susceptibility to majority of common antibiotics tested and were also resistant to few antibiotics (Table 1).

Antibacterial activity of the crude cell extract of the test organism

Agar well diffusion experiment clearly demonstrated the antagonistic characteristic of crude cell extract of *V. parahaemolyticus* strain AN3 as manifested by growth inhibition of gram (-ve) indicator bacteria; *Acinetobacter* sp. strain An 2, *Aeromonas hydrophila* strain An4, *Alteromonas aurentia* strain SE 3, and gram (+ve) *S. arlettae* strain An1. Interestingly, ethyl acetate cell extract of *V. parahaemolyticus* strain AN3 caused remarkably wider inhibitory zones as compared to most of the common antibiotics tested. Interestingly, cell free supernatant as well as ethyl acetate (control) was unable to show any inhibitory effect on the growth of indicator bacteria. This clearly revealed that these antibacterial organic metabolites are present inside the cells of *V. parahaemolyticus* strain AN3 without being secreted out (Figures 1a, b, c, d and Table 1).

GC-MS analysis of the crude cell extract

GC-MS analysis of crude ethyl acetate cell extract of *V. parahaemolyticus* strain AN3 clearly revealed the presence of several important antimicrobial organic metabolites which included indole, phenyl acetic acid, N-(3-methyl-1,2,4-oxadiazol-5-yl)-1-pyrrolidine, carboximidamide,

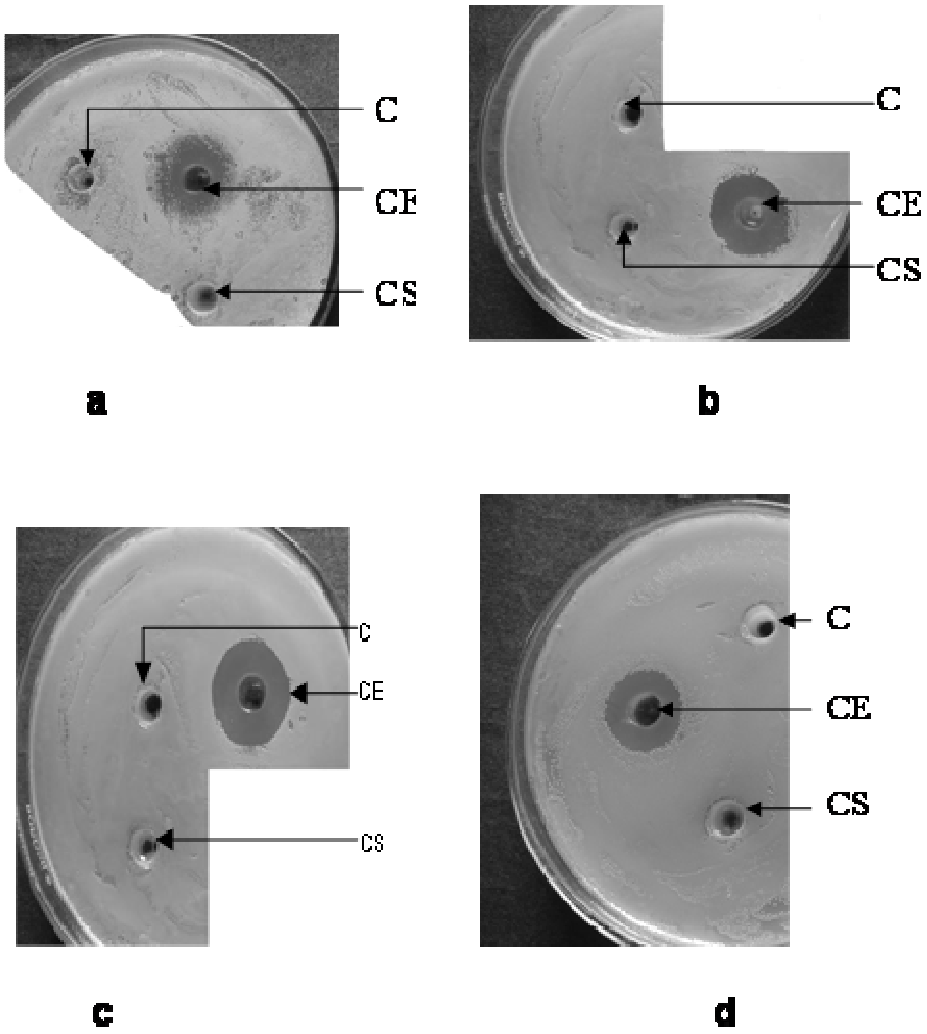

Figure 1. Antibacterial activity of the crude cell extract of the *V. parahaemolyticus* strain An3 on indicator bacteria. a. *S. arlettae* strain An1; b. *Acinetobacter* sp. strain An2, c. *A. hydrophila* strain An 4; d. *A. aurentia* strain SE3; C, Control (ethyl acetate); CE, cell extract; CS- culture supernatant.

pyrrolopyrazines, tetramethyl pyrazine and other phenolic compounds (Figures 2a - g and Table 2).

DISCUSSION

Marine microorganisms including bacteria, fungi and microalgae have received increasing attention during past few years due to increasing re-isolation of previously discovered compounds. Novel and unique structures of the secondary metabolites produced by marine organisms also have forced researchers to view the marine environment from different perspective and marine microorganisms as new biomedical sources. Until now, more extensive and focussed efforts to discover new antibiotics have involved the terrestrial environment mainly due to ease of availability, isolation and culture conditions. By the end of 2008, approximately 3000 microbial metabolites were reported from marine environment (Rahman et al., 2010).

GC-MS analysis of crude ethyl acetate cell extract of *V. parahaemolyticus* strain An3 demonstrated an interesting concoction of compounds with significant antimicrobial activity. A well known antagonistic compound has already been reported from *V. parahaemolyticus* as Vibrindole A, a bis-indole derivative (Bell et al., 1994) along with other indole derivatives (Kobayashi et al., 1994; Velury et al., 2003). In addition, other metabolites such as phenyl acetic acid, pyrrolidine carboximidamide, pyrrolopyrazines, tetramethyl pyrazine and phenolic compounds are also well known antimicrobials tested against different bacteria, fungi and other microbes (Kim et al., 2004; Somers et al., 2005; Chaudhary et al., 2006; Kumar et al., 2008; Farzaliev et al., 2009; Roy et al., 2010).

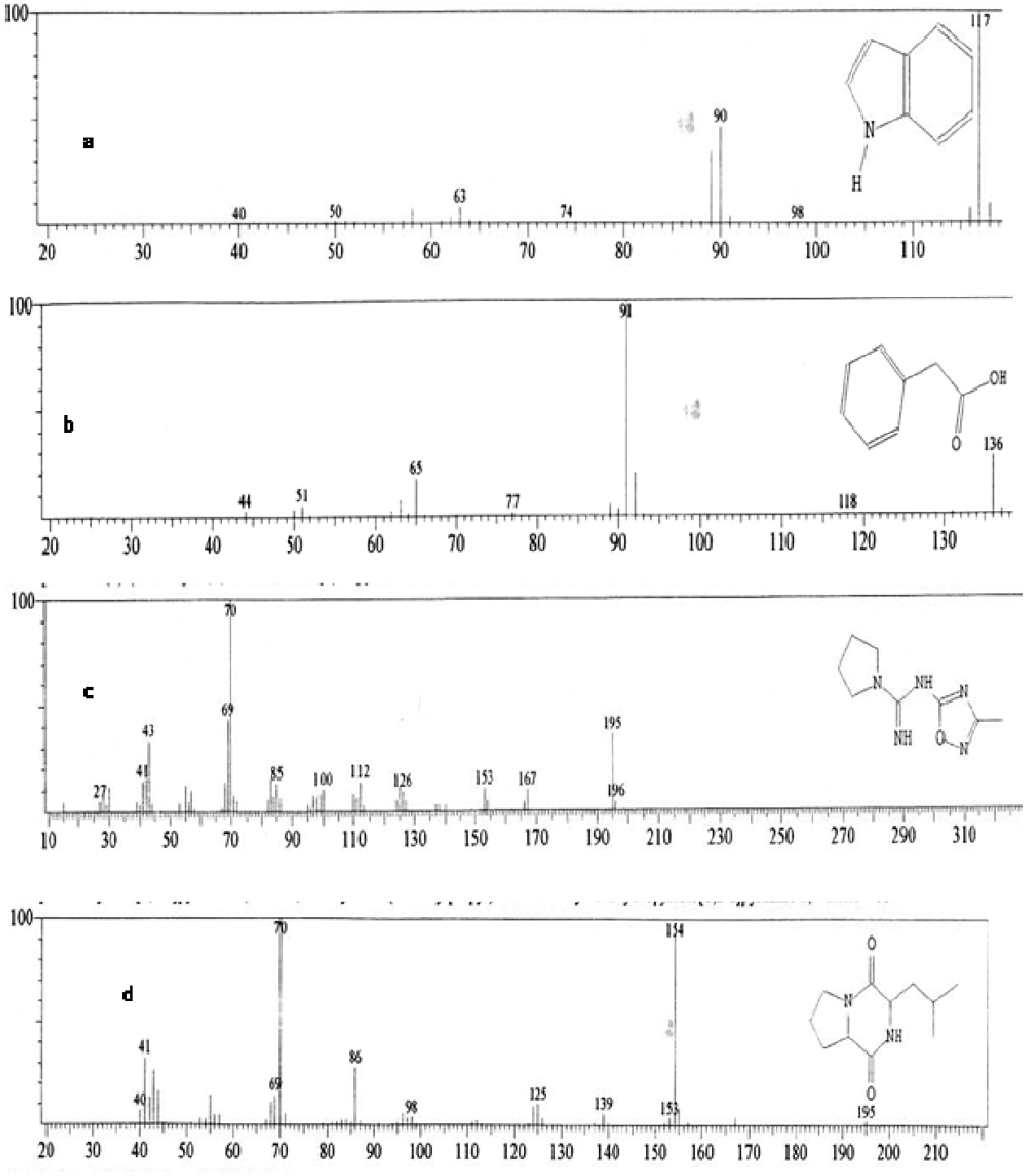

Figure 2. Structure and GC-MS spectrum of major antibacterial metabolites present in crude cell extract of *V. parahaemolyticus* strain An3: **a.** indole (Peak 117); **b.** phenyl acetic acid (Peak 91); **c.** N-(3-methyl-1,2,4-oxadiazol-5-yl)-1-pyrrolidinecarboximidamide (Peak 70); **d.** pyrrolo- (1,2-a)pyrazine-1,4- dione hexahydro-3(2-methylpropyl)(Peak 70); **e.** phenol, 4-(1,1,3,3-tetramethyl butyl) (Peak 135.2); **f.** nonyl-phenol (Peak 149); **g.** tetramethyl pyrazine (Peak 54).

Currently, the treatment of infections is compromised worldwide by the emergence of multi-drug resistant bacteria. In general, bacteria have the genetic ability to transmit and acquire resistance to drugs used as therapeutic agents. One way to prevent antibiotic resistance is by using new compounds which are not based on existing

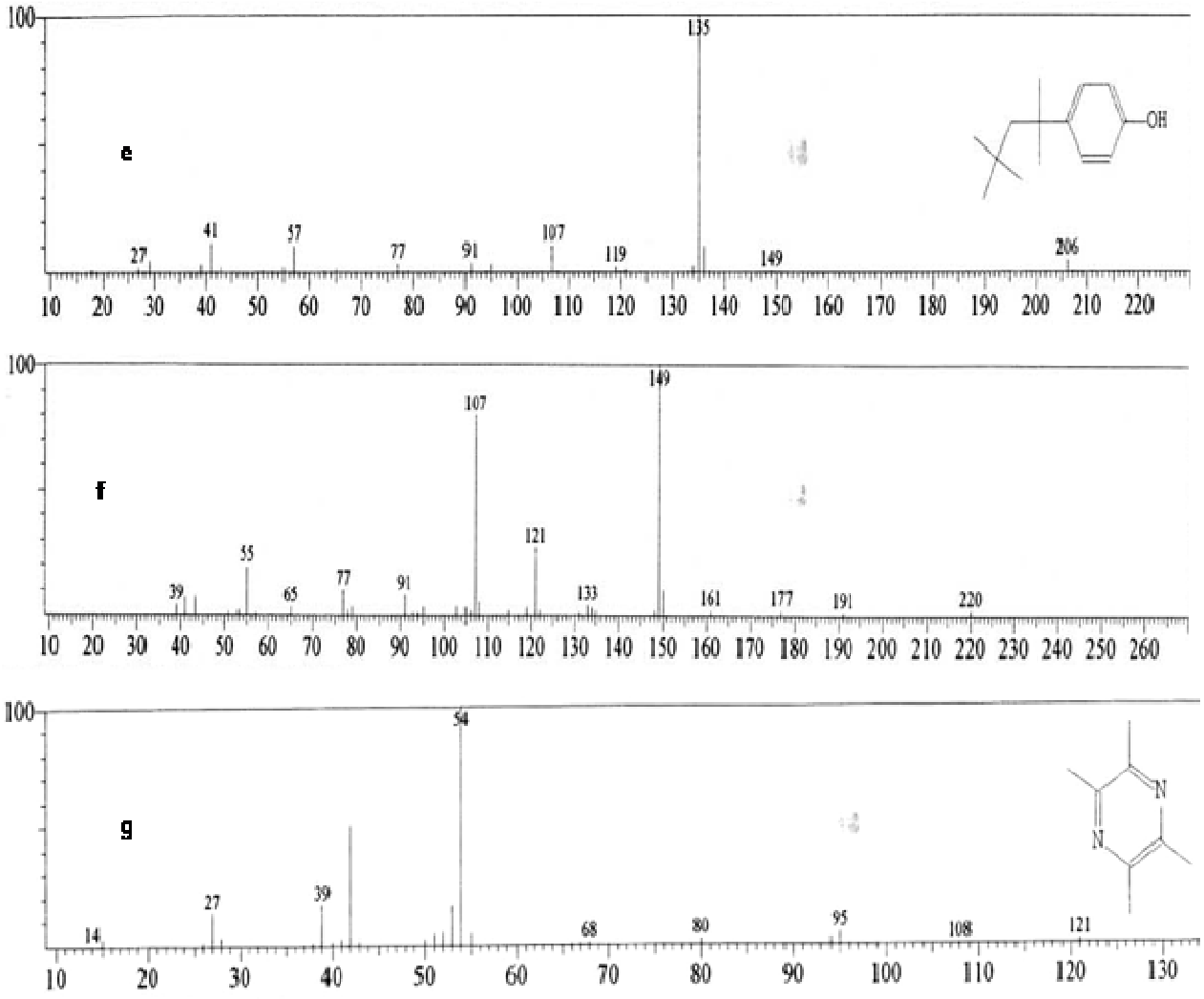

Figure 2. Contd.

Table 2. Major antibacterial metabolites present in ethyl acetate cell extract of *V. parahaemolyticus* strain An3.

Antibacterial metabolite	Retention time (mins)	% in test volume
Indole	9.842	3.0
Phenyl acetic acid	9.3	2.0
N-(3-methyl-1,2,4-oxadiazol-5-yl)-1-Pyrrolidinecarboximidamide	19.75	1.86
Pyrrolo-(1,2-a)pyrazine-1,4- dione, hexahydro-3(2-methylpropyl)	20. 033	1.3
Phenol, 4- (1,1,3,3-tetramethyl butyl)	15.425	0.76
Nonyl-phenol	16.492	0.6
Tetramethyl pyrazine	6.775	0.23

synthetic antimicrobial compounds. In the present investigation, we have tried to explore the production of novel antimicrobials in order to prevent antibiotic resistance in pathogenic bacteria.

Our primary interest should be practical management and control of infectious pathogens in newly developed aquaculture sites and human infections. It is interesting to note that even pathogenic strains can be used as biocontrol agents against other pathogens due to the inherent presence of antimicrobial compounds in the form of secondary metabolites. Thus antibacterial metabolites synthesized by the test organism may serve as source of

valuable antimicrobial drugs to control pathogenic bacterial strains causing fish and human diseases**.**

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