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Full Length Research Paper

Extraction and detection of quorum sensing N- acyl homoserine lactones from shrimp pathogen *Vibrio harveyi* and antagonistic effect of terrestrial plants against its growth

Sudalayandi Kannappan[1]*, Krishnamoorthy Sivakumar[1], Prasannakumar Patil[1] and Richard Kennedy R.N.[2]

[1]Genetics and Biotechnology Unit, Central Institute of Brackishwater Aquaculture (CIBA - ICAR), 75, Santhome High Road, Raja Annamalai Puram, Chennai - 600 028, Tamilnadu, India.
[2]Floriculture Research Station (Tamil Nadu Agricultural University), Thovalai - 623 302, Kannyakumari District, Tamilnadu, India.

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Vibrio harveyi **strains were isolated from the water samples of low salinity. Isolates were confirmed by both the presence of *vhh* gene using polymerase chain reaction and bio-chemical tests. *V. harveyi* was grown in LB medium and its spent culture was treated with ethyl acetate. The organic layer was pooled and dried by Roto - Vapour with vacuum at 30˚C. The dried residues were dissolved with 50 % acetonitrile and water. The presence of N-acyl homoserine lactones (AHL) compounds from the residues of crude extract and the standard "3 - Oxo hexanoyl homoserine lactone" were analyzed separately using Liquid chromatography and mass spectrometry (LC-MS). Different peaks were detected and peaks corresponding to 214.4 m/z were identified as N- (3-oxohexanoyl)-L-homoserine lactones in the sample. Different unknown peaks were also identified. Aqueous extracts of 47 terrestrial plants were evaluated against luminescence disease causing *V. harveyi,* through "Agar well diffusion assay". Ten plant extracts showed zone of inhibition (5 to 7 mm) on *V. harveyi*. Extracts from *Phyllanthus niruri* and *Cynodon dactylon* showed the highest zone of inhibition (8.3 mm) against *V. harveyi* followed by *Calotropis gigantean* and *Costus speciosus* (7.3 mm). Plant extracts were reported for the presence of alkanes, alkenes, aromatics, primary and secondary amines etc, by Fourier transform infra red spectroscopy (FT-IR). The antagonistic activity may be due to by the presence of these functional compounds in the extracts. Therefore, terrestrial plants could be used to control aquatic bacterial pathogens, which can also be extended to control human bacterial pathogens.**

Key words: *V. harveyi,* extraction of N-acyl homoserine lactones (AHL), analysis by Liquid chromatography and mass spectrometry (LC-MS), terrestrial plants, antagonism, Fourier transform infra red spectroscopy.

INTRODUCTION

Aquaculture continues to be the fastest-growing animal food producing sector. In shrimp grow-out practices, higher stocking densities always cause diseases by aquatic pathogenic bacteria, viruses and environmental contaminants

(Darshanee et al., 2011). Among the bacterial disease, vibriosis is one of the major disease problems in aquaculture (Christopher et al., 2010). *Vibrio harveyi* is causing bio-luminescence in both the shrimp larviculture and grow-out practices (Srinivasan and Ramasamy, 2009) that cause complete mortality especially among the postlarvae of shrimp hatcheries (Kannapiran et al., 2009). *V. harveyi* produce many virulence factors such as protease, phospholipase, haemolysin, cysteine protease, metallo protease, serine protease and chitinase (Defoirdt et al., 2010).

Many Gram-negative bacteria produce AHLs for a type of intercellular signaling known as quorum sensing, which is commonly associated with their pathogenic or communal behavior (Miller and Bassler, 2001). N- Acyl homoserine lactones (AHL) are one of the important signal molecules for *V. harveyi's* cell to cell communication. AHL can vary in their chain length from C4 to C18. In addition, AHLs can have different degrees of unsaturation at the C-7 or C-8 position, as well as oxidation at the 3rd position (Marketon et al., 2002). There are many antibiotics used to control bio-luminescence causing *V. harveyi* but they are ineffective due to microbial resistance developed among the bacteria. Therefore an alternative bio preservative agent has to be explored from bio resources. The quest for plants with medicinal properties continues to receive attention by scientists, particularly of ethno botanical significance for a complete range of biological activities (Ganthiraja et al., 2009). Several plants and herb species has potential for antimicrobial and antiviral properties (Zaika, 1988) and this has risen about the future of phyto-antimicrobial agents (Das et al., 1999).

According to World Health Organization (WHO), more than 80% of the world's population rely on traditional medicine for their primary healthcare needs and the outlook for the use of antimicrobial drugs in the future is still uncertain (Muruganantham et al., 2009; Anago et al., 2011). The problem of microbial resistance is growing consequently; measures must be taken to develop research for better understanding about the mechanisms of resistance. Plants generally produce many secondary metabolites which constitute an important source of inhibiting many pathogens. Identification and characterization of secondary metabolites is very important. In this regard, the present study was made an attempt to find out the possible antibacterial agents from indigenous terrestrial plants against shrimp pathogen *V.harveyi* from southern India.

MATERIALS AND METHODS

Isolation of *V. harveyi*

Water samples of less salinity (3 to 17 PSU, Practical salinity Unit) were collected from the Muttukadu Experimental Station of CIBA (ICAR), Chennai, India. The samples were pre-enriched in alkaline peptone water (APW) and serially diluted with normal saline (0.85 % NaCl, w/v) and 0.1 ml of enriched inoculums was surface spread on Thiosulphate citrate bile salts sucrose agar medium (TCBS), Seawater complex agar (SWC) and *V. harveyi* selective agar medium (VHSA) separately. In VHSA, bio-luminous colonies were observed after 20 h of incubation at 30°C. Then, the colonies were repeatedly streaked on VHSA (Harris et al., 1996). The isolates were again confirmed by various bio-chemical tests such as arginine dihydrolase (-), lysine (+), ornithine decarboxylase (+), gelatinase (+), Voges proskauer (-) and D-glucosamine (-), etc (Abraham and Palaniappan, 2004).

PCR assay for *vhh* gene

The DNA was extracted from wild *V. harveyi* strains (isolated from seawater) and used for PCR. DNA was isolated from type strain *V. harveyi* (MTCC 3438) by cetyl trimethyl ammonium bromide (CTAB) method and used as positive control. Then, these isolates were confirmed for the presence of *vhh* gene (*V. harveyi* hemolysin) using primer designed by Darshanee et al. (2011). The following combination of master mix was used: distilled water (20.85 µl); DMSO (1.25 µl), dNTPs (0.6 µl); Taq polymerase (0.3 µl); Primer: 0.8 to 1 µl (each); buffer (3 µl). PCR reaction conditions were followed as cycles: 30; 94ºC/1min; 55ºC/1 min; 72ºC/1 min; 72ºC/10 min. DNA Ladder (100 bp) was used as molecular marker. 1% agarose gel was used for visualization of DNA bands. 10 µl of PCR product was loaded to each well and electrophoresis was done at 80 v and visualized in gel documentation unit.

Preparation of plant extract

The crude bioactive compounds from plants were extracted based on the methods described by Srinivasan et al. (2009) with slight modifications. A total of 47 terrestrial plants were collected from Kanyakumari district of Tamilnadu.

The plants were washed in 1% $KMnO_4$ (w/v) to remove the epiphytes, sand and other extraneous matters. Later, the plants were shadow dried at room temperature, then pulverized using sterile pestle and mortar after words stored at -20ºC till further use. One g of the powder was mixed with 10 ml of sterile distilled water and shaker incubated at 250 rpm for 12 h/28ºC. Then the extracts were filtered through Whatman No. 1 filter paper, the volume was minimized to 1 % and then oven dried at 30°C/ 2 days. Later the extracts were neutralized to pH 7.0 with 0.1 N NaOH.

The extracts were tested for their zone of inhibition against *V. harveyi* (Doughari and Manzara, 2008) and stored at 4°C for further use.

*Corresponding author. E-mail: sudalikanna@gmail.com. Tel: + 91- 44 - 24616948, + 91 – 9677039103. Fax: + 91- 44 – 2461031.

Abbreviations: AHL, N-acyl homoserine lactones; **TCBS,** thiosulphate citrate bile salts sucrose agar; **VHSA,** *Vibrio harveyi* selective agar; **LCMS,** liquid chromatography and mass spectrometry; **PSU,** practical salinity Unit; **FTIR,** fourier transform infra red spectroscopy; **TIC,** total ion current; **RT,** retention time; **SWC,** seawater complex agar.

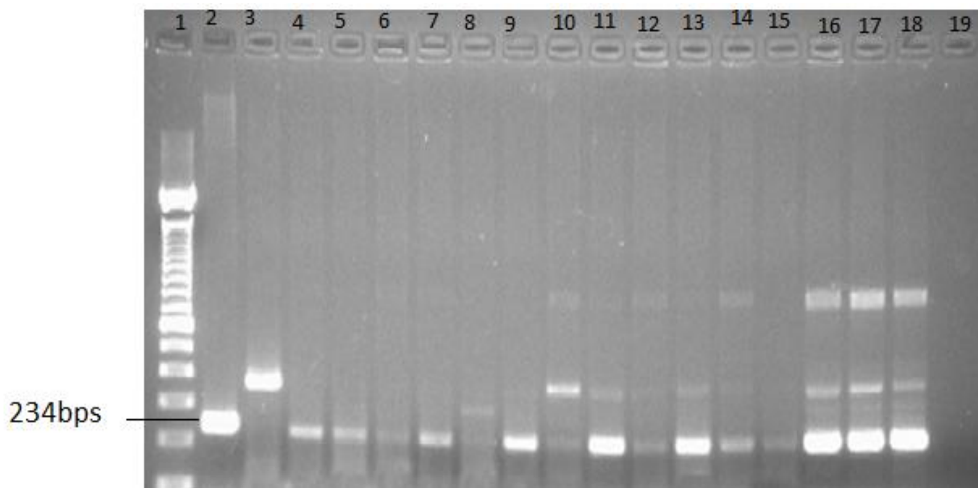

Figure 1a. Identification of *V. harveyi* isolates by the presence of *V. harveyi* hemolysin gene. 1, DNA ladder (100 bp); 2, 4 to 7, 9 to 15, *V. harveyi* sample*;* 16 to 18*, V. harveyi* MTCC 3438 control.

Antagonism of plant extract against *V. harveyi* by well diffusion method

V. harveyi strain was prepared by transferring 25 µl from the stock cultures (-80°C) to 25 ml of Luria Bertani (LB) broth and then shaker incubated at 120 rpm for 24 h (OD 1.80). LB molten agar was prepared and mixed with *V. harveyi* (10^9cfu / ml) and later, 30 µl was transferred into sterile Petri plates. The medium was allowed to solidify. 8 mm size wells were made into the agar plates using sterile pipette tips (Ravi kumar et al., 2011). The bottom of the well was sealed with soft agar (0.8 %). Plant extracts (300 µl) were filled in the well and the plates were refrigerated for 2 h and then incubated for 24 h at 37°C. The zones of inhibitions were measured (Rossi et al., 2011).

Extraction of N-Acyl homoserine lactone (AHL) compounds

1 ml of *V. harveyi* was taken as an initial inoculum and added to 1000 ml of LB broth and kept in a shaker incubator (100 rpm, 28˚C) for 24 h or until OD reached 1.80. Then, the whole inoculum was aseptically transferred into a sterile centrifuge tubes and centrifuged at 10,000 rpm for 15 min and cell pellets were discarded. The supernatant was filtered through 0.2 µm membrane filter to remove the cell debris. Later, 600 ml of filtrate was mixed with 300 ml of ethyl acetate (2: 1 ratio) and shaker incubated for 10 min. Then, the mixture was allowed to stand for 5 min in a separating funnel to get two immiscible layers (organic layer and aqueous layer). The upper layer was organic layer and the bottom layer was aqueous layer. The organic layer was collected in a sterile container and the remaining aqueous layer was extracted twice as described above. The entire organic layer was pooled and dried by Roto - vapour instrument with a help of vacuum at 30°C. The dried residues were dissolved with 50 % of acetonitrile and water, mixed thoroughly and stored at -20°C for further analysis using LC-MS (Ty A Gould et al., 2006). AHL compound 3 - Oxo hexanoyl homoserine lactone (3-Oxo HSL) was procured from sigma and used as reference standard.

Liquid chromatography mass spectrometry (LC-MS)

LCMS was performed by “AB Sciex 3200 Q TRAP Linear Ion trap quadrupole” (Liquid chromatography and Mass Spectrometry). The parameters were ion spray voltage of 5500 V, probe temperature of 500°C, curtain gas of 25 units, Gas source of 1 of 40 units to 2 of 40 units, medium units of CAD gas, De-clustering potential of 25 V, entrance potential of 6.50 V, collision energy potential of 16.86 V and collision exit potential of 2V. Nitrogen was used as the collision gas. Multiple reaction monitoring experiments were conducted using the same.

Fourier transform infra red spectroscopy (FTIR)

A drop of the plant extract was placed on one of the KBr plates. Then, the second plate was placed on top and a quarter turn was made to obtain a nice even film (Nicolet Impact 400 FT-IR Spectrophotometer). The plates were placed into the sample holder and the spectrum was run (Muruganantham et al., 2009) and various functional compounds were detected from this plant extract.

RESULTS

100 *Vibrio* strains were isolated from low saline water and 26 of them were identified as *V. harveyi,* following various biochemical tests and by the presence of vhh gene. The expected size was 234 bps (Figure 1a). The aqueous extracts of 47 terrestrial plants were tested against bioluminescence causing *V. harveyi.* Out of 47 plants, only 10 plant extracts (Table 1) showed zone of inhibition (5 to 7 mm). Among the plant extracts tested, *C. gigantea, P. niruri, L. inermis, L. camara* and *C. dactylon* was most inhibitory to *V. harveyi*. The extracts of *P. niruri* and *C. dactylon* showed the highest zone of inhibition (8.3 mm)

Table 1. Zone of inhibition produced by aqueous extracts of various terrestrial plants against *V. harveyi.*

Plant Specie	Diluent used	Zone of inhibition (mm)
Phyllanthus niruri	Water	8.33 ± 0.31
Calotropis gigantean	Water	7.33 ± 0.23
Lantana camara	Water	6.66 ± 0.13
Lawsonia inermis	Water	8.33 ± 0.29
Cynodon dactylon	Water	5.33 ± 0.21
Strychnos nux vomica	Water	4.3 ± 0.19
Acacia pennata	Water	5.33 ± 0.13
Polyscios flicifolia	Water	5.66 ± 0.21
Costus speciosus	Water	7.23 ± 0.21
Emblica Officinalis	Water	3.33 ± 0.12

Values are average of 5 determinations, represented in mm, zones are excluding the spots.

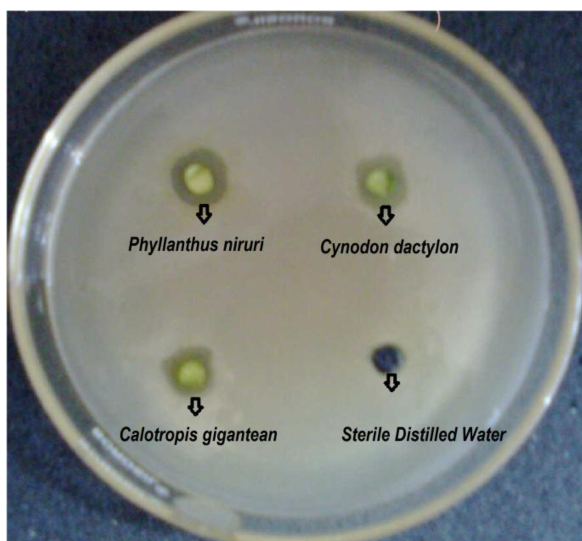

Figure 1b. Antagonism of various terrestrial plant extracts against *V. harveyi*

against *V. harveyi* followed by *C. gigantean* and *C. speciosus* (7.3 mm). The lowest zone of inhibition was showed by aqueous extract of *E. officinalis* (3.3 mm) and *Strychnos nux vomica* (4.3 mm) against *V. harveyi* (Figure 1b). The antagonistic activities of crude plant extracts are not due to their pH because the extracts were neutralized and then tested against *V. harveyi.* The plant extracts that showed inhibitory zones were subjected to analysis using Fourier Transform Infra red Spectroscopy (FT-IR). All the four plant extracts were found to have various functional groups of compounds such as primary, secondary and aliphatic amines, amides, alkanes, aliphatic amines, alkynes and alkyl halides as common except *C. gigantean*, which has particularly aromatic functional group of compounds (Table 2 and Figure 4). The presence of AHL com-pounds from the residues of crude extract and standard 3 - Oxo hexanoyl homoserine lactone (3 - Oxo HSL) (Sigma) were sepa-rately subjected for LC-MS analysis. Different peaks corresponding to each m/z reported were showed (Figures 2 and 3). The peak corresponding to 214.4 m/z (The *m* refers to the molecular or atomic mass and *z* to the number of elementary charges carried by the ion) was identified as N- (3-oxohexanoyl) -L- homoserine lactone (3 - Oxo HSL) in the sample. 3 Oxo- C8-HSL compounds were reported as 242.3 m/z and 3 Oxo- C9 HSL compounds were reported as 256.4 m/z. But other unknown peaks reported in the sample may be unknown compounds which were not compared with other AHL standards. The total ion current (TIC) chromatogram re-presents the summed intensity across the entire range of masses being detected at every point in the analysis. The range is typically several hundred mass-to-charge units or more. Here different peaks showed correspon-ding m/z ratio. The peaks were 214.4, 242.3, 256.4, 304.5, 332.5, 413.5, 429.5 and 463.5 m/z according to MS spectrum.

DISCUSSION

AHLs were extracted from the cell culture supernatant by organic solvents such as ethyl acetate (McClean et al., 1997). Alternatively, AHLs can be extracted with other solvent such as methylene chloride. In order to prevent lactone hydrolysis during subsequent storage, the crude extract was not acidified with the addition of acetic acid (Yates et al., 2002). Initially, samples and standards were analysed by SIM mode of LC-MS. The standard 3 - Oxo hexanoyl homoserine lactone was showed as peak with retention time (RT) of 5.19 min, correspondending to

Table 2. Various functional groups of compounds detected from the terrestrial plant extracts by FT-IR.

Plant extract	Frequency (cm^{-1})	Bond	Functional group
Phylanthus niruri	3402.1	N-H stretch	Primary, Secondary amines, amides
	2923.8	C-H stretch	Alkanes
	1635.5	N-H bend	Primary amines
	1249.7	C-N stretch	Aliphatic amines
	1041.4	C-N stretch	Aliphatic amines
	617	-C (triple bond) C-H: C-H bend	Alkynes
		C-Br stretch, C-Cl stretch	Alkyl halides
Calotropis gigantean	3402.1	N-H stretch	Primary, Secondary amines, amides
	2923.8	C-H stretch	Alkanes
	2376.1	Unknown	unknown
	1627.8	N-H bend	Primary amines
	1411.7	C-C stretch (in- ring)	Aromatics
		C-N stretch	
	1103.2	-C (triple bond) C-H: C-H bend	Aliphatic amines
	624		Alkynes
Lantana camara			Primary, Secondary amines, amides
	3409.8	N-H stretch	Alkanes
	2923.8	C-H stretch	Alkenes
	1643.2	-C=C- stretch	Aromatics
	1442.6	C-C stretch (in- ring)	
	1257.4	C-N stretch	Aromatic amines
	1064.6	C-N stretch	Aliphatic amines
	617	-C (triple bond) C-H: C-H bend	Alkynes
Lawsonia inermis	2923.8	C-H stretch	Alkanes
	1635.5	N-H bend	Primary amines
	1373.2	Unknown	Unknown
	1242	C-N stretch	Aliphatic amines
	1033.7	C-N stretch	Aliphatic amine
Cynodon dactylon	2923.8	C-H stretch	Alkanes
	1635.5	N-H bend	Primary amines
	1380.9	Unknown	Unknown
	1249.7	C-N stretch	Aliphatic amines
	1056.9	C-N stretch	Aliphatic amine

molecular mass of 236. The sample was crude, hence there is a possibility for the presence of other bacterial metabolites as well as fragments of AHL because of ionization. AHL compounds were produced by more than 70 bacteria but the structure difference is only in acyl chain length from C4 to C18. In addition, AHLs can have different degrees of unsaturation at the C-7 or C-8 position, as well as oxidation at the 3 position (Marketon et al., 2002). Ty A.Gould et al. (2006) also found that the peak corresponding to 214.4 m/z may be either 3- Oxo hexanoyl homoserine lactone moiety (3- Oxo C6 HSL) or N-heptanoyl homoserine lactone moiety (C7 HSL). Then the peak corresponding to 242.3 m/z may be either 3-Oxo octanoyl homoserine lactone moiety (3-Oxo C8 HSL) or C9 HSL moiety. The peak corresponding to 256.4 m/z may be either 3-Oxo C9 HSL moiety or decanoyl homoserine lactone (C10 HSL). Other peaks were not identified with using suitable standards. The abundant of these peaks were showed due to the absence of pretreatment of samples during extraction (Ty A. Gould et al., 2006) to remove unwanted metabolites for better detection of peaks (Figure 3).

Sample Name: "HMSL 100 PPB" Sample ID: "" File: "001.wiff"
Peak Name: "HS-236.0 / 192.0" Mass(es): "236.0/192.0 amu"
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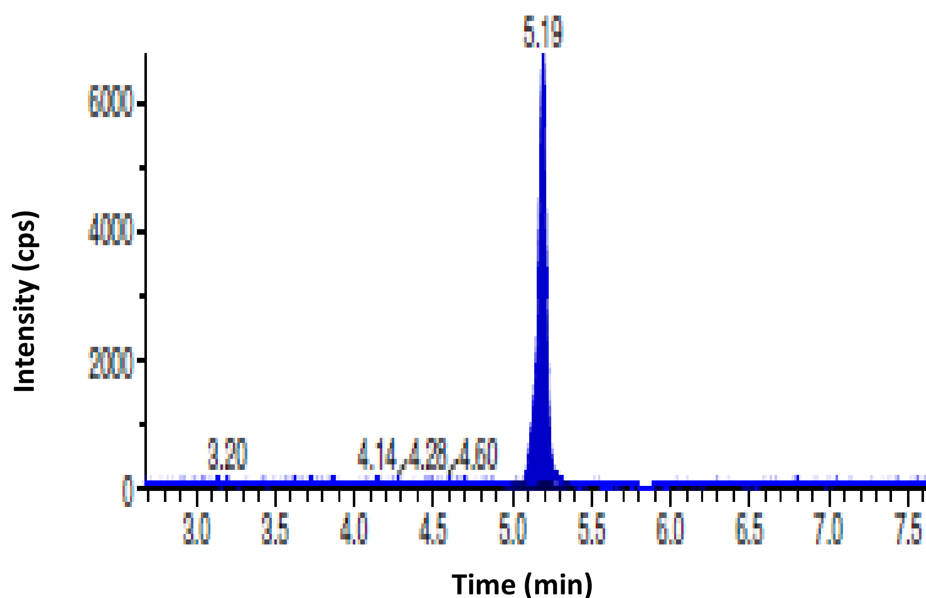

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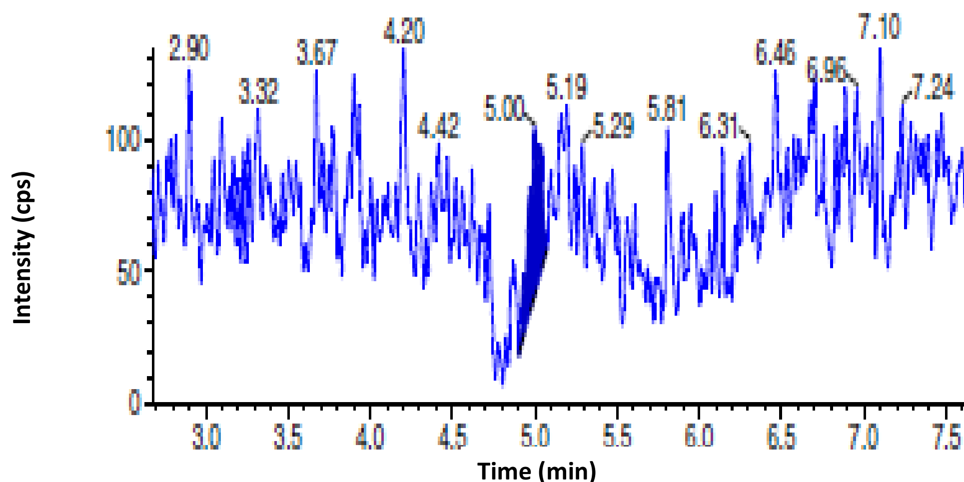


Figure 2. Standard, Determination of AHL compounds from the crude ethyl acetate treated spent culture extract of *V. harveyi* using LC-MS showing selected ion monitoring (SIM) mass chromatogram of standard. **Sample,** Acyl homoserine lactones showing selected ion monitoring (SIM) mass chromatogram from the crude ethyl acetate treated spent culture of *V. harveyi.*

+Q1: 0.000 to 1.404 min from Sample 9 (SCAN SAMPLE Q1) of L-HOMOSERINE-LACTONE.wiff (Turbo Spray) Max. 3.1e6 cps.

3-Oxo-C6-HSL
3-Oxo-C8-HSL
3-Oxo-C9 HSL
304.5
413.5
332.5
391.5
429.5
463.5
Intensity, cps
m/z, amu

Figure 3. Total ion current mass spectrum of crude ethyl acetate extract of spent culture from *V. harveyi.*

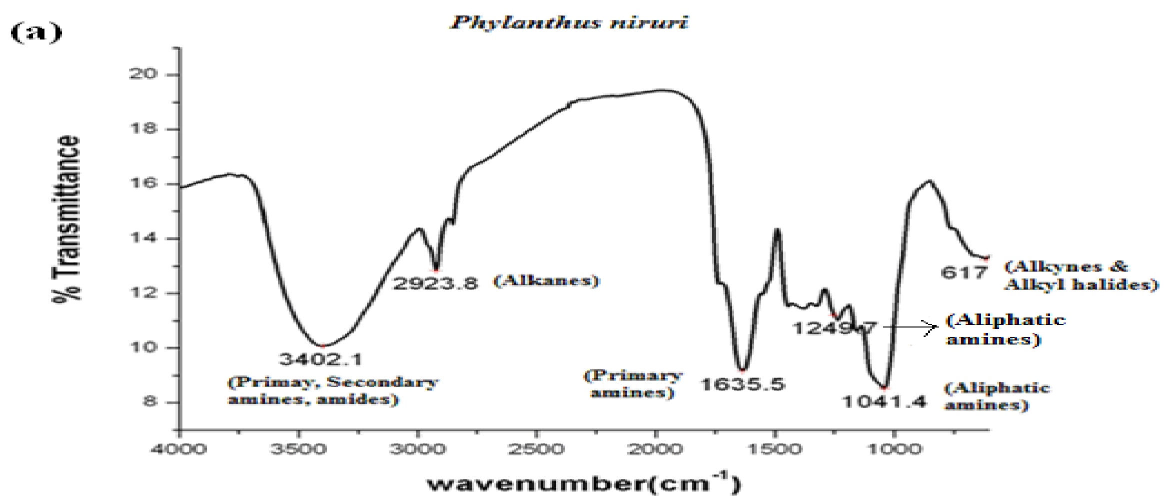
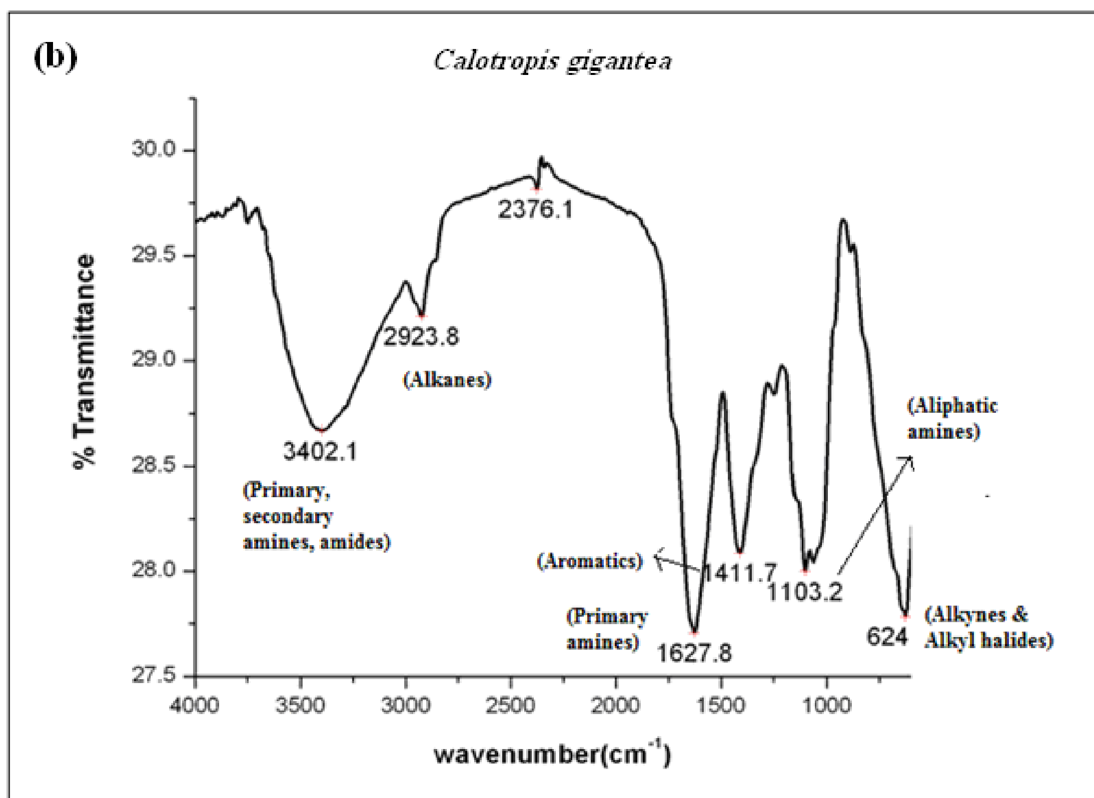
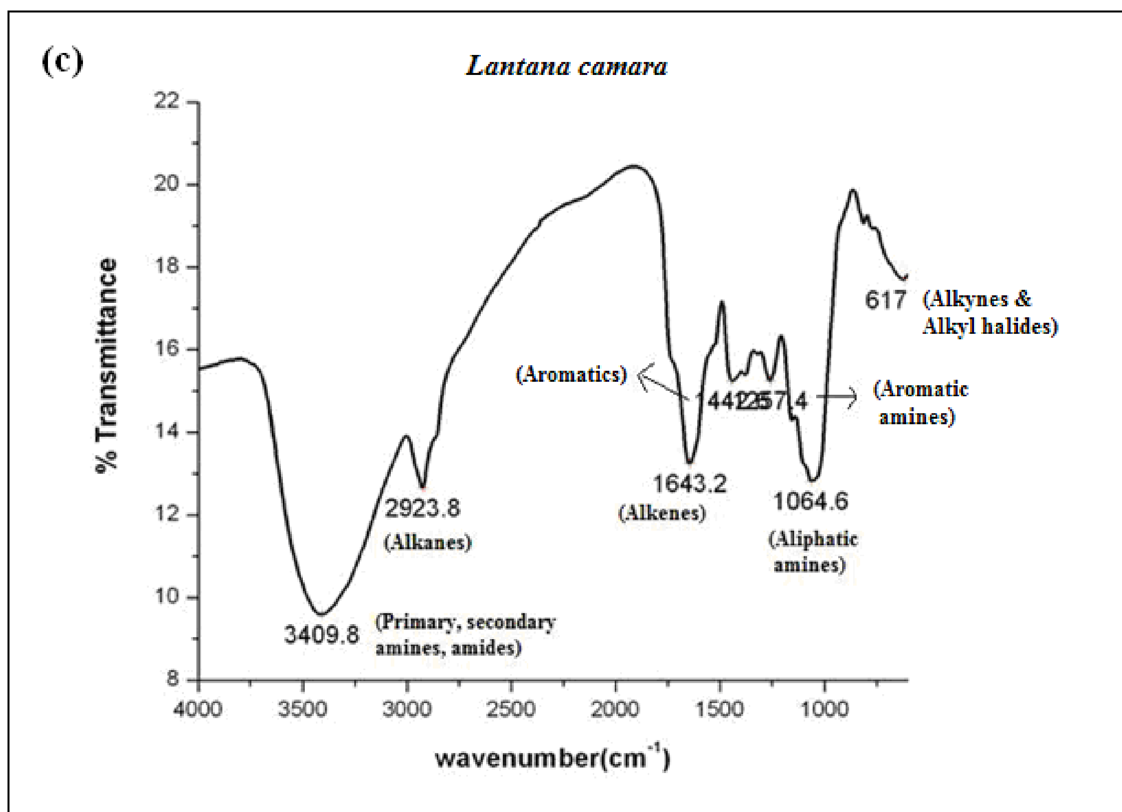


Figure 4. FT-IR Spectra of (a) *Phylanthus niruri* (b) *Calotropis gigantea* (c) *Lantana camara* (d) *Lawsonia inermis* and (e) *Cynodon dactylon.*


(b)
Calotropis gigantea
% Transmittance
wavenumber(cm⁻¹)
2376.1
2923.8
(Alkanes)
3402.1
(Primary, secondary amines, amides)
(Aliphatic amines)
(Aromatics)
1411.7
1103.2
(Primary amines)
1627.8
624
(Alkynes & Alkyl halides)


(c)
Lantana camara
% Transmittance
wavenumber(cm⁻¹)
617
(Alkynes & Alkyl halides)
(Aromatics)
(Aromatic amines)
2923.8
(Alkanes)
1643.2
(Alkenes)
1064.6
(Aliphatic amines)
3409.8
(Primary, secondary amines, amides)

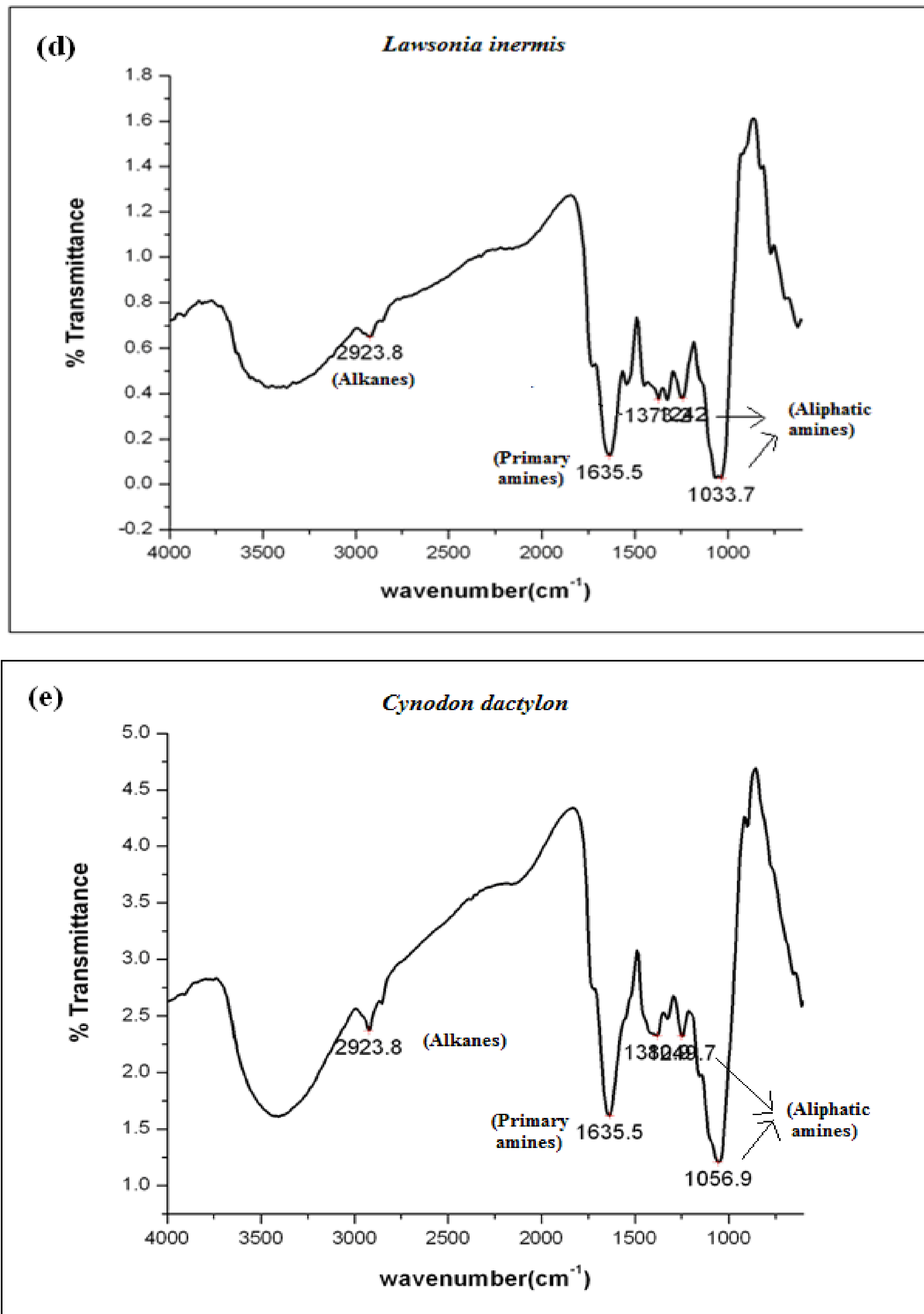


Figure 4. Contd.

This study confirms that terrestrial plants such as *P. niruri*, *C. gigantean*, *L. camara*, *L. inermis* and *C. dactylon* had antagonistic activity (3 to 8 mm) against *V. harveyi.* Sridevi et al. (2011) reported antibacterial activity of herbal extract against *V. harveyi.* Leaf extract of *R. mucronata* had *in vitro* antibacterial activities against multi resistant *Vibrio* spp (Baskaran and Mohan, 2012).

FT-IR results show a different range of active constituents like primary, secondary amines, amides, alkenes, and alkynes. These are the primary functional groups present in many plants as secondary metabolites; like alkaloids, terpenoids and phenolic compounds having potential activity. Among the five plants, *P. niruri* and *L. inermis* showed higher zone of inhibition on *V.harveyi*. Therefore, terrestrial plants could be used to control aquatic bacterial pathogens. This can also be extended to control human bacterial pathogens. Crude extracts of terrestrial plants such as *P. niruri*, *C. gigantean*, *L. camara*, *L. inermis* and *C.dactylon* showed antagonism against *V. harveyi* due to their various functional compounds. But the stability and changes of AHL compounds produced by *V. harveyi* due to treatment with crude plant extracts is still under investigation.

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