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Antagonism of marine macro alga *Kappaphycus alvarezii* extract against luminescence disease causing *Vibrio harveyi* during *Penaeus monodon* larviculture

Krishnamoorthy Sivakumar, Sudalayandi Kannappan*, Masilamani Dineshkumar and Prasanna Kumar Patil

Genetics and Biotechnology Unit, Central Institute of Brackishwater Aquaculture (Indian Council of Agricultural Research), 75, Santhome High Road, RajaAnnamalaiPuram, Chennai - 600 028, Tamilnadu, India.

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Bio-luminescence disease is caused by *Vibrio harveyi,* it is one of the major diseases in shrimp hatcheries and grow-out practices. *V. harveyi* produces virulence factors responsible for pathogenicity. The use of antibiotics as therapy for luminescence causes development of antibiotic resistance among the bacterial strains. Henceforth, it is indispensable to use bio-inhibitory agents as substitute for antibiotics from marine resources. Hence, in this study, marine macro alga *Kappaphycus alvarezii* was collected, shadow-dried, pulverized and crude compounds were extracted. This algae extract at 300 µg level gave zone of inhibition (8.6 mm) against *V. harveyi* through "agar well diffusion assay". The 300 µg treated with *V. harveyi* in LB broth and virulence factors produced were monitored for five days. Results show that in the control, higher level of lipolysis, thermonuclease, proteolysis, phospholipase were observed, in the treatment, moderate level was observed. The production of luminescence was reduced to 6.0, 7.0, 9.7 and 16.0 counts per second (CPS) observed by luminometer for 4 days when compared with the control (39.6, 50.3, 59.3 and 63.6 CPS). Further, the algae extract at 200 µg/ml was tested during larviculture for 30 days. The extract gave 29.70% reduction in the cumulative percentage of mortality. This extract was characterized by FTIR, GC-MS and detected various functional compounds like alcohols, phenols, alkenes, esters and ethers, etc. These compounds may be responsible for antagonism against *V. harveyi* and its virulence factors. This study confirms that the crude *K. alvarezii* extract may be a bio-inhibitory agent to control luminescence disease causing *V. harveyi.*

Key words: *Kappaphycus alvarezii* extract, antagonism, *Vibrio harveyi*, virulence factors, shrimp postlarvae, mortality reduction.

INTRODUCTION

Bacterial diseases have been reported to be a major restrictive issue in the production of both shrimp and its larvae in the grow-out practices. Among the *Vibrios*, the luminescence disease causing *Vibrio harveyi* is one of the most important pathogens, capable of causing heavy mortality among marine fish and penaeid shrimp (Vezzulli et al., 2010). In the past two decades, mass mortalities caused by *V. harveyi* infections were frequently reported (80-100%) in hatcheries and grow-out ponds (Zhou et al., 2012; Raissy et al., 2011). *V. harveyi* has been customary

*Corresponding author. E-mail: sudalikanna@gmail.com. Tel: +91- 44-24616948 or +91-9677039103. Fax: +91-44-24610311.

Figure 1. Marine macro alga *K. alvarezii* collected from the Eastcoast of India.

to produce extra-cellular products which indicate its pathogenicity due to virulence factors like biolumine-scence, proteases, phospholipases, lipases, sidero-phores, chitinases and hemolysin (Soto-Rodriguez et al., 2012). The uses of antimicrobial chemicals, especially antibiotics for controlling aquatic diseases, have led to the advent of additional virulence as well as resistance among bacterial pathogens (Rahman et al., 2010).

This problem has compelled exploring an alternative bio-agent from marine algae, which is effectively biodegradable and eco-friendly. Marine organisms had potential to represent an inexhaustible reservoir of bio-active compounds to be used in pharmaceuticals, medicine, food industries and cosmetics (Badea et al., 2009).

The chemical composition of macro algae were reported such as proteins with all essential amino acids, minerals and vitamins. The red alga *Kappaphycus alvarezii* is one of the economically important tropical seaweed, which is in high demand for carrageenan. The biochemical constituents such as carbohydrates, lipids, proteins, fat, amino acids, fatty acids, phenols and sterols were estimated from *Kappaphycus* sp. (Rajasulochana et al., 2012). Crude extracts and the polyphenol content of *Kappaphycus* sp. exhibited reducing power with hydroxyl radical scavenging activity higher than that of standard antioxidants (Holdt and Kraan, 2011).

The antibacterial activity of *Sargassum ilicifolium* and *K. alvarezii* was reported against animal borne bacterial pathogens (Rebecca et al., 2012). Bioactivity of diverse compounds extracted from seaweeds such as brown, red and green alga plays an imperative role in preventing diverse diseases and has antioxidant, antiviral and antimicrobial properties (Patra et al., 2009). The anti-microbial activities of marine macro algae are always associated with their secondary metabolites like terepe-noids, phlorotannins or phenolic lipids (Prabha et al., 2013). The antibacterial activity of seaweed *K. alvarezii* and *K. striatum* were tested against bacterial pathogens (Prasad et al., 2013).

Though, several studies confirmed the biological activity of marine alga, *K. alvarezii*, is not considerably studied against disease causing *V. harveyi*. Thus, the present study aimed to discover the antago-nism of crude marine macro algae, *K. alvarezii* extract, characterization of various photo-chemical by FTIR and GC-MS and test against luminescent disease causing *V. harveyi* during *Penaeus monodon* larviculture.

MATERIALS AND METHODS

Isolation of *V. harveyi*

V. harveyi strains were isolated from *P. monodon* larviculture tanks. The isolates were identified using standard morphological, physiological and biochemical tests and then confirmed by PCR (Sivakumar and Kannappan, 2013). The pathogenicity of *V. harveyi* isolates were confirmed for lysis of RBC by spotting in 3% sheep blood agar (Hi-media, India). The isolates were re-confirmed again by *V. harveyi* selective agar (VHSA) (Harris et al., 1996) and then stored in Luria-Bertani (LB) broth with sterile glycerol (15% v/v) (Hi-media, India).

Collection of macro alga

Macro alga *K. alvarezii* was collected (Figure 1) in the intertidal zone of Mandapam (Latitude 9.2886°N; Longitude 79.1329°E) region, Ramanathapuram District, Tamilnadu, India (Figure 2). *K. alvarezii* was washed in freshwater in 1% $KMnO_4$ (w/v) solution to remove the epiphytes, sand and other extraneous matter and then shadow dried. *K. alvarezii* was later pulverized using a mechanical grinder, weighed and used for extracting fatty acid extract.

Solvent extraction

Ethyl acetate solvent was used for extracting crude compounds from the alga at 30°C, called “cold extraction”. Algal extract was prepared by taking 1.0 g of alga powder and mixed with 10.0 ml of solvent and shaker incubated at 30°C for 96 h at 50 rpm. Then the extract was filtered by Whatman filter paper No. 1, rotary evaporated (30°C) under vacuum and stored at 4°C for further use. The subsequent extract was liquefied with 5 mg/ml of 30% (v/v) DMSO (Dimethyl Sulfoxide) and intended for antagonism against *V. harveyi* (Sivakumar and Kannappan, 2013).

Estimation of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) of crude extract, *K. alvarezii* was determined by Islam et al. (2008).

Antibacterial assay

Antibacterial activity of crude extract, *K. alvarezii* was ascertained against *V. harveyi* by the “agar well diffusion assay” (Sivakumar and Kannappan, 2013).

Figure 2. Map showing Mandapam region (Latitude 9.2886°N; Longitude 79.1329°E), India where macro alga *K. alvarezii* was collected.

Effect of crude *K. alvarezii* extract against the growth and virulence factors of *V. harveyi*

K. alvarezii extract (300 µg/ml) was added into 100 ml of LB medium. Active 24 h old *V. harveyi* of 500 µl (1.8 OD) was inoculated into LB medium and shaker incubated at 28°C/100 rpm/5 days. The growth (at 600 nm) with various virulence factors such as luminescence (counts per second-CPS), proteolytic, lipolyitc, phospholipase, thermonuclease, crude bacteriocin (660 nm), exopolysaccharide (520 nm) and protease produced (440 nm) by *V. harveyi* were measured. In plate assay the activity was measured based on the hydrolysis of medium and it was rated with the qualitative parameters like weak, moderate, high and very high (Table 1). Cell surface hydrophobicity was observed by salt aggregations test (SAT) and cell adhesion was observed by bacterial adhesion to hydrocarbons test (BATH) (Soto-Rodriguez et al., 2012). SAT test was determined as the lowest molarity of ammonium sulphate (0.05-4.0 M) that caused visible agglutination of a test organism. Each test was performed in triplicates and values were expressed in average of three determinations with SD.

Fourier transform infra red spectroscopy (FTIR) analysis

The shadow dried *K. alvarezii* was ground as fine powder using a pestle and mortar. The FTIR spectra was recorded using BRUKER IFS 66 model spectrometer in the region 4000 - 400 cm^{-1} by employing the standard KBr pellet technique (D'Souza et al., 2008).

Gas chromatography and mass spectrometry analysis

Gas chromatography-mass spectrometry (GC-MS) analysis was performed by using Agilent GC-MS-5975C with the triple-axis detector equipped with an auto sampler. The GC column used was fused silica capillary column (length 30 m x diameter 0.25 mm x film thickness 0.25 µm) used with helium at 1.51 ml for 1 min as a carrier gas. The mass spectrometer was operated in the electron impact (EI) mode at 70 eV in the scan range of 40-700m/z. The split ratio was adjusted to 1:10 and injection volume was 1 µl. The injector temperature was 250°C; oven temperature was 70°C/3 min, which rose to 250°C @14°C min^{-1} (total run time, 34 min). The temperature of the transfer line and of the ion source was set to a value of 230°C and the interface temperature at 240°C, respectively. Full mass data was recorded from 50-400 Dalton per second and scan speed was 2000. Mass start time was at 5 min and end time at 35 min. Peak identification of crude *K. alvarezii* extract was performed by comparison with retention times of standards and the mass spectra obtained was compared with those available in the NIST libraries (NIST 11- Mass Spectral Library 2011 version) with an acceptance criterion of a match above a critical factor of 80% (Musharraf et al., 2012).

Challenge of crude *K. alvarezii* extract against *V. harveyi* during larviculture of *P. monodon*

The plastic tubs were washed with 1% $KMNO_4$ solution and filled with 20 L of low saline water (@20 Practical salinity units-PSU). Disease free postlarvae (PL 10) of *P. monodon* procured from shrimp hatchery were acclimatized at 20 PSU for 5 days under laboratory conditions at 29 ± 1°C with continuous aeration. The average body weight of PL ranged from 16 to 17 mg and stocked @1000 numbers in each tubs. The control tub was inoculated with *V. harveyi* (10 ml of 1.80 OD) alone. The treatment tub was inoculated with *V. harveyi* and 200 µg (2g/10 L) of crude *K. alvarezii* extract. Third tub was considered as another control where crude *K. alvarezii* extract was added at 200 µg per ml with PL. The fourth tub was additional control for PL with neither *V. harveyi* nor extract were added. The aeration was given in each tub to provide oxygen level not more than 4 mg/L. The PL feed was given twice @15% of body weight. The water quality parameters such as temperature,

salinity and pH were determined once in five days. The mortality of PL was counted every day. No water exchange was given for all the tubs till 30 days. The water samples were collected once in five days by sterile water bottles. The total heterotrophic bacteria and *V. harveyi* counts were enumerated using selective medium. All the experimental tubs were top covered to avoid any external contaminations. For each experiment, the triplicate was maintained and the values are expressed as average of three determinations (Kannappan et al., 2013).

RESULTS

MIC of *K. alvarezii*

The MIC of *K. alvarezii* extract was established at 40 µg concentration. The extract showed a zone of inhibition (8.6 mm) at 300 µg level against *V. harveyi*. Whereas, 200 and 100 µg level of concentrations showed less zone of inhibition as 4.6 and 2.3 mm respectively.

Effect of *K. alvarezii* extract on the changes of growth and virulence factors produced by *V. harveyi*

The treatment reduced the growth of *V. harveyi* (OD) from 1st to 5th day. The highest OD difference was observed on 2nd day (0.184) and lowest was on 4th day (0.182) as compared to the control (Figure 3a). But, growth reduction on *V. harveyi* was noticed in all treatment days. The maximum reduction on bacteriocin production (OD) was observed on 4th and 5th days (0.172 and 0.174) and minimum (0.027) was observed on 2nd day as compared to the control (OD 1.746 and 1.744 on 4th and 5th and 1.647 on 2nd days). But, reductions on crude extra cellular protein was noticed in all the treatment days (Figure 3b). The production of exopolysaccharide (EPS) was reduced in the treatment as 0.261, 0.301, 0.304, 0.007 and 0.299 for 1st to 5th days as compare to control (OD 1.963, 2.391, 2.134, 1.851 and 1.813) (Figure 3c). The maximum reductions of protease level were as 0.054 and 0.080 in the treatment as compared to the control (OD 0.091 and 0.104 respectively) on 1st and 5th days (Figure 3d).

In the treatment, moderate levels of phospholipase, proteolysis, lipolysis and thermonuclease were noticed on 1st to 5th day as compared to the control (very high) activity. In SAT test, the control *V. harveyi* revealed strong hydrophobic activity for 1st to 5th day whereas, the treated one showed moderate hydrophobic activity for 1st to 5th day. Similar way, BATH assay also exhibit strong level of hydrophobic nature for control from 1st to 5th day. When crude extract of *K. alvarezii* was treated with *V. harveyi*, the production of luminescence was reduced to 6.0, 7.0, 9.7 and 16.0 CPS for four days as compared to the control (Figure 3e). The maximum reduction on luminescence was reported during the 4th day (16.0 CPS) and minimum reduction was found during the 1st day (6.0 CPS) when compared with the control (39.6, 50.3, 59.3, 63.6 CPS).

FTIR of *K. alvarezii*

The FTIR spectrum of *K. alvarezii* is shown in Figure 4. Various functional groups were identified and compared with standard Library data. FTIR spectrum showed the presence of some important functional groups such as alcohols, phenols, α- and β-unsaturated esters, α and β-unsaturated aldehyde, α and β-unsaturated ketones, esters, ethers, alkanes, alkenes, primary amines, nitro compounds, aromatics and carboxylic acids, alkyl halides, aliphatic amines, aldehydes etc. (Table 2).

GC-MS of *K. alvarezii*

GC-MS analysis on the crude ethyl acetate extract of *K. alvarezii*, was found to have a mixture of volatile compounds. Fatty acid methyl esters were investigated quantitatively by gas-chromatography-tandem mass spectrometry in multiplereactions monitoring mode and thus allows a better signal resolution without a preliminary fractionation of the extract. A total of 34 peaks were observed with retention times as shown in Figure 5. Chemical constituents were identified using spectrum data base NIST 11 software installed in GC-MS. The GC-MS analysis of *K. alvarezii* extract revealed that the main chemical-constituent was “n-hexadecanoic acid” (tR = 18.20, 18.87 min) (38.43, 9.90% respectively) and followed by 5-eicosene (tR = 18.52 min, 9.39%), heptadecane (tR = 15.46 min, 5.58%) and 1-octadecene (tR = 15.47 min, 3.18%) (Figure 6). It is possible that bioactive compounds primarily consisting of “n-Hexadecanoic acid”(tR = 18.20, 18.87 min) (38.43, 9.90%, respectively) may be involved in biological activity (Table 3).

Challenging of *K. alvarezii* extract against *V. harveyi* during larviculture of *P. monodon*

When *K. alvarezii* extract was challenged against *V. harveyi* during *P. monodon* larviculture for 30 days. The reduction of cumulative percentage of mortality on PL was noticed as 29.70% as compared to control (76.30%). Two trails were maintained as negative controls to distinguish if any influence of extract affects PL under larviculture. However, it was noticed that treatment does not affect the PL as compared to control (76.30%) which showed less reduction on cumulative percentage mortality with extract and PL (29.56%) and with PL alone (28.39%). The weight of the PL was measured for both control and treatments and not much weight difference was observed both in the treatment and control. On the 30th day, the average weight of the PL was 271.5 and 267.9 mg for control and treatment, respectively.

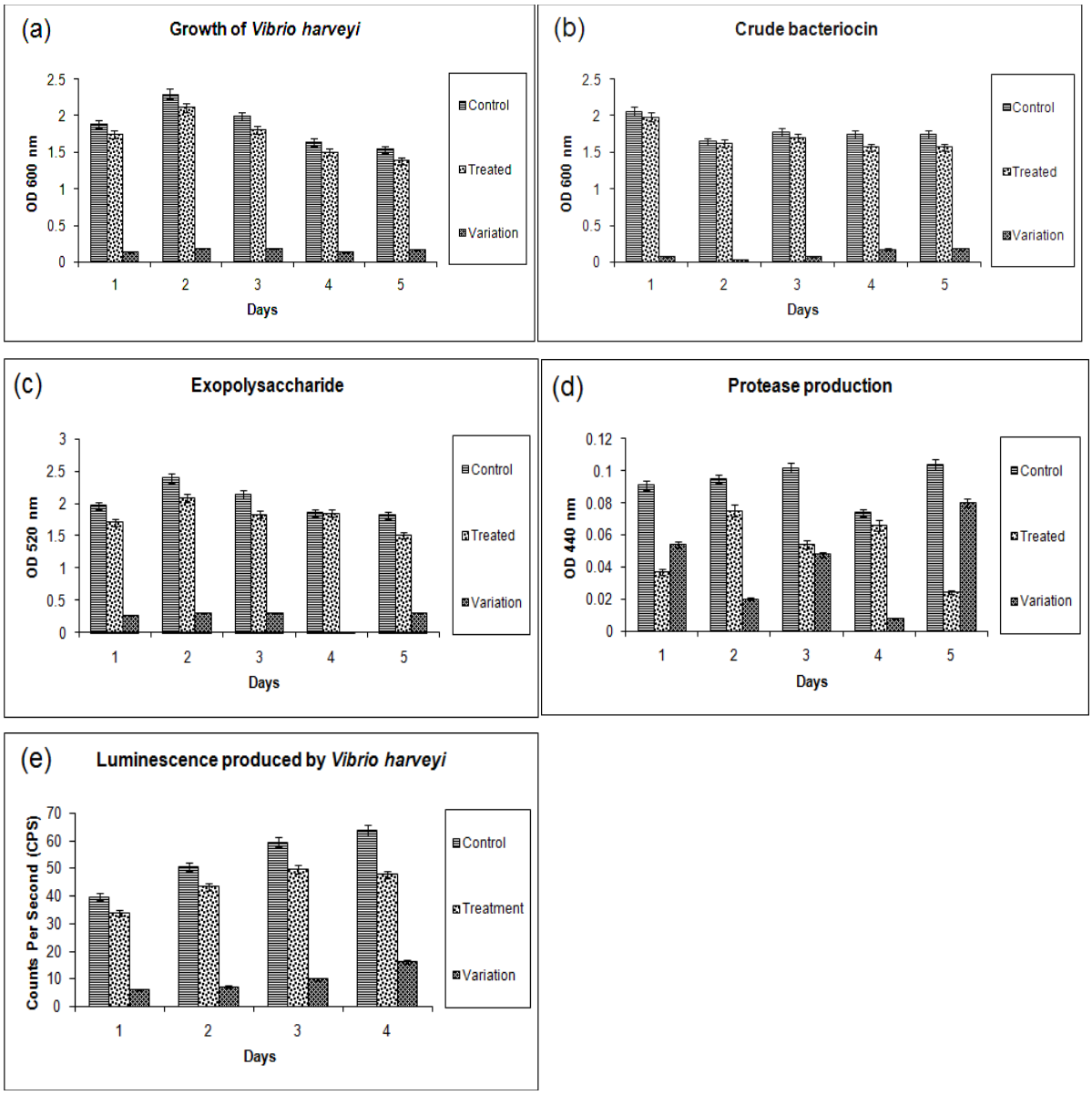


Figure 3. Crude extract of *K. alvarezii* against the changes of growth and virulence factors produced by *V. harveyi* in LB broth for five days.

The total heterotrophic and *V. harveyi* counts were observed for every sampling. Luminescent *V. harveyi* counts were observed in VHSA medium under darkroom. The maximum decrease on *V. harveyi* count was observed on 5th, 10th, 15th, 20th and 25th days, and the mean values for treatment were 6.55×10^4, 3.21×10^4, 3.15×10^3, 4.85×10^3 and 8.30×10^3 cfu/ml as compared to the control (1.17×10^5, 2.62×10^5, 7.40×10^4, 2.02×10^4 and 2.53×10^4 cfu/ml, respectively). Various water quality parameters like temperature, salinity and pH observed in every sampling were presented in Table 4. No much changes of water quality parameters both in treatment and control was observed. But, in the treatment, and with extract alone slight brownish color formation was noticed as compare to the control due to the unpurified nature of extract.

DISCUSSION

The FTIR analysis of *K. alvarezii* was observed for the presence of various functional groups of compounds like proteins, fatty acids, sterols and β-carotene. These compounds can be the active agents responsible for the antibacterial effect and may also serve as food product, because it contains rich proteins, cholesterol, β-carotene

Table 1. Effect of *K. alvarezii* extract on the changes of virulence factors produced by *V. harveyi.*

Day	Virulence factor											
	Proteolysis activity		Phospholipase activity		Lipolysis activity		Thermonuclease activity		Cell surface hydrophobicity			
									SAT (M)		BATH (%)	
	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated
1	++++	++	++++	++	++++	++	++++	++	0.51± 0.02	1.25± 0.04	97.54±4.21	48.14± 1.39
2	++++	++	++++	++	++++	++	++++	++	0.56± 0.02	1.33 ± 0.03	97.53±3.61	48.01 ± 2.16
3	++++	++	++++	++	++++	++	++++	++	0.62 ± 0.02	1.36 ± 0.05	96.31± 3.19	46.96± 1.84
4	++++	++	++++	++	++++	++	++++	++	0.78± 0.03	1.44± 0.04	95.73 ± 2.87	45.82± 1.71
5	++++	++	++++	++	++++	++	++++	++	1.00± 0.04	1.53 ± 0.06	95.15± 4.19	43.19± 1.91

Control, *V. harveyi* untreated with crude extract; treated, *V. harveyi* treated with crude extract of *K. alvarezii*; Activity of *V. harveyi*+ = weak; ++ = moderate; +++ = high; ++++ = very high; SAT test (0.0 to 1.0 molarity (M) = strongly hydrophobic, 1.0 to 2.0 M = moderately hydrophobic; 2.0 to 4.0 M = weakly hydrophobic, and >4.0 M = not hydrophobic); BATH-test (>50% partitioning = strongly hydrophobic, 20 to 50% partitioning = moderately hydrophobic; and <20% partitioning = not hydrophobic).

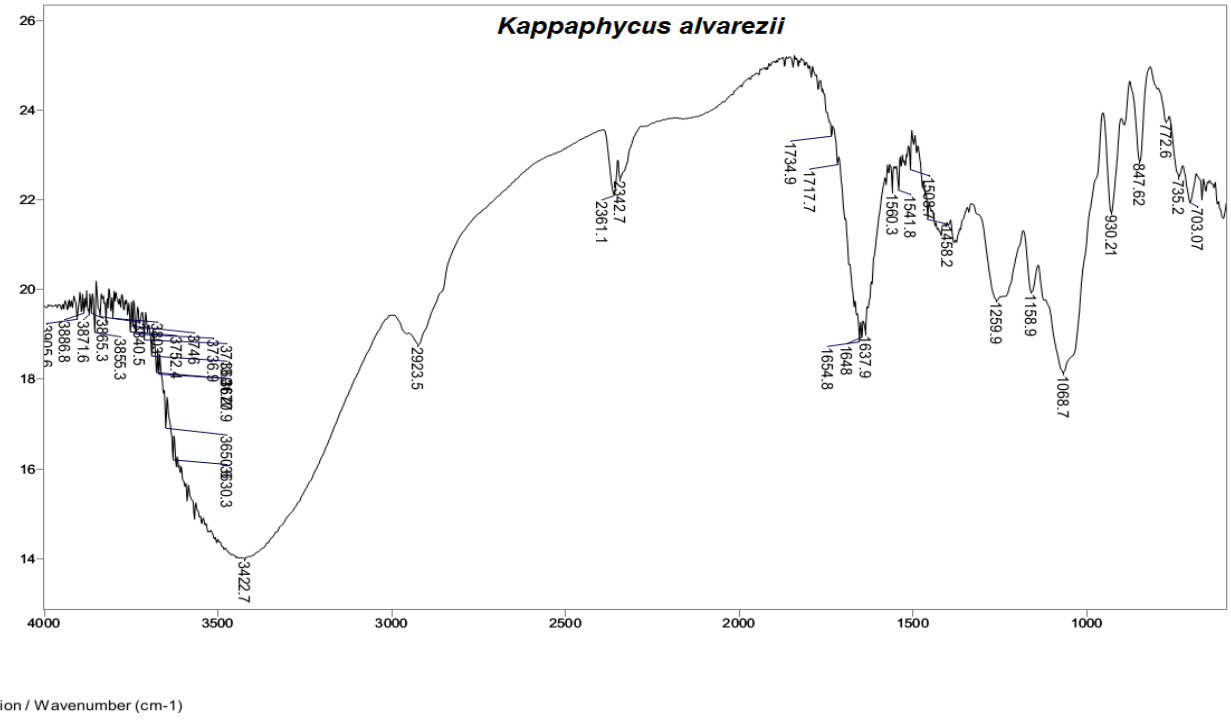


Figure 4. FTIR spectrum of shadow dried *K. alvarezii* powder.

Table 2. The wave number (cm^{-1}) of dominant peak obtained from the FTIR absorption spectra of *K. alvarezii.*

Frequency (cm^{-1})	Bond	Functional groups
3630.3	O-H stretch, free hydroxyl	Alcohols, phenols
3422.7	O-H stretch, H-bonded	Alcohols, phenols
2923.5	O-H stretch	Carboxylic acids
	C-H stretch	Alkanes
1734.9	C=O stretch	Carboxylic acids, carbonyls (general), aldehydes, saturated aliphatic
1717.7	C=O stretch	Carboxylic acids, carbonyls (general), α, β-unsaturated esters
1654.8	-C=C- stretch	Alkenes
1648	-C=C- stretch	Alkenes
	N-H bend	Primary amines
1637.9	N-H bend	Primary amines
1541.8	N-O asymmetric stretch	Nitro compounds
1508.7	N-O asymmetric stretch	Nitro compounds
1458.2	C-C stretch (in-ring)	aromatics
	C-H bend	Alkanes
1259.9	C-N stretch	Aromatic amines
	C-O stretch	Alcohols, carboxylic acids, esters, ethers
	C-H wag (-CH_2X)	Alkyl halides
1158.9	C-O stretch	Alcohols, carboxylic acids, esters, ethers
	C-H wag (-CH_2X)	Alkyl halides
	C-N stretch	Aliphatic amines
1068.7	C-O stretch	Alcohols, carboxylic acids, esters, ethers
	C-N stretch	Aliphatic amines
930.21	=C-H bend	Alkenes
	O-H bend	Carboxylic acids
847.62	=C-H bend	Alkenes
	N-H wag	Primary, secondary amines
	C-H "oop"	Aromatics
	C-Cl stretch	Alkyl halides
772.6	=C-H bend	Alkenes
	N-H wag	Primary, secondary amines
	C-H "oop"	Aromatics
	C-Cl stretch	Alkyl halides
735.2	=C-H bend	Alkenes
	N-H wag	Primary, secondary amines
	C-H "oop"	Aromatics
	C-Cl stretch	Alkyl halides
703.07	=C-H bend	Alkenes
	N-H wag	Primary, secondary amines
	C-H "oop"	Aromatics
	C-Cl stretch	Alkyl halides

and fatty acids (Rajasulochana et al., 2009). The anti-microbial effect of *K. alvarezii* extract was proved against many bacterial pathogens (Bibiana et al., 2012). Sivakumar and Kannappan (2013) have proved that extract of *K. alvarezii* at 300 µg gave 8.6 mm zone of inhibition against *V. harveyi.* Recently, Prasad et al. (2013) determined the antibacterial activity of *K. alvarezii* and *K. striatum* extracts against Gram positive and negative bacteria. Consequently, in the present study also, *K. alvarezii* extract, reduced the growth of *V. harveyi.* Silva et al. (2013) has reported that the marine macro algae exhibit bioactivity against virulent and antibiotic resistant *Vibrio* species.

Marine algae are rich sources of bioactive agents (Rangaiah et al., 2010). In this study, the reductions on crude bacteriocin produced by *V. harveyi* was noticed in all the days of treatment by *K. alvarezii* extract. In the present study, *K. alvarezii* extract reduced EPS and

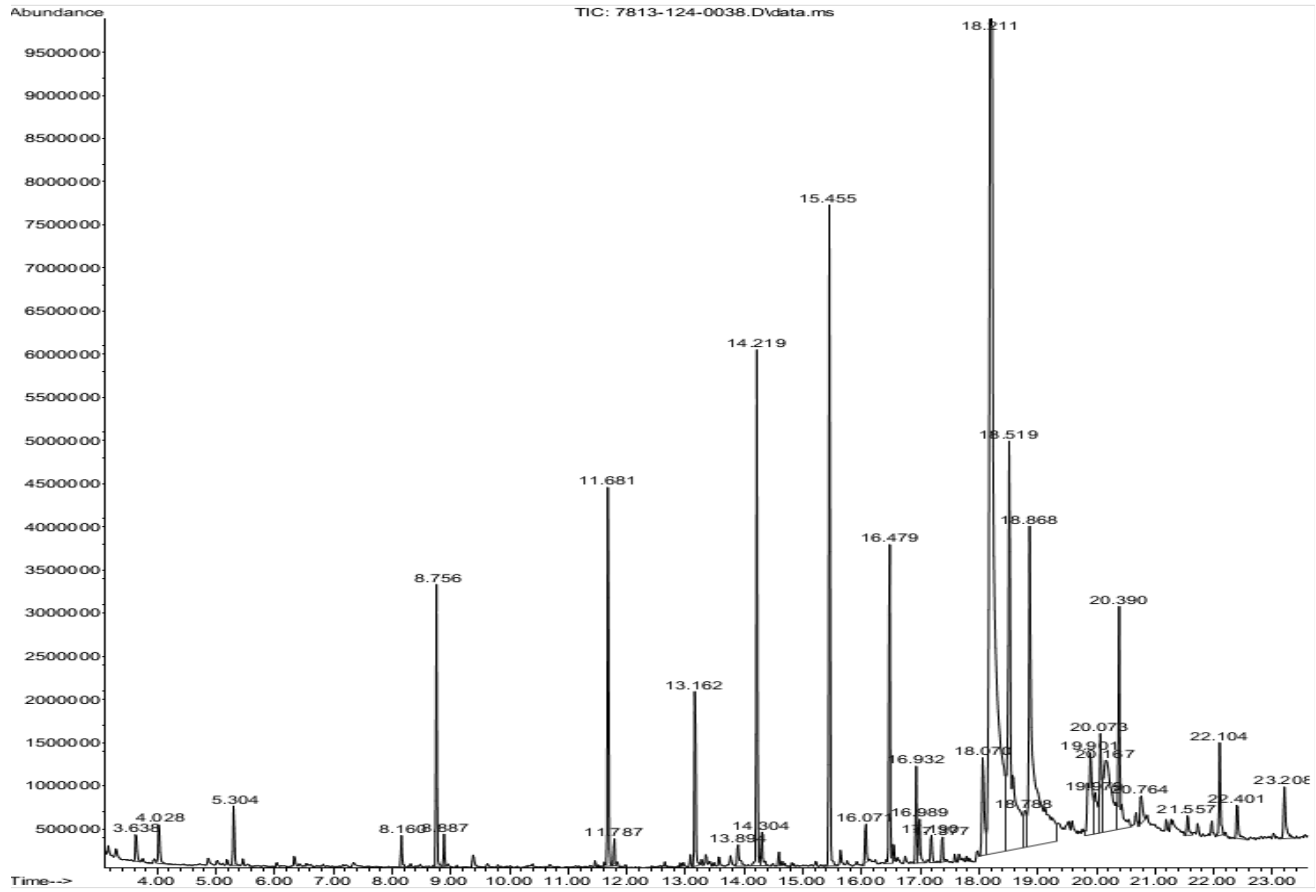
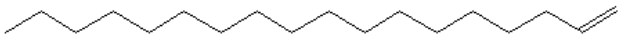


Figure 5. GC-MS chromatogram of the crude extract of *K. alvarezii.*

Structure of Heptadecane ($C_{17}H_{36}$) detected by GC-MS from *K. alvarezii.*

Structure of Octadecene ($C_{18}H_{36}$) detected by GC-MS from *K. alvarezii.*



Structure of n-Hexadecanoic acid ($C_{16}H_{32}O_2$) detected by GC-MS from *K. alvarezii.*

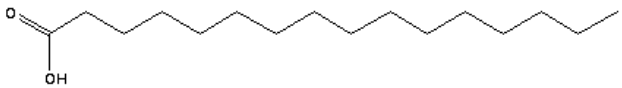


Figure 6. Major compounds isolated from *K. alvarezii.*

protease produced by *V. harveyi* as compared to control. Liu et al. (1997) has purified 38 kDa cysteine protease of *V. harveyi* isolated from shrimp and suggested that protease plays a key role in developing infections among the tiger shrimps. The moderate level of reduction on proteolysis, phospholipase, lipolysis and thermonuclease level of *V. harveyi* were observed in all the treatment days as compared to the control.

Table 3. GC-MS profile of *K. alvarezii.*

Retention time (min)	Compounds name	Peak area (%)	Molecular formula	Molecular weight
3.63	Styrene	0.34	C_8H_8	104.14
4.03	Anisole	0.50	C_7H_8O	108.13
5.30	1-Decene	0.61	$C_{10}H_{20}$	140.26
8.15	4H-1,3-Benzodioxin	0.32	$C_8H_8O_2$	136.14
8.75	1-Dodecene	2.20	$C_{12}H_{24}$	168.31
8.88	1-Dodecane	0.25	$C_{12}H_{26}$	170.33
11.68	1-Tetradecene	3.52	$C_{14}H_{28}$	196.37
11.78	Tetradecane	0.25	$C_{14}H_{30}$	198.38
13.16	Phenol, 2,4-bis(1,1-dimethylethyl)	1.76	$C_{14}H_{22}O$	206.32
13.89	1-[p-Chlorophenyl]-3-[4-[[3-[1-Pyrrolidinyl]propyl]amino]-6-[trichloromethyl]triazinyl]guanidine	0.37	$C_{18}H_{22}Cl_4N_8$	492.23
14.21	Cetene	4.03	$C_{16}H_{32}$	224.42
14.29	Hexadecane	0.42	$C_{16}H_{34}$	226.44
15.46	Heptadecane	5.58	$C_{17}H_{36}$	240.46
16.07	Tetradecanoic acid	0.40	$C_{14}H_{28}O_2$	228.37
16.47	1-Octadecene	3.18	$C_{18}H_{36}$	252.48
16.92	Bicyclo[3.1.1]heptanes, 2,6,6-trimethyl	0.84	$C_{10}H_{18}$	138.24
16.98	2-Pentadecanone, 6,10,14-trimethyl	0.51	$C_{18}H_{36}O$	268.47
17.18	1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester	0.34	$C_{16}H_{22}O_4$	278.34
17.37	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	0.28	$C_{20}H_{40}O$	296.53
18.07	Palmitoleic acid	1.57	$C_{16}H_{30}O_2$	254.40
18.20	n-Hexadecanoic acid	38.43	$C_{16}H_{32}O_2$	256.42
18.52	5-Eicosene, (E)-	9.39	$C_{20}H_{40}$	280.53
18.78	Oleic acid	0.74	$C_{18}H_{34}O_2$	282.46
18.87	n-Hexadecanoic acid	9.90	$C_{16}H_{32}O_2$	256.42
19.90	Heptafluorobutyric acid, n-tetradecyl ester	2.19	$C_{18}H_{29}F_7O_2$	410.41
19.97	Cis-Vaccenic acid	0.88	$C_{18}H_{34}O_2$	282.46
20.07	Cyclohexane, 1-(1,5-dimethylhexyl)-4-(4-methylpentyl)-	1.58	$C_{20}H_{40}$	280.53
20.16	Heptafluorobutyric acid, pentadecyl ester	3.91	$C_{19}H_{31}F_7O_2$	424.43
20.38	E-15-Heptadecenal	2.75	$C_{17}H_{32}O$	252.43
20.76	Oleic acid	0.45	$C_{18}H_{34}O_2$	282.46
21.56	2(1H)-Naphthalenone, 3,4,4a,5,6,7-hexahydro-4a-[(methylamino) methyl]-, ethylene acetal	0.29	$C_{14}H_{23}NO_2$	237.33
22.09	Cyclotetracosane	0.90	$C_{24}H_{48}$	336.63
22.40	Z-10-Methyl-11-tetradecen-1-ol propionate	0.50	$C_{18}H_{34}O_2$	282.46
23.20	5-Methylthieno[3,2-b]pyridine	0.83	C_8H_7NS	149.21

The cell surface hydrophobicity tests in whole treatment days exhibit moderately hydrophobic activities as compared to the control. Recently, the inhibitory effect of crude extracts of marine algae such as *Skeletonema costatum*, *Ulva fasciata* and *K. alvarezii* showed reduction on luciferase and luminescence produced by *V. harveyi* (Sivakumar and Kannappan, 2013). So, the current study also revealed the same reductions on luminescence.

The FTIR spectra of *K. alvarezii,* which was corroborated with the FTIR values reported for *Chlorella vulgaris* (Duygu et al., 2012). Dean et al. (2010) identified various functional compounds like protein, polysaccharide and lipids from micro algae using FTIR. The absorption spectrum of FTIR analysis showed with 5 important peaks due to aldehyde, esters, methylene and methyl groups with hydroxyl bond absorption. These groups were present in the lipids of many microalgae

Table 4. Challenging *K. alvarezii* extract against *V. harveyi* with the reduction in the cumulative percentage mortality on *P. monodon* postlarvae.

Day	Cumulative percentage mortality				Treatment tubs (cfu/ml)		Control tubs (cfu/ml)		Average weight of postlarvae (mg)		Water quality parameters for treatment and control tubs			
	Control tubs with *V. harveyi*	Treatment tubs extract with *V. harveyi*	Tubs with extract and PL alone	Tubs with PL alone	Total plate count	*V. harveyi*	Total plate count	*V. harveyi*	Treatment tubs	Control tubs	Temp (°C)	Salinity (PSU)	pH in control tubs	pH in treatment tubs
0	0.00	0.00	0.00	0.00	2.78×10^{6}	2.14×10^{6}	2.59×10^{6}	1.86×10^{6}	16.9 ± 3	17.5 ± 2	29.0±1.0	20±0.5	8.50±0.2	8.30±0.2
5th	13.66±0.3	07.06±0.1	2.39±0.1	3.23±0.1	3.30×10^{4}	6.55×10^{4}	1.45×10^{5}	1.17×10^{5}	60.5 ± 4	63.3 ± 4	29.5±1.0	20±0.5	8.40±0.2	8.40±0.2
10th	26.05±0.9	15.61±0.4	6.19±0.2	6.03±0.2	3.56×10^{4}	3.21×10^{4}	2.74×10^{5}	2.62×10^{5}	121.3 ± 6	126.1 ± 5	29.0±1.0	20±0.5	8.30±0.2	8.30±0.2
15th	35.63±1.1	23.36±0.7	12.05±0.5	13.33±0.5	2.26×10^{4}	3.15×10^{3}	1.46×10^{4}	7.40×10^{4}	158.1 ± 7	162.3 ± 5	30.0±1.0	20±0.5	8.50±0.2	8.50±0.2
20th	47.33±1.5	29.19±1.3	18.13±0.6	17.43±0.5	1.01×10^{4}	4.85×10^{3}	7.80×10^{4}	2.02×10^{4}	208.5 ± 9	201.1 ± 5	30.0±1.0	21±0.5	8.30±0.2	8.30±0.2
25th	62.13±2.3	38.53±1.4	24.69±0.9	23.86±1.0	6.00×10^{4}	8.30×10^{3}	1.88×10^{4}	2.53×10^{4}	249.2 ± 8	251.3 ± 9	31.0±1.0	21±0.5	8.10±0.2	8.20±0.2
30th	76.30±2.9	46.60±1.1	29.56±1.0	28.39±1.0	1.36×10^{4}	9.95×10^{3}	2.06×10^{4}	1.53×10^{4}	267.9 ± 7	271.5 ± 8	30.0±1.0	21±0.5	8.30±0.2	8.00±0.2

Values of average of three determinations with standard deviation (SD).

species (Yin et al., 2011).

The total chromatogram of *K. alvarezii* extract is showed in Figure 5. The peaks of the identified compounds and their relative percentages are summarized in Table 3. Many species of microalgae which produces major constituents like tetradecanoic acid, hexadecanoic acid, octadecanoic acid methyl esters etc. (Musharraf et al., 2012). Marine algae exhibit a higher level of fatty acid compounds which show potential bioactivity (Manilal et al., 2010). Therefore in the present study, biological activity of *K. alvarezii* against *V. harveyi* was due to the presence of chemical-constituents such as n-hexadecanoic acid (tR = 18.20, 18.87 min) (38.43, 9.90% respectively) and followed by 5-eicosene, (E) - (tR = 18.52 min) (9.39%). It is possible that bioactive compounds primarily consisting of n-hexadecanoic acid (tR =18.20, 18.87 min) (38.43, 9.90% respectively) may be involved in biological activity. More recently an auto inhibitor (named 15-hydroxyeicosapentaenoic acid) has also been identified from *S. costatum* (Imada et al., 1992).

Challenge of *K. alvarezii* extract against *V. harveyi* during *P. monodon* postlarvae revealed 29.70% reduction in the cumulative percentage of mortality as compared to control (76.30%). Similarly, the ethyl acetate fractions of *Acanthus ilicifolius* gave 27.74% of reduction in the cumulative percentage of mortality as compared to the control (61.06%). Saptiani et al. (2012) has reported that ethyl acetate, n-butanol fractions of crude *A. ilicifolius* extract controlled *P. monodon* postlarvae from *V. harveyi* infections.

Marine algae are potential source for wide range of polyunsaturated fatty acids (PUFA), carotenoids, phycobiliproteins, polysaccharides and phycotoxins (Chu, 2012) etc. It was reported that lipids inhibit microorganisms by disrupting cellular membrane (Bergsson et al., 2011) of microbes. In microalgae, fatty acids are accumuated predominantly in the lipids that constitute the cell membranes and during cellular disintegration large quantities of fatty acids are released from cellular lipids by host lipolytic enzymes (Wichard et al., 2007). Further, the fatty acids are dreadfully antimicrobial and similar protection may be afforded to microalgae under stress from pathogenic microbes. However, these fatty acids may further affect the expression of bacterial virulence factors, which are important for the establishment of an infection.

Davies and Marques (2009) proved that saturated and unsaturated fatty acids can prevent initial bacterial adhesion and subsequent biofilm formation. Further they postulated that fatty acids of chain length more than 10 carbon atoms would persuade lysis of bacterial protoplasts. Owing to the harsh environments in which many macro algae exist, they have developed effective defense mechanisms with rich source of bioactive compounds, including polysaccharides, polyphenols, fatty acids and peptides, with dissimilar

structures and activities from those found in terrestrial plants (Tierney et al., 2010). Recent findings show that the main substances biosynthesized by algae are fatty acids, steroids, carotenoids, polysaccharides, lectins, mycosporine-like amino acids, halogenated compounds, polyketides and toxins (Cardozo et al., 2007). So, the marine macro algae *K. alvarezii* extract also will have immense applications in aquaculture.

In conclusion, this study proves that the crude extract of *K. alvarezii* inhibited the growth and modulated the virulence factors produced by *V. harveyi*. This extract also controlled the mortality caused by *V. harveyi* during shrimp larviculture. Based on this study, the *K. alvarezii* extract can be used as alternative bio-inhibitors for the aquaculture practices. Application of such bio-products would reduce the side effects by applying the synthetic compounds with reduced cost and eco-friendly nature.

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