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RESEARCH ARTICLE

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Protective effect of mangrove (*Rhizophora apiculata*) leaves extract in shrimp (*Penaeus monodon*) larvae against bio-luminescent disease-causing *Vibrio harveyi* bacteria

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Abstract

Application of chemicals for monitoring if *Vibrio* outbreaks can enhance resistance in mangrove (*Rhizophora apiculata*) leaves extract was tested against bioluminescence disease-causing *Vibrio harveyi* bacteria. An inhibitory zone of 12 mm was observed at 350 µg/mL. The growth of *V. harveyi* was decreased from 0.783 to 0.533 (OD_{600}) as compared to control (0.970 to 1.031 OD) against *R. apiculata* extract. The extract was treated against virulence produced by *V. harveyi*, the crude bacteriocin values decreased from 1.653 to 1.574 OD as compared with control. Further, extract 200 µg/mL was challenged against *V. harveyi* (10 mL at 1.8 OD_{600}) during larviculture of *Penaeus monodon* for 30 days. The mortality increased from 9.0 to 68.5% in the control, but in the treatment the mortality varied from 0 to 57.8% till 30th day. The difference in the decrease of mortality in the treatments was 10.6%. *V. harveyi* count was decreased from 1.46×10^5 to 3×10^3 cfu/mL, respectively in the treatment for the 30th day as compared to control (1.33×10^5 to 9.2×10^3 cfu/mL). *R. apiculata* leaves extract was reported to have various functional groups of compounds as determined by Fourier Transform Infrared Spectroscopy (FTIR). The GC-MS analysis revealed that the *R. apiculata* extract contains compounds such as 1, 2-diacetate, cyclododecane, 2-chloropropionic acid and squalene. These compounds might be responsible for the antagonism against *V. harveyi*. Hence, crude extracts of *R. apiculata* can be used as a non-antibiotic agent to control shrimp disease caused by *V. harveyi* during larviculture.

Additional keywords: leaves extract of *Rhizophora*; antagonism; marine *Vibrio*; shrimp larviculture.

Abbreviations used: BATH (bacterial adhesion to hydrocarbon); CPM (cumulative percentage mortality); CPS (counts per second); DMSO (dimethyl sulfoxide); MIC (minimum inhibitory concentration); OD (optical density); PSU (practical salinity unit); SAT (salt aggregation test).

Authors' contributions: Conceived designed and performed the experiments, analyzed the data and documentation: SK & KS. Contributed reagents/materials/analysis tools: SK. Supplied the samples: SS. All authors read and approved the final manuscript.

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Introduction

Vibrio is a primary and virulent bacterial pathogen responsible for large-scale mortality in all stages of *Penaeid* shrimp hatchery and grow-out practices. Unsuitable application of chemicals for regulating *Vibrio* outbreaks leads the result in the advancement of resistance among bacteria (Vaseeharan *et al*., 2010). Many technologies have been habituated to manage bio-luminescent bacteria existing in the shrimp grow-out and larviculture systems. "Green water culture system" is a pioneering technique used in the aquaculture in which the shrimps or fish are co-cultured with profuse microalgae such as *Chlorella*, but this is yet to be demonstrated to be pragmatic in shrimp grow-out practices. Probiotic bacteria has been widely used for monitoring various fish diseases in shrimp grow-out practices, but high quantities are necessary to become commercial (Defoirdt *et al*., 2007). Consequently, the exploration for alternate methods to control diseases

caused by resistant *Vibrio* is an imperative challenge for the sustainable development of aquaculture. It is possible that instead of chemicals, alternative bio-inhibitors obtained from marine plants can be used to control resistant marine *Vibrio*.

Rhizophora spp such as *Rhizophora mucronata*, *R. mangle*, and *R. apiculata* have been reported for the potential bioactive substances for the benefit of the society (Suryati & Hala, 2002). *R. mucronata* extracts have been reported for antagonism against multi-drug resistant *V. harveyi* and *V. campbellii* (Baskaran & Mohan, 2012). Similarly, *R. apiculata* showed antibacterial activity by tannin extract of barks (Lim *et al*., 2006) and explored the potential of absorbent capacity of heavy metal ions (Oo *et al*., 2009). Immuno-stimulant activity and better chemoprotectant against cholera toxin (CTX) induced toxicity in mice was studied with methanol extracts of *R. apiculata* (Vinod Prabhu & Guruvayoorappan, 2012) and also used for human ailments such as angina, dysentery (Joel & Bhimba, 2010). According to Dhayanithi *et al*. (2015) extracts of *R. apiculata* could improve the immune system in *Clown* fish against *V. alginolyticus*. Hence, the present study evaluated the antagonism of ethyl acetate extract of leaves of *R. apiculata* against growth and virulence factors of *V. harveyi* for protecting *Penaeus monodon* larvae against bio-luminescent disease-causing *V. harveyi* during shrimp larviculture.

Material and methods

Isolation and identification of *V. harveyi*

V. harveyi was isolated from water samples collected from the Muttukadu Experimental Station (MES) of ICAR-CIBA, Muttukadu at Chennai. The isolates were identified by various bio-chemical tests such as arginine dihydrolase (-), lysine (+), ornithine decarboxylase (+), gelatinase (+), Voges-proskauer (-), D-glucosamine (-) (Abraham & Palaniappan, 2004; Ananda Raja *et al*., 2017a) and compared with the characteristics of standard type strain *V. harveyi* ATCC 25919. The isolates were re-confirmed further by streaking in *V. harveyi* selective agar (VHSA) and stored in VHSA slants at 4°C (Harris *et al*., 1996).

Sampling of mangrove leaves and preparation of extracts

Leaves of mangrove plant (*R. apiculata*) were collected from the mangrove forest at Pichavaram in Tamil Nadu, India (Lat 11º27'N; Lan 79º 47'E). The leaves were washed with 10 mg/L of $KMnO_4$ for 10 min to remove the epiphytes, sand and other extraneous matters, then cleaned with fresh water, later shade-dried at room temperature (RT), pulverized using sterile pestle and mortar and finally stored at -20ºC till further use. The powder (2 g) was extracted with 100 mL of ethyl acetate using Soxhlet apparatus. The extracts were neutralized to pH 7 using 0.1 N NaOH or HCl and filtered through Whatman No.1 filter paper. The extracts were dried at 42ºC using a hot air oven. For cold extraction, the *R. apiculata* extract was prepared by mixing 1.0 g of powder with 10 mL of ethyl acetate and then shaker-incubated at 37°C at 50 rpm for 96 h. The extract was filtered through Whatman filter paper No.1, then rotary evaporated (30°C) under vacuum and stored at 4°C for further use. The pH was neutralized as described earlier, the resultant extract was liquefied with 5 mg/mL of 30% (v/v) dimethyl sulfoxide (DMSO) and used for testing antagonism against *V. harveyi* (Sivakumar & Kannappan, 2013).

Antibacterial assay

Antibacterial activity was ascertained against *V. harveyi* through "agar well diffusion assay" as described by Das *et al*. (2005). Cells of *V. harveyi* (50 µL of 10^8 cfu/mL of 18 h old broth culture) were inoculated into sterile Petri dishes. The LB agar (35 mL) was poured into plates and allowed to solidify at room temperature (RT) for 1 h. Two wells (6 mm) were made on the LB agar plates using a sterile steel borer. The wells were sealed at the bottom using 10 µL of 1% soft sterile agar and filled with 200 µL of *R. apiculata* leaves extract. The plates were incubated at 37°C for 48 h and zones of inhibitions of bacteria around the well were measured (Das *et al*., 2015) excluding the well. Antimicrobial activity of this crude extract was determined by dissolving in 30% Dimethyl Sulfoxide (DMSO) at various concentrations. DMSO was used as negative control. Similarly, the extract obtained through "cold extraction" was also tested. The inoculated plates were incubated at 37ºC for 24 h and zones of inhibitions were measured.

Estimation of minimum inhibitory and bactericidal concentrations (MIC & MBC)

The minimum inhibitory concentrations (MIC) for the *R. apiculata* leaves extract was evaluated as described by Islam *et al*. (2008). Dilution methods were used to determine the MIC of *R. apiculata* extract. In dilution tests, *V. harveyi* was tested for their ability to produce visible growth on a series of LB agar plates. Various concentrations of extracts (5.0 to 50 µg) were examined and lowest concentration of the extract which

inhibited the visible growth of *V. harveyi* was known as the MIC. The plates were incubated at 37°C for 24 h and 20 µL of *V. harveyi* (1.8 OD or 2.19×10^7 cfu/mL) was tested for the MIC on LB agar medium. The MBC was evaluated as the lowest concentration of a crude plant extract required to kill 99.9% of 20 µL of *V. harveyi* (1.8 OD or 2.19×10^7 cfu/mL).

Impact of *R. apiculata* leaves extract against the growth and virulence produced by *V. harveyi*

R. apiculata extract at 300 µg/ mL was added to 100 mL of LB medium. A volume of 500 µL of active 24 h old *V. harveyi* (1.8 OD) was inoculated into LB broth and incubated at 37°C in 100 rpm for 5 days under shaking condition. Every day, 3 mL of *V. harveyi* inoculum was taken out and the growth of *V. harveyi* was measured at OD 600 nm. Various virulence factors such as proteolytic, lipolytic, phospholipase, thermonuclease activities, crude bacteriocin production, exopolysaccharide (EPS) and proteases produced by *V. harveyi* with growth were estimated. Salt aggregation test (SAT) were carried out for cell surface hydrophobicity and cell adhesion was examined by bacterial adhesion to hydrocarbons test (BATH) (Soto-Rodriguez *et al*., 2012). Each test was performed in triplicate and values were expressed in average along with standard deviation.

Cell lysate preparation and estimation of luciferase with luminescence

Luciferase produced by *V. harveyi* was tested using the luciferase assay kit (LUC1, Technical Bulletin MB-260, Sigma, USA) and read by a luminometer (Victor TM X3, Perkin Elmer, USA). *V. harveyi* cells were harvested by centrifugation at 10,000 rpm for 5 min. The pellet was re-suspended in 333 µL of 1X cell lysis buffer per mL of *V. harveyi* and incubated for 10 min at 25°C. The suspension was centrifuged at 12,000 rpm for 1 min at 4°C. The supernatant was removed and stored in ice. Luciferase substrate (lyophilized, luciferase assay substrate was suspended in luciferase assay buffer) as cell lysate containing luciferase was equilibrated to 25°C before use. Cell lysate (20 µL) was added to 100 µL of the luciferase substrate and mixed well. Readings were recorded in 10 seconds for light emission by the luminometer (Victor X3- Perkin Elmer) and expressed as counts per second (CPS, *i.e.,* photons per second). The light intensity was nearly constant for 20 seconds. The LB broth medium and 1X lysis buffer were used as negative control for luciferase assay. For the estimation of luminescence, *V. harveyi* cells were harvested by centrifugation at 10,000 rpm for 5 min and its spent culture medium was used (Kannappan *et al*., 2013) and estimated by luminometer.

Fourier transform infra-red spectroscopy (FT-IR) analysis

The shade dried leaves of *R. apiculata* powder was subjected to FT-IR BRUKER IFS 66 model spectrometer (Fig. 2) in the region 4000-400 cm^{-1} by employing 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 autosampler. The GC column used was fused with silica capillary column (length 30 m × diameter 0.25 mm × film thickness 0.25 mm) 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 40-700 m/z. The split ratio was adjusted to 1:10 and injection volume was 1 µL. The injector temperature was 250°C; the oven temperature was kept at 70°C for 3 min, raised to 250°C at 14°C/min (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, full mass data was recorded between 50-400 Dalton/s and scan speed 2000. Mass start time was at 5 min and end time at 35 min. Peak identification of crude *R. apiculata* 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).

Effect of leaves extract of *R. apiculata* on *V. harveyi* during *P. monodon* larviculture

Plastic tubs were washed with 10 mg/L of $KMnO_4$ solution (w/v) for 10 min and filled with 20 L of low saline water at 20 Practical Salinity Units (PSU). Disease-free (Ananda Raja *et al*., 2017b) postlarvae (PL 10) of *P. monodon*, obtained from a shrimp hatchery were acclimatized at 20 PSU for 5 days under laboratory conditions at 37 ± 1°C with continuous aeration. The average body weight of PL ranged from 17 to 18 ± 0.2 mg and stocked at 1000 numbers per tubs. The first control tub was inoculated with *V.*

harveyi (10 mL of 1.8 OD) alone. The second tub was considered as treatment and inoculated with *V. harveyi* 2 gm/10L of crude *R. apiculata* extract. The third tub was considered as control where crude *R. apiculata* extract was added at 200 µg /mL alone with PL. The fourth tub was a control for PL, where neither *V. harveyi* nor extract was added. Aeration was provided for each container ensuring that oxygen level did not go above 4 ppm. The PLs were fed twice a day and the feed requirement per day was 15% of their body weight. All the experimental tubs were covered with plastic lid at the top to avoid external contamination. The water temperature, salinity and pH were recorded every 5 days. Experiments were carried out in triplicates. The mortality of PL was counted daily. No water exchange was given for the entire containers till 30 days. The total heterotrophic and *V. harveyi* counts were enumerated using LB and *V. harveyi* selective agar media under spread plate method (Traifalgar *et al*., 2009; Biswas *et al*., 2012).

Statistical analysis

The data were analyzed and expressed as means along with the standard deviation. Analysis of variance (ANOVA) (SPSS, ver. 16.0) was carried out to assess the treatments (p<0.05). Cumulative percentage mortality was calculated as CPM = [cumulative frequency / total number of observations (n)] × 100.

Results

Antagonism of leaves extract of *R. apiculata*

Crude extracts of 200, 250, 300, 350 and 400 µg/mL showed 6, 8, 10, 12 and 14 mm inhibitory zones respectively (excluding the well size 8 mm). As a positive control, 10 µL of oxytetracycline (250 mg/25 mL) showed a zone of inhibition of 23 mm whereas the DMSO negative control had no effect. Cold extraction of the crude extracts showed 5, 6, 7, 8, and 9 mm inhibitory zones respectively. The MIC for the crude extract against *V. harveyi* was 6 mm at 200 µg/mL and MBC for *V. harveyi* was 12 mm at 300 µg/mL (Fig.1).

Effect of leaves extract of *R. apiculata* on the growth and virulence of *V. harveyi*

The crude *R. apiculata* extract reduced the growth of *V. harveyi* (OD) from the fifth day. The highest OD difference compared to control was observed on the 15th day (0.402) and the lowest on the 10th day (0.041). The production of luminescence was decreased to 31, 44, 34, 47, 35 and

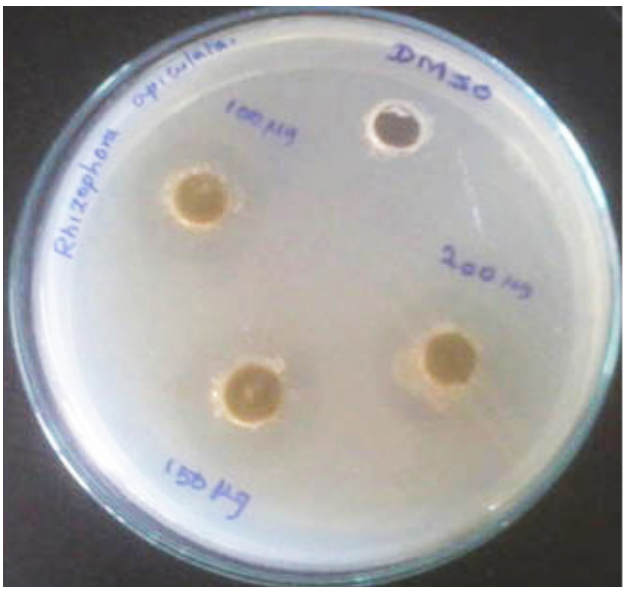

Figure 1. The extract of *R. apiculata* leaves showing antagonism against *V. harveyi*

51 CPS at days 5, 10, 15, 20, 25 and 30, respectively compared to control (Table 1). The maximal reduction on luminescence was detected on the 30th day (51 CPS) and the minimal reduction on the 5th day (31 CPS). Production of intracellular luciferase was decreased during the study. The maximal decrease was observed on the 30th day (45 CPS) and a minimal decrease was observed on the 10th day (15 CPS). The maximum decrease of bacteriocin production (OD) was observed on the 20th and 30th days (0.385 and 0.382) and the minimum (0.18) was observed on the 15th day. The maximal decrease of protease occurred on the 30th day. The *R. apiculata* extract treatment was associated with a weak level (+) of phospholipase production by *V. harveyi* as compared with very high phospholipase (+++) production by the control for all 30 days (Table 1). In the SAT test, *V. harveyi* revealed strong hydrophobic activity for the 5th to 30th days in the control whereas the treatment showed moderate to weak hydrophobic activities.

FTIR of leaves extract of *R. apiculata*

The FTIR spectrum of dried powder of *R. apiculata* leaves (Fig. 2) and functional groups identified were compared with the FTIR standard library data. FTIR spectrum showed the presence of significant functional groups such as aromatics, alkanes, alcohol, carboxylic acids, esters, ethers, aliphatic amines and alkyl halides (Table 2).

GC-MS of leaves extract of *R. apiculata*

GC-MS analysis on the crude ethyl acetate extract of *R. apiculata* revealed a mixture of volatile compounds. Fatty acid methyl esters were investigated quantitatively

Table 1. Effect of *R. apiculata* leaves extract on the decrease of virulence produced by *V. harveyi.*

Virulence factors	Days					
	5	10	15	20	25	30
Luciferase (CPS)						
Treatment	133	111	115	152	122	125
Control	151	126	138	182	150	170
Salt aggregation test (SAT) (moles/L) [a]						
Treatment	1.50	1.50	1.75	1.75	2.50	2.00
Control	0.50	0.75	0.50	0.50	0.75	0.50
Bacteriocin OD at 660 nm						
Treatment	1.653±0.011	1.702±0.021	1.835±0.031	1.664±0.050	1.764±0.056	1.653±0.009
Control	1.901±0.088	2.005±0.035	2.015±0.059	2.049±0.011	1.947±0.020	2.035±0.063
Protease OD at 440 nm						
Treatment	0.191±0.019	0.176±0.007	0.218±0.004	0.142±0.029	0.129±0.017	0.138±0.023
Control	0.267±0.009	0.214±0.006	0.271±0.020	0.190±0.014	0.163±0.016	0.240±0.034
Growth OD at 600 nm						
Treatment	0.794±0.025	0.259±0.012	0.578±0.042	0.274±0.017	0.651±0.034	0.528±0.012
Control	0.970±0.016	0.300±0.004	0.980±0.020	0.446±0.016	0.844±0.010	0.972±0.018
Luminescence produced (CPS)						
Treatment	126	124	156	140	135	116
Control	157	168	190	187	170	167
Phospholipase activity [b]						
Treatment	+	+	+	+	+	+
Control	+++	+++	++++	++++	++++	++++

[a] SAT test (0.0 to 1.0 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). [b] Activity of *V. harveyi*: + = weak, ++ = moderate, +++ = high, ++++ = very high.

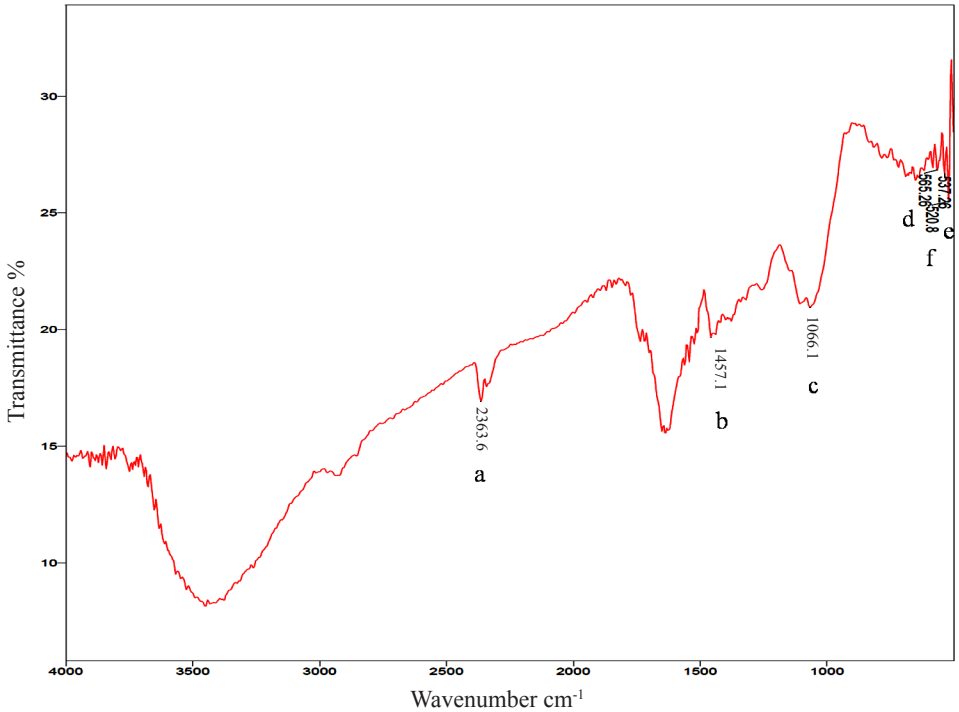

Figure 2. FTIR spectrum of shade-dried leaves powder of *R. apiculata*. Dominant peaks: a, unknown; b, aromatics, alkanes; c, alcohols, carboxylic acids, esters, ethers, aliphatic amines; d, alkyl halides; e, alkyl halides; f, alkyl halides.

Table 2. Wave number (cm^{-1}) of dominant peaks obtained from the FTIR absorption spectra (Fig. 2) of leaves extract of *R. apiculata*

Dominant Peaks	Frequency (cm^{-1})	Bond	Functional group
a	2363.6	Unknown	Unknown
b	1457.1	C–C stretch (in–ring) C–H bend	Aromatics Alkanes
c	1066.1	C–O stretch C–N stretch	Alcohols, carboxylic acids, esters, ethers Aliphatic amines
d	565.26	C–Br stretch	Alkyl halides
e	537.26	C–Br stretch	Alkyl halides
f	520.8	C–Br stretch	Alkyl halides

by GC-MS in multiple reactions monitoring mode and thus allowing for a better signal resolution without a preliminary fractionation of the extract. A total of 21 peaks were observed with retention times. The main chemical-constituent reported was squalene (69.2 peak area %), tocopherol (6.49%), vitamin E (6.34%), hexadecanoic acid (5.01%), octadecatrienoic acid (3.19%) and bicyclo heptane (2.39%). In the present study, the biological activity of the leaves extract of *R. apiculata* on *V. harveyi* may be due to the presence of these chemical constituents (Table 3). Chemical constituents were identified using spectrum database NIST 11 software installed in GC-MS.

Effect of leaves extract of *R. apiculata* on *V. harveyi* during *Penaeus monodon* larviculture

During the experiment, mortality varied from 0 to 57.8% till 30th day in the treatment. The cumulative percentage mortality increased in the control from 9 to 68.5% for the 5th to the 30th day. The difference in the overall decrease of cumulative percentage mortalities among the treatment tank were 10.6% compared to control. The growth of postlarvae uniformly increased from 2- 3 mg in the 20th day and a 10 ± 0.2 mg increase was noticed on the 25th day of larviculture. *V. harveyi* counts were decreased in the treatment from 1.46×10^5

Table 3. GCMS profile of *R. apiculata* leaves extract

Peak No	Retention time (min)	Compound	Peak area (%)	Chemical formula	Molecular weight
1	8.547	1-Undecanol, acetate	0.79	$C_{13}H_{26}O_2$	214.193
2	9.288	Glycerol 1,2-diacetate	1.27	$C_7H_{12}O_5$	176.068
3	10.435	2,2'-Sulfinyldiethanol	1.78	$C_4H_{10}O_3S$	138.035
4	11.467	Cyclododecane	0.96	$C_{12}H_{24}$	168.188
5	12.047	Methyl (3,3-difluoro-2-propenyl) silane	0.69	$C_4H_8F_2Si$	122.036
6	12.933	Phenol, 2,4-bis (1,1-dimethylethyl)	1.05	$C_{14}H_{22}O$	206.167
7	13.994	5-Octadecene, (E)-	1.07	$C_{18}H_{36}$	252.282
8	15.838	Tetradecanoic acid	0.50	$C_{14}H_{28}O_2$	228.209
9	16.245	1-Nonadecene	1.43	$C_{19}H_{38}$	266.297
10	16.695	Bicyclo [3.1.1] heptane, 2,6,6-trimethyl-, (1α,2β,5α)	2.39	$C_{10}H_{18}$	138.141
11	16.956	Phthalic acid, isobutyl trans-dec-3-enyl ester	0.67	$C_{22}H_{32}O_4$	360.23
12	17.145	3-Chloropropionic acid, undec-2-enyl ester	0.84	$C_{14}H_{25}ClO_2$	260.154
13	17.915	n-Hexadecanoic acid	5.01	$C_{16}H_{32}O_2$	256.24
14	18.278	2-Chloropropionic acid, octadecyl ester	1.00	$C_{21}H_{41}ClO_2$	360.28
15	19.411	Phytol	1.62	$C_{20}H_{40}O$	296.308
16	19.541	Ethanol, 2-(9,12-octadecadienyloxy)-, (Z,Z)-	0.64	$C_{20}H_{38}\ 0_2$	310.287
17	19.599	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	3.19	$C_{18}H_{30}O_2$	278.225
18	20.297	Phytol, acetate	0.68	$C_{22}H_{42}O_2$	338.318
19	25.031	Squalene	61.59	$C_{30}H_{50}$	410.391
20	26.701	γ-Tocopherol	6.49	$C_{28}H_{48}O_2$	368.2984
21	27.340	Vitamin E	6.34	$C_{29}H_{50}O_2$	430.381

to 3×10^3 cfu/mL on the 30th day as compared to control (1.33×10^5 to 9.2×10^3cfu/mL). In the treatment group, the total heterotrophic bacterial (THB) counts decreased from 1.51×10^5 cfu/mL to 5.2×10^3 cfu/mL as compared to control (1.39×10^5 to 7.2×10^3 cfu/mL) (Table 4).

Discussion

Crude extracts obtained from both cold & hot extraction processes showed a significant inhibition on *V. harveyi*. The hot extract was greater than cold extraction in terms of inhibition. Correspondingly, Gurudeeban *et al*. (2013) have reported antimicrobial activity of *R. mucronata* leaves extracts. The chemical constituents of *R. apiculata* were screened and various phytochemicals such as 2-(2-ethoxyethoxy) ethanol (26.45%) and Kaur-16-ene (3.37%), benzophenone (16.09%) and 2-(2-ethoxyethoxy) ethanol (7.82%) constituents were found (Abidin *et al*., 2013). Manilal *et al*. (2010) have also reported that methanol extract of *R. apiculata* exhibited antimicrobial activity against shrimp-borne pathogens such as *V. harveyi, V. vulnificus, V. alkaligens, V. alginolyticus* and *V. parahaemolyticus.* It has again been confirmed in multidrug-resistant *V. harveyi* and *V. cambellii* by Ravikumar *et al*. (2010) with leaf extract of *R. mucronata.* Present study revealed that the solvent also play vital role in the extraction of phytochemical, the ethyl acetate extracts of leaves of *R. apiculata* were screened against *V. harveyi*. Similarly, Annapoorani *et al*. (2013) have reported in leaf extracts of *R. apiculata* against human pathogens such as *Pseudomonas aeruginosa*, *Enterobacter* spp and *Staphylococcus aureus*. The present work is also in agreement with Annapoorani *et al*. (2013) and inhibition could be due to the phytochemicals of phenolic group, alkaloids, steroids, triterpenes, flavonoids, catechin, tannin and anthro-quinone etc.

The decrease of virulence was also concordant with previous reports. Methanol extract of *R. apiculata* and *R. mucronata* (1 mg/mL) showed the virulence factors reduction of protease, pyocyanin pigments and biofilm produced by *P. aeruginosa* (Musthafa *et al*., 2013). *R. apiculata* extract treatment made *V. harveyi* strongly hydrophobic as BATH difference was 68.9 and 43.9. *R. apiculata* extract reduced 31 to 44 CPS of luminescence as compared to control (157 to 190 CPS). The bark extract obtained from *R. annamalayana* (at 1.0 mg/mL) decreased the bioluminescence produced by *V. harveyi* MTCC 3438 and also decreased its growth to 99% (Gao & Xiao, 2012). Present study agrees with the presence

Table 4. Effect of leaves extract of *R. apiculata* against the cumulative percentage mortality decrease in *P. monodon* postlarvae caused by *V. harveyi.*

Parameters	Days						
	0	**5th**	**10th**	**15th**	**20th**	**25th**	**30th**
Cumulative percentage mortality (CPM)							
Control (PL with *V. harveyi*)	0	9.36±0.3	19.13±0.9	27.47±1.2	39.13±1.6	48.82±2.1	68.50±2.3
Treatment (PL with extract and *V. harveyi*)	0	5.31±0.2	14.56±0.3	22.36±1.5	31.76±1.4	40.91±1.7	57.88±2.5
Control (PL with extract)	0	3.91±0.13	6.83±0.5	11.69±0.4	17.08±0.6	21.57±0.6	26.59±1.1
Control (PL alone)	0	3.09±0.1	6.31±0.2	11.63±0.5	17.89±0.5	22.36±1.0	27.19±1.0
Treatment tubs (cfu/mL)							
Total plate count	1.51×10^5	1.19×10^4	1.7×10^4	8.0×10^4	1.36×10^4	3.6×10^3	5.2×10^3
V. harveyi count	1.46×10^5	8.7×10^3	8.5×10^4	1.9×10^4	2.17×10^3	1.83×10^3	3.0×10^3
Control tubs (cfu/mL)							
Total plate count	1.39×10^5	1.7×10^5	1.9×10^4	2.09×10^5	1.38×10^4	9.0×10^3	7.2×10^3
V. harveyi count	1.33×10^5	2.75×10^5	2.18×10^5	1.17×10^3	2.0×10^3	1.82×10^4	9.2×10^3
Average weight of PL (mg)							
Treatment tubs	18.9±2	62.5±5	136.3±8	169.6±10	218.9±11	269.3±14	293.6±23
Control tubs	18.7±3	59.6±9	133.3±7	164.8±13	221.9±12	259.2±16	291.9±21
Water quality parameters (Treatment and Control)							
Temperature (°C)	31±1.0	31±1.0	30±1.0	31±1.0	31±1.0	30±1.0	31±1.0
Salinity (PSU)	20±0.5	20±0.5	20±0.5	20±0.5	21±0.5	21±0.5	21±0.5
pH in control tubs	8.30±0.2	8.40±0.2	8.20±0.2	8.30±0.2	8.20±0.2	8.10±0.2	8.30±0.2
pH in treatment tubs	8.30±0.2	8.40±0.2	8.30±0.2	8.40±0.2	8.30±0.2	8.20±0.2	8.30±0.2

Values are the average of three determinations with standard deviation (SD). Significant ($p<0.05$) differences were found between the leaves extract of *R. apiculata* treated with *V. harveyi* and control. PL: postlarvae, PSU: practical salinity unit (1 PSU = 1g/kg).

of similar kind of compound pattern also reported earlier by Abidin *et al*. (2013) and Satyavani *et al*. (2015), in the extracts of leaves of *R. apiculata* were reported to contain 18 phyto-compounds, major compounds 1-adamantly–p-me-thylbenzalimine, clivorin, 4-butyl pyridine, 1-oxide, acetamide and p-aminodiethylaniline, also the *R. apiculata* leaves were reported to contain 2-(2- ethoxy-ethoxy) ethanol (26.45%) and Kaur-16-ene (3.37%) (Selvaraj *et al*., 2014), whereas the bark of *R.apiculata* contains phenolic compounds like lyoniresinol-3α-*O*-β-arabinopyranoside, lyoniresinol-3α-*O*-β-rhamnoside, afzelechin-3-rhamnoside and butylated hydroxy anisole which exhibited antioxidant activities (Halim *et al*., 2013). Hong *et al*. (2011) also reported essential oils, higher alkanes, acids, alcohol and esters from *R. apiculata*. The cumulative percentage mortality decrease in postlarvae caused by *V. harveyi* was found to be 10.6% on treating with *R. apiculata* extract till 30 days. The crude extract obtained from the bark of *R. apiculata* was found to contain tannin that show antimicrobial activity (Shamsuddin *et al*., 2013) and proved to be non-lethal to brine shrimp. But Sivakumar *et al*. (2014) have reported that when *Ulva fasciata* extract was tested on *V. harveyi* during *P. monodon* larviculture, the decrease on cumulative percentage of mortality on postlarvae caused by *V. harveyi* was found to be 32.4%. Methanol extract from *R. mucronata* also showed inhibition against *V. harveyi* (Ramesh *et al*., 2014). When shrimps were fed with *R. apiculata* leaves during shrimp grow–out practices, the survival and biomass was increased significantly (Nga *et al*., 2006). The values observed from the bio-assay of *R. apiculata* extract against the *V. harveyi* during *P. monodon* larviculture revealed significant differences ($p < 0.05$) between the *R. apiculata* extract treated *V. harveyi* infected cultures and control.

Our results indicate that the ethyl acetate extracts of *R. apiculata* inhibited growth and modulated virulence factors produced by *V. harveyi*. This extract also controlled the mortality caused by *V. harveyi* during shrimp larviculture. Based on this study, *R. apiculata* extract could be used as an alternative bio-product for aquaculture practices.

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