

EFFECTS OF A MARINE DIATOM *SKELETONEMA COSTATUM* EXTRACT ON LUMINESCENCE DISEASE CAUSING *VIBRIO HARVEYI* DURING *PENAEUS MONODON* LARVICULTURE

KRISHNAMOORTHY SIVAKUMAR¹ AND SUDALAYANDI KANNAPPAN*

Crustacean Culture Division, ICAR, Central Institute
of Brackishwater Aquaculture, 75 Santhome High Road, MRC Nagar,
Chennai - 600 028, Tamil Nadu, India

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Abstract

The crude extract of *Skeletonema costatum* obtained through cold extraction method was characterized for its phytochemicals, using FTIR and GC-MS. The antagonism of *S. costatum* extract was tested against *Vibrio harveyi*, its various virulence factors, and also tested against *Vibrio harveyi* during *Penaeus monodon* larviculture. *S. costatum* extract, controlled the growth of *V. harveyi*. The zones of inhibition were 12.6 ± 0.2 , 6.6 ± 0.1 and 3.7 ± 0.1 mm at 300, 200 and 100 μg , respectively. The production of bio-luminescence was also reduced to 10.0, 11.0, 20.0, 25.0 CPS. The extract contained fatty acids such as 16-octadecanoic acid (48.05%) and pentadecanoic acid (18.94%) as evidenced by GC-MS. *S. costatum* extract revealed various compounds *viz.*, alcohols, phenols, etc, which are responsible for controlling *V. harveyi* and virulence factors. During *P. monodon* larviculture, *S. costatum* extract at 200 $\mu\text{g/ml}$ showed reduction ($p < 0.05$) in the cumulative percentage of mortality caused by *V. harveyi* ($35.20 \pm 0.6\%$) as compared to control ($76.30 \pm 0.8\%$). It can be concluded that *S. costatum* extract may be used to control disease-causing *V. harveyi* during shrimp larviculture.

Introduction

Worldwide demand for shrimps has remarkably improved and sea catches are deficient to meet out, consequently, shrimps are grown in an intensive way. Development of aquaculture has been distinctive in displaying the overpowering modifications in global fish/shrimp production for many years. The advancement in aquaculture resulted in the emergence of growing unknown diseases (Tandel *et al.* 2017). Opposing influences of aquatic diseases on the production of global aquaculture are distressing. Amongst disease-causing agents, *Vibrio harveyi*, a bio-luminescent bacterium has been found to cause 80 - 100% mortality among the postlarvae of shrimp hatcheries (Raissy *et al.* 2011). In order to govern shrimp's health various types of chemicals are used on a large scale. Controlling of bacterial diseases by preservatives during shrimp culture practices, may develop resistance among them. Therefore, alternate eco-friendly resources may be explored to control shrimp diseases. Seaweeds produce plentiful bioactive molecules which may be valuable as an antimicrobial agent against aquatic bacteria. The leaves extract of a mangrove plant, *Rhizophora apiculata* was evidenced to control *Vibrio harveyi* (Kannappan *et al.* 2018) during shrimp larviculture. The crude extract of the marine herb, *Sesuvium portulacastrum* and macro algae, *Ulva* spp. has been established to control *V. harveyi* (Dineshkumar *et al.* 2017a) and other bacteria during shrimp larviculture.

Skeletonema costatum, a marine diatom, inhabited near the shore waters of the sea, forms the feeding ground for crustacean larvae which has been designated to have many bio-active compounds (Prariono *et al.* 2013) and its antibacterial activity has been tested on fish and shellfish borne bacterial pathogens (Naviner *et al.* 1999). The crude extract of *S. costatum* was found to be inhibitory on *V. anguillarum* and other pathogens such as *Klebsiella pneumonia* and *Salmonella*

*Author for correspondence: <sudalikanna@gmail.com>. ¹ICAR-Krishi Vigyan Kendra, Tamil Nadu Veterinary and Animal Sciences University, Kattupakkam-603 203, Tamil Nadu, India.

typhi (Shanmugapriya and Ramanathan 2011). *V. harveyi* has been well-known as one of the disease-causing bacteria for various fish/shrimps as it produces virulence factors such as bioluminescence, proteases, phospholipases, lipases, siderophores, chitinases and haemolysins (Soto-Rodriguez *et al.* 2012).

Lipids and fatty acids from marine algae have been recognized to antagonize microbes by disruption of their cell membrane (Bergsson *et al.* 2011). Maintaining primary productivity in the fish/shrimp grow-out ponds through microalgae had shown the inhibitory effect against luminous *V. harveyi* (Tendencia *et al.* 2006). The present investigation was focussed on the antagonism of crude extract, *S. costatum* against growth and virulence of the luminescence disease-causing *V. harveyi* during shrimp larviculture. Characterization of various functional compounds from the extract by Fourier transform infrared spectroscopy (FTIR), Gas chromatography and mass spectroscopy (GC-MS) and then testing against luminescence disease causing *V. harveyi* during *P. monodon* larviculture was carried out.

Materials and Methods

Vibrio harveyi strains were isolated from the infected *Penaeus monodon* and then identified and confirmed by PCR (Maiti *et al.* 2009). The strains were stored in Luria-Bertani (LB) broth with sterile glycerol (15% v/v) and named them as *V. harveyi* 1 to *V. harveyi* 20 for further use. *V. harveyi* (MTCC 3438) was used as a reference strain.

Skeletonema costatum was collected from the Muttukadu lagoon, Chennai, India using 10 µm plankton net and then observed under the microscope. The cells were observed as cylindrical with long tubular structure and then mass cultured in out-door FRP tanks (5000 litres) using Guillard's medium (F/2 medium, Guillard and Ryther 1962) with aeration at 28°C. The cells were collected using a plankton net (10 µm). The collected algae were shade dried, then stored at 4°C, later used for extraction. One gram of dried *S. costatum* powder was mixed in 10ml of ethyl acetate and shaker incubated at 30°C @ 50 rpm/96 hrs for extraction of compounds called as "Cold extraction method" (Das *et al.* 2005). Afterwards, the extracts were filtered by Whatman filter paper (No.1), rotary evaporated at 30°C/3 hrs and then stored in darkness at 4°C. For antibacterial activity, the extract was liquefied with 5 mg/ml of 30% (v/v) DMSO (Dimethyl sulfoxide) and tested against *V. harveyi* through Agar well diffusion assay (Chellaram *et al.* 2011). DMSO @200 µl of 30% (v/v) was used as control. MIC was evaluated against *V. harveyi*. *S. costatum* cells alone were evaluated against *V. harveyi* for antagonism (Yamasaki *et al.* 2011). Ten µl of *V. harveyi* (2.19×10^7 cfu/ml) and 100 ml of *S. costatum* cells (10^5 cells/ml or 0.151 OD₇₀₀) was inoculated in F/2 medium and co-cultured for 5 days. For inhibition, 2 ml of co-culture broth was analyzed for mean differences of *V. harveyi* counts between the control and treatments as observed in darkroom as well as determined using *Vibrio harveyi* Selective Agar (VHSA) medium. The mean differences of *S. costatum* counts were estimated by Sedgewick counting chamber. Agar overlay method (Chellaram *et al.* 2011) was used to study about antagonism of *S. costatum* cells against *V. harveyi*. Initially *S. costatum* was grown (10^5 cells/ml or 0.151 OD₇₀₀) in F/2 medium, then centrifuged and spotted (50 µl) on agar containing F2 medium and incubated for 96 hrs/28°C. *V. harveyi* cells were grown (2.19×10^7 cfu/ml) overnight in LB broth at 28°C, and inoculated (20 µl) into semi-solid LB agar medium and poured over the spotted *S. costatum* cells. After 24 hrs of incubation, the zone of inhibition around *S. costatum* indicated as an inhibition on *V. harveyi*.

Extract of *S. costatum* (300 µg/ml) was taken in 100 ml of LB medium. *V. harveyi* cells (500 µl of 2.19×10^7 cfu/ml) were inoculated and incubated at 28°C/100 rpm/5days in a shaker incubator. The growth of *V. harveyi* with virulence factors such as luminescence, protease, crude bacteriocin, exopolysaccharide was measured by the spectrometer. The Phospholipase,

proteolysis, lipolysis and thermonuclease were tested based on the hydrolysis of the medium in the plate assay. The activities were marked with qualitative parameters as weak, moderate, high and very high. Surface hydrophobicity was determined by salt aggregation test (SAT) as the lowest molarity of ammonium sulfate (0.05 - 4.0 M) that caused visible agglutination by *V. harveyi* and Cell adhesion to hydrocarbons Test (BATH) (Soto-Rodriguez *et al.* 2012). For estimation of luminescence, the spent culture of *V. harveyi* was used and reading was observed in 10 sec as light emission by Luminometer (Perkin Elmer) after shaking the plates and expressed as counts per second (i.e., photons per second). For determining various functional compounds, the shade dried extract was powdered and compounds were detected and recorded using BRUKER IFS 66 model FTIR spectrometer (D'Souza *et al.* 2008).

GC-MS analysis was performed (Elumalai and Sakthivel 2013) using JEOL system (Joel, GCMate II, Akishima, Tokyo, Japan) equipped with a mass selective detector with a capillary column (30 m × 0.25 mm × 0.25 mm) using helium at 1 ml/min as a carrier. Peaks identification from crude *S. costatum* extract was identified by comparison with retention times of standards. The mass spectra obtained were compared with those available in the installed NIST libraries.

PCR tested postlarvae (PL17) of *P. monodon*, procured from a private shrimp hatchery was acclimatized to saline water @20 PSU (Practical Salinity Unit) for 5 days/28°C. The challenge tests were carried out in 3 plastic tubs (50 litres), disinfected prior with 10 mg/l of KMnO₄ (w/v)/10 min and filled with 20 litres of saline water (20 PSU). The control tub was inoculated with *V. harveyi* alone (10 ml of 1.80 OD₆₀₀). The second tub was treatment, where PL was inoculated with *V. harveyi*. Then 200 µg of *S. costatum* extract per ml (2 gm/10 litre) of saline water. The third tub was a control where *S. costatum* extract was added (200 µg/ml) in saline water alone with PL and without *V. harveyi* (Kannappan *et al.* 2018). The PL feed (CPTM) was given twice/day @15%. The experimental tubs were covered at the top to avoid contamination (Traifalgar *et al.* 2009). The mortality of PL was ascertained by counting the number of dead larvae. The total heterotrophic bacteria and *V. harveyi* counts were determined using a selective medium. All the experiments were executed in triplicate and expressed as means with SD. Data obtained were analyzed by ANOVA using SPSS software version 16.0. The significance level was kept at $p < 0.05$.

Results and Discussion

The biomass of *S. costatum* cell density showed 2.56 ± 0.2 g/l (WWB) and 0.216 ± 0.01 g/l in (DWB) in out-door culture. The extract of *S. costatum* showed a zone of inhibition of 12.9 ± 0.2 , 6.6 ± 0.1 and 3.7 ± 0.1 mm at 300, 200 and 100 µg, respectively against *V. harveyi* (Table 1). In control, DMSO did not show any inhibition. This study revealed that 200 µg of *S. costatum* extract was inhibitory to *V. harveyi* (6.8 ± 0.2 mm) and the same concentration of the extract was found to inhibit the growth of *V. harveyi* during shrimp larviculture. When *S. costatum* cells (50 µl) alone (9.13×10^5 cells/ml) were spotted on an agar plate, 17.30 ± 0.1 mm was observed as an inhibitory zone against the growth of *V. harveyi*. Marine microalgae such as *Chlorella vulgaris*, *Spirulina platensis* and *Nannochloropsis bacillaris* also showed antagonism against *Vibrio* sp. (Dineshkumar *et al.* 2017b).

During co-culture, *S. costatum* reduced the growth of *V. harveyi* from 2.32 - 0.47 logs from 1st to 5th day whereas, in control, it varied from 3.38 - 3.11 log. The differences from 1st day to 5th day ranged from 1.06 - 2.64 logs. During co-culture, *S. costatum*, varied from 8.32×10^5 to 9.13×10^5 cells/ml from 1st to 5th days, but in control, *S. costatum* showed from 8.29×10^5 to 9.09×10^5 cells/ml. The difference among the cells was observed as 3.0×10^3 to 4.0×10^3 cells/ml from 1st to 5th day (Table 2). This study confirmed that *S. costatum* cells reduced the load of *V.*

harveyi, 1.06 - 2.64 log as compared to control (3.38 - 3.11 log). Similarly, Tendencia *et al.* (2006) had reported inhibition on *V. harveyi* (3.0 logs) by co-culturing with *Chlorella* spp. While treating *V. harveyi* with *S. costatum* extract, the growth of *V. harveyi* reduced from 1st to 5th day. The highest growth difference was observed on 3rd day (0.375 OD) and lowest on 1st day (0.223) as compared to control. The maximum reduction on bacteriocin production was 0.231 OD on 3rd day and minimum (0.051 OD) was observed on 2nd day (Table 3). However, the reduction of crude extracellular protein released was noticed daily in all the treatments.

Table 1. Antagonism of *S. costatum* extract against *V. harveyi* through agar well diffusion assay.

Extract	Zone of inhibition (mm)			
	100 µg	200 µg	300 µg	DMSO
<i>S. costatum</i>	3.7 ± 0.1	6.6 ± 0.1	12.9 ± 0.2	0.0 ± 0.0

Table 2. Growth of *S. costatum* during co-culture with *V. harveyi*.

Tests	Cell counts (cells/ml)					
	Inoculation day	Day 1	Day 2	Day 3	Day 4	Day 5
<i>S. costatum</i> (Control)	8.12×10^5	8.29×10^5	8.52×10^5	8.75×10^5	8.89×10^5	9.09×10^5
<i>S. costatum</i> and <i>V. harveyi</i>	8.15×10^5	8.32×10^5	8.54×10^5	8.81×10^5	8.92×10^5	9.13×10^5
Difference in cell counts	-3×10^3	-3.0×10^3	-2.0×10^3	-6.0×10^3	-3.0×10^3	-4.0×10^3

The production of luminescence by *V. harveyi* was reduced to 10.0, 11.0, 20.0, 25.0 CPS for 4 days, compared to control (39.6, 50.3, 59.3 and 63.6) (Fig. 1). The maximum reduction on luminescence was observed on 4th day (25 CPS) and minimum on day 1 (10.0 CPS). In treatments, a moderate level of reduction on phospholipase and proteolysis was noticed from 1st to 5th day. However, moderate level of lipolysis and thermonuclease activities were observed on 1st to 3rd, 4th and 5th days as compared to control (very strong). In the SAT test, *V. harveyi* as a control revealed strong hydrophobic activity from 1st to 5th day whereas, in treatment *V. harveyi* showed moderate hydrophobic activity from 1st to 5th day.

Microalgae possess extracellular products that can influence the microbes and their surroundings (Natrah *et al.* 2014). *Skeletonema marinoi* and other diatoms are well-known to produce toxic polyunsaturated aldehydes (PUAs) in response to cell damage that can affect different organisms including bacteria (Taylor *et al.* 2009). *V. harveyi* was well-known to produce extracellular compounds that are deliberated as the essential virulence determinants of *V. harveyi* (Soto-Rodriguez *et al.* 2012). In microalgae, fatty acids get congregated in the lipids of cell membranes which will be released by host lipolytic enzymes during cellular disintegration. Further, fatty acids are active antimicrobial compounds and related protection may be afforded to microalgae under stressful conditions from pathogenic microbes (Rangaia *et al.* 2010). Here, exopolysaccharide and protease production by *V. harveyi* was slowly reduced due to the extract of *S. costatum* as compared to control. The differences of pathogenicity among *V. harveyi* isolates revealed that proteases, hemolysins, phospholipase and other exotoxins may exert substantial roles in the virulence of *V. harveyi* (Austin and Zhang 2006).

The FTIR spectrum of *S. costatum* had shown different functional groups such as alcohols, phenols, unsaturated esters, aldehydes and ketones, esters, ethers, alkenes, primary amines, nitro compounds, aromatics and carboxylic acids, etc. (Fig. 2). Similar values were reported for

Chlorella vulgaris (Duygu *et al.* 2012). The absorption spectrum of FTIR analysis exhibited 5 important peaks like aldehyde (C=O), esters (C-O), methylene (CH₂), methyl (CH₃), and hydroxyl bond absorptions (O-H), which were also sturdy in lipid content for many microalgae species (Yin *et al.* 2011). Marine algae produce fatty acids like tetradecanoic acid, hexadecanoic acid, octadecanoic acid methyl esters etc. (Musharraf *et al.* 2012) that show bioactivity. In this study, the antagonism of *S. costatum* against *V. harveyi* may be due chemical constituents such as 16-octadecanoic acid, methyl ester (48.05%) followed by pentadecanoic acid, 13-methyl, methylester (18.94%).

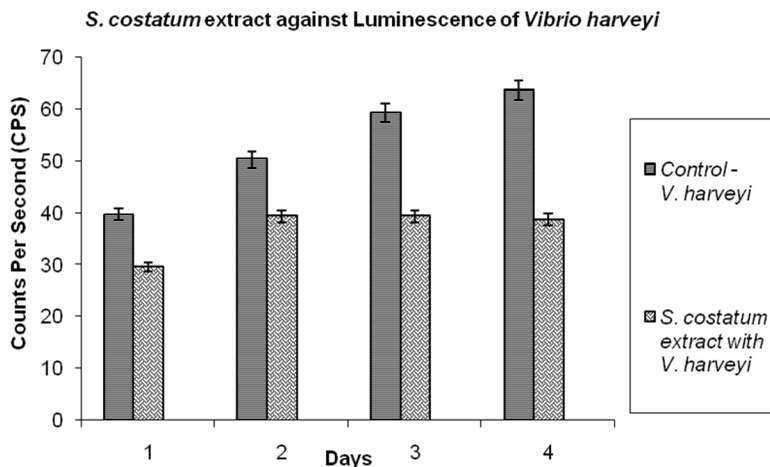


Fig. 1. Effects of *S. costatum* extract against luminescence produced by *V. harveyi*.

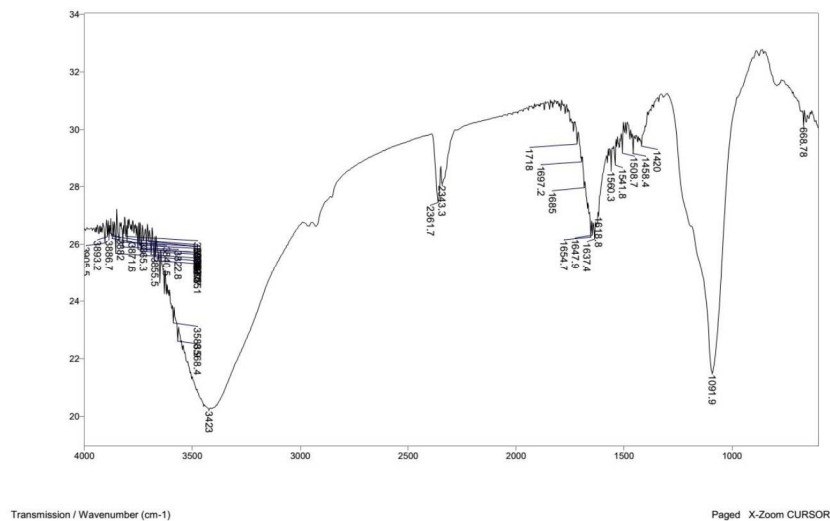


Fig. 2. FTIR spectrum of *S. costatum*.

By GC-MS analysis, the extract of *S. costatum* was found to have a mixture of volatile compounds. A total of 10 peaks were observed with respective retention times in GC-MS chromatogram (Fig. 3). The main chemical-constituent was 16-octadecanoic acid, methyl ester (tR = 10.30 min) (48.05%) followed by pentadecanoic acid, 13 methyl, methyl ester (tR = 9.40 min)

Table 3. Effects of *S. costatum* extract against virulence factors of *V. harveyi*.

Days	Virulence factors analyzed											
	Proteolysis		Phospholipase		Lipolysis		Thermonuclease		Cell surface hydrophobicity			
	Control	Treated	Control	Treated	Control	Treated	Control	Treated	SAT (M)		BATH (%)	
1	++++	++	++++	++	++++	++	++++	++	0.94 ± 0.03	1.09 ± 0.04	88.13 ± 3.33	43.33 ± 1.31
2	++++	++	++++	++	++++	++	++++	++	0.89 ± 0.02	1.13 ± 0.03	86.66 ± 3.66	40.11 ± 1.65
3	++++	+	++++	+	++++	++	++++	++	0.92 ± 0.04	1.26 ± 0.05	83.33 ± 2.56	33.33 ± 1.12
4	++++	+	++++	+	++++	++	++++	++	0.95 ± 0.03	1.39 ± 0.04	78.63 ± 2.61	30.66 ± 1.00
5	++++	+	++++	+	++++	++	++++	++	0.98 ± 0.03	1.43 ± 0.05	73.76 ± 1.91	26.56 ± 1.11

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).

Table 4. Effects of *S. costatum* extract against *V. harveyi* in *P. monodon* larviculture.

Day	Cumulative % mortality (CPM)				Treated tubs (cfu/ml)		Control tubs (cfu/ml)		Average wt. of postlarvae (mg)		Water quality			
	Control tubs with <i>V. harveyi</i>	Treated tubs with <i>V. harveyi</i>	Tubs with extract and PL alone	Tubs with PL alone	Total plate count (cfu/ml)	<i>V. harveyi</i> (cfu/ml)	Total plate count (cfu/ml)	<i>V. harveyi</i> (cfu/ml)	Treated tubs	Control tubs	Temp. (°C)	Salinity (PSU)	pH in control tubs	pH in treated tubs
0	0.00	0.00	0.00	0.00	2.14×10^6	1.78×10^6	2.59×10^6	1.86×10^6	18.9 ± 3	18.3 ± 4	29.0 ± 1.0	20 ± 0.5	8.40 ± 0.2	8.30 ± 0.2
5 th	13.66 ± 0.3	07.77 ± 0.2	2.39 ± 0.1	3.23 ± 0.1	1.03×10^5	4.60×10^4	1.45×10^5	1.17×10^5	67.5 ± 5	68.1 ± 5	29.5 ± 1.0	20 ± 0.5	8.50 ± 0.2	8.40 ± 0.2
10 th	26.05 ± 0.9	16.56 ± 0.3	6.19 ± 0.2	6.03 ± 0.2	2.12×10^5	1.81×10^4	2.74×10^5	2.62×10^5	129.4 ± 4	133.6 ± 6	29.0 ± 1.0	20 ± 0.5	8.40 ± 0.2	8.30 ± 0.2
15 th	35.63 ± 1.1	20.36 ± 0.5	12.05 ± 0.5	13.33 ± 0.5	8.85×10^4	4.90×10^3	1.46×10^4	7.40×10^4	168.5 ± 9	167.6 ± 8	30.0 ± 1.0	21 ± 0.5	8.20 ± 0.2	8.50 ± 0.2
20 th	47.33 ± 1.5	27.05 ± 0.7	18.13 ± 0.6	17.43 ± 0.5	1.05×10^4	4.70×10^3	7.80×10^4	2.02×10^4	218.6 ± 11	217.7 ± 13	30.0 ± 1.0	21 ± 0.5	8.00 ± 0.2	8.30 ± 0.2
25 th	62.13 ± 2.3	34.13 ± 1.1	24.69 ± 0.9	23.86 ± 1.0	9.20×10^4	6.80×10^3	1.88×10^4	2.53×10^4	249.7 ± 15	251.5 ± 13	31.0 ± 1.0	21 ± 0.5	8.20 ± 0.2	8.20 ± 0.2
30 th	76.30 ± 2.9	41.10 ± 1.5	29.56 ± 1.0	28.39 ± 1.0	6.40×10^4	4.20×10^3	2.06×10^4	1.53×10^4	277.4 ± 10	279.8 ± 12	30.0 ± 1.0	21 ± 0.5	8.10 ± 0.2	8.00 ± 0.2

(18.94%). When *S. costatum* extract was tested against *V. harveyi* during larviculture, the reduction on the cumulative percentage of mortality (CPM) in *P. monodon* larvae was 35.20 ± 0.6 , compared to control (76.30 ± 0.8). On the 30th day of sampling, the average weights of the PL were 279.8 ± 0.01 mg and 277.4 ± 0.02 mg for control and treatment, respectively and were not considerably dissimilar from one another. The maximum decrease of luminous *V. harveyi* counts, observed during 30 days ranged from 4.60×10^4 to 6.80×10^3 cfu/ml, respectively, compared to control (1.17×10^5 to 1.53×10^4 cfu/ml) (Table 4). Water quality parameters did not change significantly in treatment (Table 4) and control. However, in the treatment, with extract alone, there was the development of a slight greenish colour was noticed as compared to control, possibly due to the crude nature of the extract.

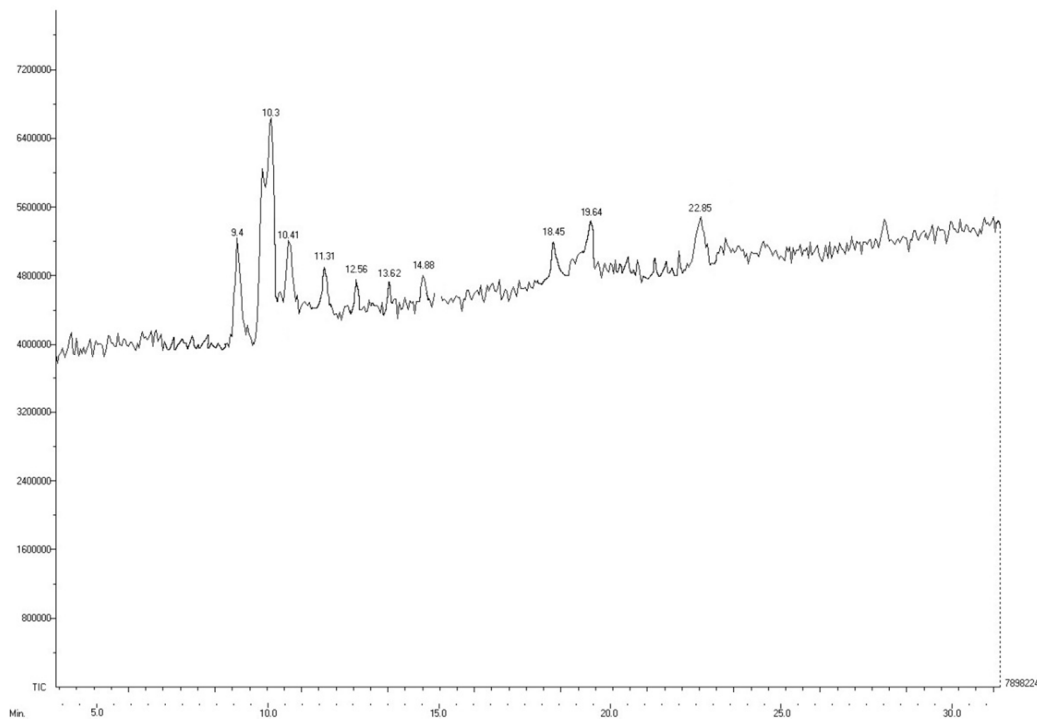


Fig. 3. GC-MS chromatogram of crude ethyl acetate extract of *S. costatum*.

Unicellular algae such as *Chaetoceros*, *Tetraselmis* spp., *S. costatum* and *Isochrysis galbana* @ 40,000 to 1,00,000 cells/ml are being used for the developmental stages of penaeid shrimps. Hence, the crude extract of *S. costatum* also has a prospective for use in shrimp larviculture. Fatty acids having a chain length of more than 10 carbon atoms would induce lysis of bacterial protoplasts and may also affect the expression of bacterial virulence factors, which are essential for the establishment of an infection. It is reported that lipids inhibit microbes by disrupting cellular membrane (Bergsson *et al.* 2011) of bacteria, fungi and yeasts. Organic extract of *S. costatum* exhibited antagonism against *V. fisheri* and *V. parahaemolyticus* (Naviner *et al.* 1999)

The outcome of this study specifies that the crude extract of *S. costatum* showed a zone of inhibition on *V. harveyi* @ 300 µg and also has decreased its virulence factors. During co-culture, *S. costatum* reduced the growth of *V. harveyi*. When *S. costatum* extract was tested against *V. harveyi* during *P. monodon* larviculture, a reduction on the cumulative percentage of mortality

(35.20 ± 0.6%) was noticed on *P. monodon* larvae. It could be inferred that *S. costatum* extract can be used as bio-agent for controlling *V. harveyi*. Application of natural bio-agent like *S. costatum* extract would moderate the undesirable contamination from applying the chemical compounds with reduced cost and eco-friendly nature. However, more studies on docking of algae molecules are necessary before using such extracts as therapeutic agents in shrimp larviculture.

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