Antifouling and toxic properties of the bioactive metabolites from the seagrasses *Syringodium isoetifolium* and *Cymodocea serrulata*

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Abstract

The present study documents the antifouling and toxic properties of seagrasses *Syringodium isoetifolium* and *Cymodocea serrulata*. For that, the seagrasses *S. isoetifolium* and *C. serrulata* were extracted individually using organic solvents viz. dichloromethane, acetone and methanol. Amongst the extracts, the maximum antimicrofouling and antimacrofouling activities were exhibited by methanol extracts of both the seagrasses. The Minimal Inhibitory Concentration (MIC) of methanolic extracts of seagrasses was ranged from 1.0 to 10 mg/ml against test biofilm bacteria and microalgal strains. Similarly, 100% fouling inhibition of limpet *Patella vulgata* was found at 6.0 mg/ml of methanolic extracts of seagrasses. The mussel *Perna indica* showed 50% of byssal production and attachment inhibition at 21.51 ± 2.03, 17.82 ± 1.07 µg/ml and the anticrustaecean activity for 50% mortality of *Artemia salina* was recorded at 732.14 ± 9.21 and 394.16 ± 5.16 µg/ml respectively for methanolic extracts of *S. isoetifolium* and *C. serrulata*. The minimal inhibitory and higher lethal concentrations of active methanol extracts shows it's less toxic nature. Based on the prolific results, methanol extracts of *S. isoetifolium* and *C. serrulata* were subjected to purification using silica gel column and thin layer chromatography. Then the active compounds of the bioassay guided fractions were partially characterized using gas chromatography coupled with mass spectroscopy (GC-MS) and keyed out that fatty acids (C_{16} to C_{24}) were the major components which responsible for the antifouling properties of the candidate seagrasses.

1. Introduction

Marine biofouling, can be defined as the growth of unwanted organisms on the surface of artificial structures immersed in water (Yebra et al., 2004; Buma et al., 2009). Biofouling causes huge material and economic costs of maintenance of mariculture, naval vessels, and seawater pipelines (Yebra et al., 2004). It is estimated that governments and industry spend over US $6.5 billion annually to prevent and control marine biofouling (Bhadury and Wright, 2004). Further, ecological implications of biofouling include increased carbon emission and potential dispersion of invasive alien species (Bellas, 2006; Floerl et al., 2009; Silkina et al., 2012).

Antifouling is the process of controlling or mitigating the settlement of fouling organisms on a surface. Commercial antifouling techniques include mechanical cleaning, biocides, toxic antifouling coatings and foul release or easy clean coatings. Amongst the above, antifouling paints containing toxic chemicals are the main strategies against biofouling in the past. Tributyltin (TBT) was the most effective component in antifouling paints which was detrimental, not readily degraded in the natural environments and had non-targeted toxicity on organisms (Konstantinou and Albanis, 2004). This property has led the International Maritime Organisation (IMO) to prohibit its application to ships since 17 September 2008 (Qian et al., 2010). The substitutes of TBT, such as Irgarol 1051 and Diuron, have also been found to be harmful to many non-target organisms (Konstantinou and Albanis, 2004; Zhou et al., 2006).

Hence, alternative and environmentally acceptable, safe and effective antifouling substances are needed for incorporation into antifouling coatings, and these may include natural products isolated from certain marine organisms (Clare, 1996). Incorporation of natural repellent products into antifouling paints has been tried by some researchers (Armstrong et al., 2000; Peppiatt et al., 2000). For this, a wide range of marine natural products have been screened for their activity concerning antimicrobial, antifungal, antialgal and antilarval properties (reviewed by Clare, 1996; Fusetani, 2004; Dobretsov et al., 2006). Compounds with antifouling potential have been studied intensively in various marine sponges (Tsouletou et al., 2002; Hellio et al., 2005) and algae (De Nys et al., 1995; Maximilien et al., 1998; Sjogren et al., 2004).
Marine natural products or crude extracts with antifouling activity have been reported from many marine organisms including marine bacteria, seaweeds, seagrasses, bryozoans, ascidians, cnidarians and sponges (Pawlik, 1992; Clare, 1996; Rittschof, 2001).

Antifouling and biological activities of marine macrophytes have been extensively studied by many researchers in various species of mangroves (Chen et al., 2008), seaweeds (Silkina et al., 2012) and seagrasses (Mayavu et al., 2009; Prabhakaran et al., 2012). Seagrasses are a rich source of secondary metabolites, particularly phenolic compounds (McMillan et al., 1980). Seagrass phenolic compounds include sulfated flavonoids, a group of conjugated metabolites for which the sulfate component is believed to represent a marine adaptation (Harborne and Williams, 1976). Phenolic compounds are well known allelopathic agents present in terrestrial plants (Swain, 1977). In support of this suggestion, polyphenolic compounds have long been associated with reduced fouling in seaweeds (Jennings and Steinberg, 1997) and it has been proposed that eelgrass chemistry alters the composition of the epiphytic community (Harrison, 1982). In addition, the total concentration of phenolic compounds in *Zostera marina* was shown to increase in response to infection by *Labyrinthula zostera* (Vergeer et al., 1995), and a phenolic compound purified from this seagrass had antifouling activity (Todd et al. 1993).

Considering the need for ecological safety and lack of information on natural antifouling compounds from marine flora, especially from seagrasses, the present work was undertaken to explore the antifouling, toxic properties of the seagrasses and also to investigate the bioactive constituents of *Syringodium isoetifolium* and *Cymodocea serrulata*.

2. Materials and methods

2.1. Seagrasses

For the present study, the following seagrass species were selected for being free of epibionts during visual examination. The fresh leaves of *S. isoetifolium* (Order: Potamogetonales, Family: Cymodoceaceae) and *C. serrulata* (Order: Potamogetonales, Family: Cymodoceaceae) were collected from the Arockiapuram coast (Lat 8° 06' 46.1" Long 77° 33' 21.9") of Kanyakumari District, Tamilnadu, India.

2.2. Extraction

Collected seagrasses were washed thoroughly with sterile seawater to remove the extraneous dirt and 3% ethanol (97% distilled water: 3% ethanol) was used to wipe off the epiphytes. Then the seagrasses were dried well in an incubator at 30 1C and finely powdered using electrical grinder. Hundred grams of each seagrass powder were extracted individually in 500 ml organic solvent, including dichloromethane, acetone and methanol. Extraction was done in darkness, at room temperature: 20 ± 2 °C. The process was repeated thrice; extracts were pooled and filtered through Whatmann no. 1 filter paper. Each filtrate was dried under reduced pressure using a rotary evaporator. Dried extracts were weighed and stored in screw cap vials for further study.

2.3. Test organisms

Antimicrofouling activity of seagrass extracts were tested against 10 biofilm bacteria such as *Pseudomonas aeruginosa* JN979983, *Halomonas aquamarina* JN561698, *Vibrio alginolyticus* JN979984, *Pantoea agglomerans* JN979985, *Serratia marcescens* JN596118, *Serratia liquefaciens* JN596115, *Vibrio fischeri* JN979986, *Vibrio parahaemolyticus* JN585666, *Shigella flexneri* JN979987 and *Aeromonas hydrophila* JN561697 collected from the microbial culture collections of Centre for Marine Sciences and Technology, Manonmaniam Sundaranar University, Rajakkamangalam, Tamilnadu, India. To screen the antimicroalgal activity, five fouling microalgal strains such as *Pleurosigma elongatum* CASMB 001, *Thalassiothrix frauenfeldii* CASMB 002, *Nitzschia sigma* CASMB 003, *Navicula longa* CASMB 004 and *Astreonellopsis glacialis* CASMB 005

were collected from the microalgal culture collections of CAS in Marine Biology, Annamalai University, Tamilnadu, India. The antimacrofouling property of the seagrass extracts was screened using the limpet *Patella vulgata* and brown mussel *Perna indica*. These animals were collected from the rocky surfaces of Manavalakurichi coast, (Lat. 8°8' 35" and Long. 77°8' 00"), Kanyakumari District, Tamilnadu, India. For cytotoxicity assay, the brine shrimp *Artemia salina* was hatched out from the cysts (San Fransisco Bay, NC, USA).

2.4. Antimicrofouling activity

2.4.1. Antibacterial assay

Antibacterial activity of seagrass extracts was assessed by following the method of Marechal et al. (2004). The assay was started with the inoculation of the same density of bacteria (2.10^8 cells/ml) using the table of Amsterdam (1996). 100 µl of each seagrass extract with the concentrations of 0.01, 0.1, 1.0, 10, 25, 50 and 100 µg/ml were poured individually in 6 wells of 96 wells plate for each bacterial assay. In addition, 6 wells free of extracts were used as a control. These plates were dried under UV chamber for 2 h to evaporate the solvent under sterile condition. 100 ml of the bacterial suspension were then added under aseptic conditions and the plates were incubated at 30 °C for 48 h to allow the bacterial growth. One plate was used for each test bacterium to decrease the risk of contamination. The least concentration of extract where no turbidity was observed in at least 4 of the 6 wells was noted as the minimum inhibitory concentration (MIC).

2.4.2. Anti-microalgal assay

Similar to the antibacterial assay, anti-microalgal assay was also conducted with the same test concentrations of seagrass extracts using 96 well plates and the assay was started with the initial cell density of 1.0×10^5 cells/ml. Then the plates were incubated under 20 lux light at 20 °C for 120 h. The least concentration of extract where no algal growth was observed in at least 4 of the 6 wells was recorded as the minimum inhibitory concentration (MIC) (Thabard et al., 2009). Based on the results, the methanolic extracts of both seagrass were selected for further study.

2.5. Antimacrofouling activity

2.5.1. Mollusc foot adherence assay

P. vulgata is a common fouling organism found on rocky shores. The mollusc foot adherence assay is a rapid and reliable assay that requires a minimum quantity of test extract to determine its effect on the settlement of mollusc *P. vulgata* by spreading and shrinking of the foot. The assay was done in triplicate as per the method of Selvin and Lipton (2002) to determine the fouling (%) and regaining (%) ability of limpet *P. vulgata*

against methanolic extracts of *S. isoetifolium* and *C. serrulata* using 1.0-6.0 mg/ml concentrations. The seawater without extract was used as the control.

2.5.2. Mussel bioassay

Mussels are one among the major fouling organisms that come under the category of hard fouling. In this context, a mussel bioassay was done by following the method of Wilsanand et al. (1999) and Murugan and Santhana Ramasamy (2003) using the brown mussel *P. indica* to explore the antifouling property of seagrass extracts. Test concentrations of seagrass extracts (0.1, 1.0, 10, 25, 50, 100, 200 and 400 µg/ml) were selected and the seawater without extract was used as the control. The assay was performed in triplicate. After 24 h, the EC_{50} (effective concentration for 50% inhibition of byssal production) and at 96 h, LC_{50} (lethal concentration for 50% mortality) were also estimated through probit analysis. The LC_{50}/EC_{50} ratio of seagrass extracts was also calculated to assess the non-toxic property.

2.5.3. Anticrustacean assay

Anticrustacean assay is a simplest screening technique to study the repelling effects of bioactive compounds against the crustaeceous fouling organisms using *Artemia* as the model organism. The cysts of brine shrimp (*A. salina*) were hatched in a conical vessel (1 L) filled with filtered seawater under constant aeration for 24-48 h. Ten active larvae (I instar) were collected from a brighter portion of the hatching chamber by using a capillary glass tube and placed in a test tube containing 10 ml of brine solution with varying concentrations (5, 10, 25, 50, 100, 250, 500, and 1000 µg/ml) of crude extract of selected seagrasses. Seawater without extract was kept as control and maintained at room temperature for 24 h under light. After 24 h of exposure, the number of larvae surviving in each test concentration was counted and the LC_{50} values were analyzed by probit analysis and the percentage of larval mortality was calculated (Meyer et al., 1982).

2.6. Chemistry of antifouling compounds

2.6.1. Fractionation

Potentially bioactive methanolic extracts (20 g) of each seagrass (*S. isoetifolium* and *C. serrulata*) were individually fractionated and purified with an Ace chromatography column (5.0 cm diameter x 61 cm length) filled with silica gel (60-200 µm mesh size) using elution gradiant of hexane (C_6H_{14}), chloroform ($CHCl_3$) and methanol (MeOH). Twenty seven fractions (150 ml) were collected separately in a step gradient elution starting with 100% hexane and ending with 100% warmed methanol. All the fractions were individually tested for bioactivity and the seventh fraction ($CHCl_3$ 75%: MeOH 25%) of both the extracts exhibited antifouling potencies. The bioassay guided column fractions were then subjected to thin layer chromatography (Merck, TLC Silica gel F_{254}) and visualized under UV chamber (282 and 326 nm) as well with p-anisaldehyde stain then the R_f values of the active compounds were documented.

2.6.2. Partial characterization

The GC_MS analysis of bioassay guided fractions of seagrass *S. isoetifolium* and *C. serrulata* were conducted individually using an Agilent GC_MS 5975 Inert XL MSD (United States) gas chromatography equipped with J&W 122-5532G DB-5ms 30 x 0.25 mm^2 0.25 µm and mass detector (EM with replaceable horn) was operated in EMV mode. Helium was used as carrier gas with the flow rate of 1.0 ml min^{-1}. The injection port temperature was operated at 250 °C. The column oven temperature was held at 80 °C for 2 min then programmed at 10 °C min^{-1} to 250 °C which was held for 0 min, and then at 5 °C min^{-1} to 280 °C which was held for 9 min. Electron impact spectra in positive ionization mode were acquired between *m/z* 50 and 550. For more accuracy, the peaks with prominent area and quality (>70%) were alone considered and the constituents were identified by comparison with the internal standards of the instrument and spectral match with NIST library.

2.7. Statistical analysis

The data were analyzed using SPSS Version 16.0 software package. The differences between the experiment and control samples were determined using one-way ANOVAs followed by Dunnett's test at 95% confidence level for all antimacrofouling assays. Using probit analysis, the EC50 (concentration at which 50% of the inhibition of byssal production in mussel compared with the control) and LC50 (concentration at which 50% of mussels and artemia naupli were dead compared with the control) values of the mussel and anticrustaecean bioassays were calculated respectively.

3. Results

3.1. Antimicrofouling activities

3.1.1. Antibacterial assay

Extracts of *C. serrulata* and *S. isoetifolium* were found to inhibit the growth of test bacteria. Among the tested solvents, the methanolic extract of *C. serrulata* and *S. isoetifolium* showed better inhibitory activity compared with other solvents against the biofilm bacteria and the MIC was 1.0 µg/ml (Table 1).

Table 1

Antimicrofouling activities and minimum inhibitory concentration of seagrass extracts.

Biofilm bacteria	***S. isoetifolium* extracts** (μg/ml)			***C. serrulata* extracts** (μg/ml)		
	D	A	M	D	A	M
P. aeruginosa JN979983	10	25	1.0	10	25	10
H. aquamarina JN561698	10	25	10	25	50	1.0
V. alginolyticus JN979984	25	50	25	NI	NI	10
P. agglomerans JN979985	25	NI	1.0	25	25	1.0
S. marcescens JN596118	10	25	10	10	50	1.0
S. liquifaecians JN596115	50	50	10	50	10	10
V. fischeri JN979986	10	NI	10	10	25	1.0
V. parahaemolyticus JN585666	NI	50	25	25	50	10
S. flexneri JN979987	25	25	1.0	25	50	25
A. hydrophila JN561697	10	50	10	10	NI	1.0
Microalgal strains						
P. elongatum CASMB 001	50	25	10	25	25	10
T. frauenfeldii CASMB 002	10	50	25	50	10	1.0
N. sigma CASMB 003	NI	10	1.0	NI	25	10
N. longa CASMB 004	50	50	25	50	10	25
A. glacialis CASMB 005	25	25	10	25	50	10

Each value is obtained from 6 replicates; D: dichloromethane; A: acetone; M: methanol NI: no inhibition.

3.1.2. Anti-microalgal assay

Extracts of *C. serrulata* and *S. isoetifolium* were also found to inhibit the growth of test microalgae. However, MIC of 10 μg/ml for test microalgae was recorded by methanolic extract of *S. isoetifolium*. Anti-microalgal activity of methanolic extract of *C. serrulata* was observed at 1.0 μg/ml (Table 1). The results implied that the methanolic extracts exhibited better antimicrofouling activities than the acetone and dichloromethane extracts.

3.2. Antimacrofouling activities

3.2.1. Mollusc foot adherence assay

During this assay, the percentage of fouling by limpet *P. vulgata* was decreased with subsequent increase in the concentration of seagrass extract from 1.0 to 6.0 mg/ml. Methanolic extracts of *S. isoetifolium* and *C. serrulata* showed 0% fouling (100% inhibition) at 6.0 mg/ml with 48.83 ± 2.05 and 45.6 ± 3.52% of limpet regaining their function when transferred to fresh seawater. The regaining ability and the behavioral changes such as spreading, shrinking, and attachment of the foot of *P. vulgata* was disturbed with increase in the concentration of seagrass extracts (Figs. 1 and 2). The variation in percentage of fouling and regaining of *P. vulgata* due to the concentration of seagrass extracts was statistically significant ($P < 0.05$-0.0001).

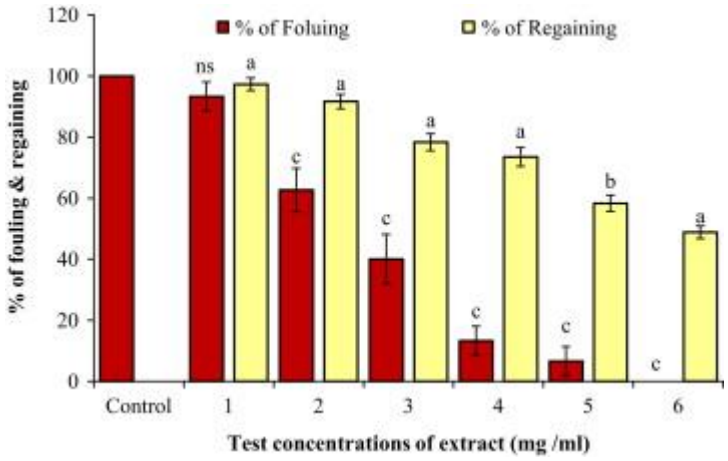

Fig. 1. Percentage of fouling and regaining of *P. vulgata* due to methanolic extract of *S. isoetifolium*. Each values are the mean ± SD of three observations. (a) $P < 0.05$; (b) $P < 0.001$; and (c) $P < 0.0001$ significant; ns: non-significant.

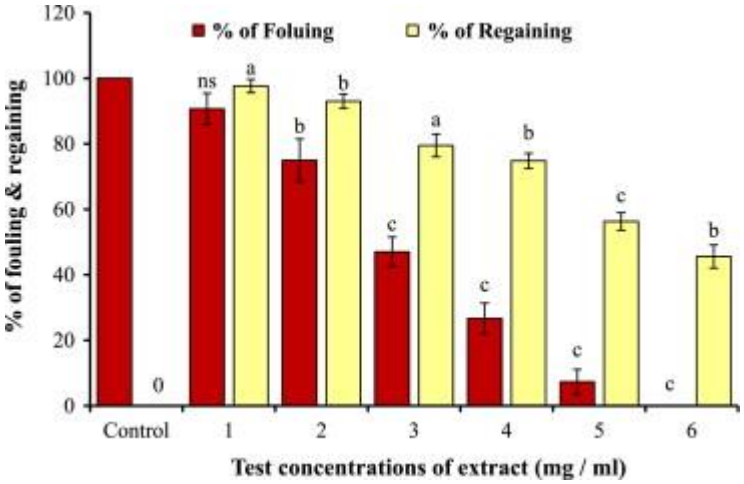

Fig. 2. Percentage of fouling and regaining of *P. vulgata* due to methanolic extract of *C. serrulata*. Each values are the mean ± SD of three observations. (a) $P < 0.05$; (b) $P < 0.001$; and (c) $P < 0.0001$ significant; ns: non-significant.

3.2.2. Mussel bioassay

The effective concentration (EC_{50}) in which 50% inhibition of byssal production and attachment for brown mussel *P. indica* was observed and the lethal concentration (LC_{50}) represented 50% mortality of mussels. The EC_{50} values were 21.51 ± 2.03 and 17.82 ± 1.07 µg/ml at 24 h for the methanolic extract of *S. isoetifolium* and *C. serrulata* respectively. The LC_{50} values of 336.5 ± 3.12 and 293.2 ± 2.46 µg/ml were recorded during 72 h of experiment for the methanolic extract of *S. isoetifolium* and *C. serrulata* respectively. The LC_{50}/EC_{50} ratios of *S. isoetifolium* and *C. serrulata* extracts were 16.45 and 15.64 (Table 2).

Table 2

EC_{50} and LC_{50} values of seagrass extracts during mussel bioassay.

Seagrass	[a]EC_{50} (µg ml 1)	[a]LC_{50} (µg ml 1)	LC_{50}/EC_{50}
S. isoetifolium	21.51 ± 2.03	336.5 ± 3.12	15.64
C. serrulata	17.82 ± 1.07	293.2 ± 2.46	16.45

[a] Are the mean ± SD of three observations.

3.2.3. Anticrustacean assay

Brine shrimp, *Artemia salina* larvae was used as a model organism to test the cytotoxic properties of selected seagrass extracts. The results indicated that the cytotoxicity of methanolic extracts of both the seagrasses were minimal with the LC_{50} values of 732.14 ± 9.21 and 394.16 ± 5.16 µg/ml respectively. Besides, the percentage mortality of *A. salina* larvae was significantly ($P < 0.05$) increased along with the hike in test concentration of extracts (Fig. 3).

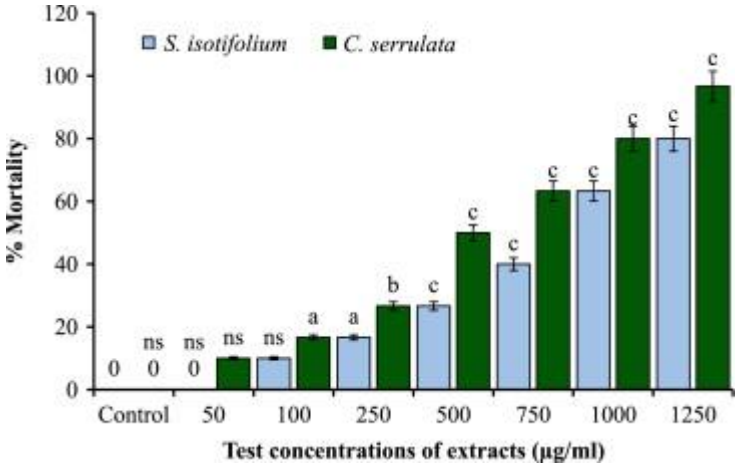

Fig. 3. Anticrustacean activity of methanolic extract of seagrasses. Each values are the mean ± SD of three observations. (a) $P < 0.05$; (b) $P < 0.001$; and (c) $P < 0.0001$ significant; ns: non-significant.

3.3. Chemistry of antifouling compounds

3.3.1. Column chromatography

In total, 27 column fractions (150 ml/fraction) were collected individually for both the seagrass extracts and evaporated under vacuum. Then the fractions were transferred to preweighed, labeled vials and tested for antifouling activity. Among the column fractions, the seventh fraction ($CHCl_3$ 75%: MeOH 25%) of *S. isoetifolium* weighing 356.2 mg and *C. serrulata* weighing 332.7 mg were expressed better antifouling activity against the test organisms.

Distribution and pattern of compounds present in the bioassay guided fractions were documented by silica gel thin layer chromatography. The bioassay guided fraction of *S. isoetifolium* recorded five compounds with the R_f values of 0.40, 0.52, 0.68, 0.89 and 0.96. Similarly, the active column fraction of *C. serrulata* registered six compounds with the R_f values of 0.48, 0.55, 0.60, 0.70, 0.88 and 0.92.

3.3.2. Partial characterization

GC-MS chromatogram of the bioassay guided column fraction of *S. isoetifolium* showed the presence of five major peaks. The respective retention times (R_t) of individual peaks recorded were 0.00-16.050, 0.00-17.531, 0.00-20.651, 0.00-20.778 and 0.00-27.960 min. The major phycoconstituents observed in the active fraction were 2-pentadecanone, 6,10,14-trimethyl, hexadecanoic acid methyl ester, 9, 12-octadecadienoic (Z,Z)-methyl ester, 9,12,15-octadecatrienoic acid methyl ester (Z,Z,Z) and 1,2-benzenedicar-boxylic acid diisooctyl ester

However, GC-MS analysis of bioassay guided column fraction of *C. serrulata* displayed six major peaks with respective retention time (R_t) ranges viz. 0.00-16.017, 0.00-16.534, 0.00-21.109, 0.00-27.898, 0.00-30.149 and 0.00-30.283 min. Thus, the active fraction unveiled the presence of 2-pentadecanone, 6,10,14-trimethyl, 1,2-benzenedicarboxylic acid butyl 1,2-methylpropyl ester, octa-decanoic acid methyl ester, 1,2-benzenedicarboxylic acid diisooctyl ester, oleic acid and erucic acid (Table 3).

Table 3

Bioactive components in bioassay guided column fractions of selected seagrasses.

RT	Name of the compound	Molecular formula	Molecular weight	*S. isoetifolium*		*C. serrulata*	
				Peak area (%)	Quality (%)	Peak area (%)	Quality (%)
16.050	2-Pentadecanone, 6,10,14-trimethyl	$C_{18}H_{36}O$	268.4	2.82	99	9.76	91
17.531	Hexadecanoic acid methyl ester	$C_{17}H_{34}O_2$	270.4	4.32	98	-	-
20.651	9,12-Octadecadienoic (Z,Z)-methyl ester	$C_{19}H_{34}O_2$	294.4	11.31	97	-	-
20.778	9,12,15-Octadecatrienoic acid methyl ester (Z,Z,Z)	$C_{19}H_{32}O_2$	292.4	6.90	95	-	-
27.960	1,2-Benzenedicarboxylic acid diisooctyl ester	$C24H_{38}O_4$	390.5	1.11	86	5.77	87
16.534	1,2-Benzenedicarboxylic acid, butyl 1,2-methylpropyl ester	$C_{16}H_{22}O_4$	278.3	-	-	4.35	86
21.109	Octadecanoic acid methyl ester	$C_{19}H_{38}O_2$	298.5	-	-	2.64	90
30.149	Oleic acid	$C_{18}H_{34}O_2$	282.4	-	-	3.45	70
30.283	Erucic acid	$C_{22}H_{42}O_2$	338.5	-	-	15.68	94

4. Discussion

As the usage of conventional antifoulants is restricted, the search towards the identification of new alternative antifouling compounds with satisfactory performance and nonpolluting nature retain growing interest (Hellio et al., 2009; Dafforn et al., 2011). Since many sessile marine organisms have developed efficient defense mechanisms against microbial epibionts, there is an increasing interest in such organisms as a source of naturally released antifouling substances (Hellio et al., 2001; Bazes et al., 2006, 2009; Marechal and Hellio, 2009; Dafforn et al., 2011; Silkina et al., 2012). Seagrasses are the submerged and sessile marine angiosperms are found to resist the attachment of epibionts.

In the present study, methanolic extracts of seagrasses such as *S. isoetifolium* and *C. serrulata* were found to show better antifouling activity by inhibiting the growth of biofilm bacteria and microalgae with the MIC's of 1.0 and 10 µg/ml respectively which were better than the MIC's of 50 and 200 µg/ml recorded by *Enhalus acoroides* against bacteria (Qi et al., 2008). Supportively, Mayavu et al., (2009) reported the antibacterial activity of crude extracts (ethanol, methanol, acetone and dichloroethane) of seagrasses. Amongst that, ethanol and methanol extracts of *S. isoetifolium* and *C. serrulata* exhibited antagonistic activity against the bacteria isolated from boat hulls.

The limpet *P. vulgata* adheres firmly to rocky surfaces or other hard substrata using the broad, flat gray-green foot and can cause extensive fouling and biodeterioration of submerged structures. The fouling inhibition in *P. vulgata* by a methanolic extract of the sea cucumber *Holothuria scabra* was found at 4.2 mg/ml concentration

(Selvin and Lipton, 2002). Accordingly, in the present study the seagrass extracts showed 100% fouling inhibition at 4.0 mg/ml concentration.

Mussels often close their shells and secrete fewer byssal threads with increasing concentration of the active extract and this may be an important criterion in improving the survivability and loss of attachment of mussels to the substrata during the experiment (Wilsanand et al. 1999). Hellio et al. (2000a, 2000b) reported inhibition of fouling organisms such as bacteria, fungi and mussels with less toxicity level by the extracts of brown algae *Sargassum muticum* and red algae *Polysiphonia lanosa*. The methanolic extract of *Sargassum wightii* at 205 µg/ml concentration (EC_{50}) inhibited the byssal production and attachment of mussel *P. indica* (Iyapparaj et al., 2012).

Methanolic extracts of *S. isoetifolium* and *C. serrulata* inhibited the byssal production and attachment at the EC50 concentrations of 21.51 and 17.82 µg/ml respectively. These EC_{50} values were better than our previous report on antimussel potentials of *S. wightii* (Iyapparaj et al., 2012). The LC_{50}/EC_{50} ratio is also known as therapeutic ratio, which is a common yardstick to measure the efficacy of a compound or extract. This ratio >50 are often considered as non-toxic, a much higher LC_{50}/EC_{50} ratio is highly recommended when selecting candidate compounds or the extracts for further study (Qian et al., 2010). Nevertheless, the LC_{50}/EC_{50} ratio of the *S. isoetifolium* and *C. serrulata* extracts were found to be <50. Despite the fact, Iyapparaj et al. (2013) reported that the cellular level and biochemical changes in mussel *P. indica* due to the toxicity of methanolic extract of *S. isoetifolium* was lower than TBT.

The cytotoxicity of the seagrass extracts was estimated by anticrustacean assay using *A. salina*. This assay also used to evaluate the bioactivity of extracts against marine fouling organisms, especially the crustaceous foulers like barnacles (Persoone and Castritsi-Catharios, 1989). The methanolic extracts of *S. iso-etifolium* and *C. serrulata* recorded a low cytotoxic property with the higher LC50 values of 732.14 ± 9.21 and 394.16 ± 5.16 µg/ml respectively. The above results are consistent with the findings of Prabhadevi et al. (1998) and Ragupathi Raja Kannan et al. (2013).

Only a few studies have been done on the bioactivity of seagrass and showed that seagrasses such as *Thalassia testudinum*, *Posidonia oceanica* and *Z. marina* had antibacterial (Harrison and Chan 1980, Devi et al., 1997a, Bhosale et al., 2002), antialgal (Harrison, 1982), antifungal (Jensen et al., 1998), antiviral (Premanathan et al., 1992), anti-inflammatory (Hua et al., 2006), toxicity (Devi et al., 1997b) and antifouling (Bhosale et al., 2002) activities. Chemical constituents of several seagrasses have also been described, including one antibiotic flavone glycoside from *T. testudinum* (Jensen et al., 1998), one sugar derivative from Ruppia maritima L. (Aquino et al., 2005), phenolic compounds from *P. oceanica* (Todd et al., 1993, Bushmann

and Ailstock, 2006), diterpenes from *R. maritima* (Della Greca et al., 2000), and steroids and fatty acids from *Zostera japonica* (Gillan et al., 1984; Sanina et al., 2004; Hua et al., 2006).

GC-MS analysis of bioassay guided fractions of *S. isoetifolium* and *C. serrulata* showed the presence of 10 lipidic metabolites i.e. fatty acids and it's esters. In agreement with the present findings, Ragupathi Raja Kannan et al. (2012) also reported the fatty acid and it's esters as bioactive compounds from the candidate seagrasses. Similarly, the lipidic metabolites like fatty acids and galactoglycer-olipids from the seaweed S. muticum reported to have antifouling potential (Poluguerne et al., 2010).

These bioactive compounds have been described to possess antimicrobial properties when derived from plants. Wagh et al. (2006) reported the antibacterial and antifungal activity of hexadecanoic acid methyl ester, 9,12-octadecadienoic acid methyl ester and octadecanoic acid methyl ester. Yayli et al. (2005) evidenced the antibacterial and antifungal activity of 2-pentadecanone 6,10,14-trimethyl. An antimicrobial property of 1,2-benzenedicarboxylic acid diisooctyl ester was described by Hema et al. (2011); however, in the present study, one of its esters 1,2-benzenedicarboxylic acid, bis (1,2- methylpropyl) ester also exhibited antifouling activity. Khoobchandani et al. (2010) reported the antibacterial activity of oleic and erucic acid. Similarly, 9,12,15-octadecatrienoic acid was exhibited antibacterial and antifungal activities (Arunkumar and Muthuselvam, 2009).

The bioactive metabolites of seagrasses *S. isoetifolium* and *C. serrulata* may responsible for the antifouling activity. Hence, these seagrasses could be used as a source in the search for an alternate and safe remedy to biofouling. Further research on the individual chemical characterization of the antifouling metabolites using LC-MS and NMR is being directed in our laboratory. Also, field validation of the isolated bioactive components is needed. Testing the physical and chemical stability of these bioactive compounds with paint components will pave the way for the development of eco-friendly antifouling coatings.

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