In vitro antibacterial, cytotoxicity and haemolytic activities and phytochemical analysis of seagrasses from the Gulf of Mannar, South India

Rengasamy Ragupathi Raja Kannan, Radjassegarin Arumugam, Palanisamy Iyapparaj, Thirunavukarasu Thangaradjou, Perumal Anantharaman*

Centre of Advanced Study in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai 608 502, Tamil Nadu, India

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ABSTRACT

It is essential to study the phytochemical constituents and toxicological properties of seagrasses when considering their food applications. Aqueous methanolic extracts of six seagrasses were evaluated for their antibacterial, cytotoxic (brine shrimp lethality assay) and haemolytic activity. Thin layer chromatography (TLC) and phytochemical analysis were used to compare the phytochemical profiles of six seagrasses. Among the six seagrasses examined, Halodule pinifolia and Cymodocea rotundata showed predominant growth inhibitory activity against all the tested human pathogens. Cytotoxicity of seagrass extracts against nauplii of Artemia salina revealed that Syringodium isoetifolium exhibited lesser toxicity with LC_{50} value of 699.096 µg/ml. Of all the seagrasses tested, H. pinifolia recorded the minimum haemolytic activity of 2.07 ± 0.63% at 1000 µg/ml concentration. Phytochemical analysis showed the presence of common plant chemical constituents which varied with respect to species. The present findings suggest the possible pharmacological applications of selected seagrasses that can be used as food ingredients.

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1. Introduction

Seagrasses are submerged marine angiosperms growing abundantly in tidal and subtidal areas of all seas except in the Polar Regions. Seagrass biomass is used as human food especially by coastal populations (Hemminga & Duarte, 2000). In folk medicine, seagrasses have been used for a variety of remedial purposes, e.g. for the treatment of fever and skin diseases, muscle pains, wounds, stomach problems, remedy against stings of different kinds of rays and tranquillizers for babies (de la Torre-Castro & Rönnbäck, 2004). In India, seagrasses are used as medicine (treatment of heart conditions, seasickness), food (nutritious seeds), fertiliser (nutrient rich biomass) and livestock feed (goats and sheep) (Newmaster 2004). In India, seagrasses are used as medicine (treatment of heart conditions, seasickness), food (nutritious seeds), fertiliser (nutrient rich biomass) and livestock feed (goats and sheep) (Newmaster et al., 2011). Seagrasses produce antimicrobial compounds that may act to reduce or control the microbial growth and there are many reports describing antibacterial (Ragupathi Raja Kannan, Arumugam, & Anantharaman, 2010a; Ragupathi Raja Kannan, Arumugam, & Anantharaman, 2012; Sreenath Kumar, Sarada, Gideon, & Rengasamy, 2008), antifungal (Arumugam, Ragupathi Raja Kannan, Arumugam, Meenakshi, & Anantharaman, 2010). Antimicrobial and phytoconstituents of seagrasses, knowledge of its toxicological information is scare, except Prabhadevi, Solimabi, DeSouza, and Kamat (1998). Therefore the present study was undertaken to evaluate the antibacterial, cytotoxicity and haemolytic property and phytochemical constituents of aqueous methanolic extract of six seagrasses.

2. Materials and methods

2.1. Seagrasses

The fresh leaves of Enhalus acoroides (Linnaeus f.) Royle, Thalassia hemprichii (Ehrenberg) Ascherson, Halodule pinifolia (Miki) den Hartog, Syringodium isoetifolium (Ascherson) Dandy, Cymodocea serrulata (R. Brown) Ascherson & Magnus and Cymodocea rotundata...
2.2. Preparation of seagrass extracts

Crude extracts were obtained by soaking 100 g dry weight (DW) of each seagrass powder individually in 2 L of aqueous methanol (1:4) for 24 h at room temperature under dark conditions. The extraction was repeated thrice, pooled and filtered through Whatmann No. 1 filter paper. Each filtrate was concentrated to dryness under reduced pressure using a rotary flash evaporator. The dry aqueous extracts were lyophilized and stored in a refrigerator until further analysis.

2.3. Pharmacological screening

2.3.1. Antibacterial activity

2.3.1.1. Preparation of bacterial culture. Human bacterial pathogens \textit{Staphylococcus aureus}, \textit{Vibrio cholerae}, \textit{Shigella dysenteriae}, \textit{Salmonella paratyphi} and \textit{Shigella boydii} used in this study were received from Department of Microbiology, Rajah Muthiah Medical College and Hospital, Annamalai University, Annamalainagar, Tamil Nadu, India. Bacterial strains were grown in Muller Hinton broth for 24 h at 37 °C. Before using the culture for antibacterial assay, culture broth was serially diluted using above sterile fresh broth medium to get a cell number of $10^9$ ml^{-1}.

2.3.1.2. Disc diffusion assay. The standard disc diffusion method described by Bauer, Kirby, Sherris, and Tvak (1966) was followed. Whatman filter paper (No. 1) discs of 6 mm diameter were impregnated with 10 µl of the solution containing crude extract (at a concentration of 100 mg/ml) and fraction isolated from TLC (at 10 mg/ml) were prepared using dimethyl sulfoxide (DMSO). These discs were evaporated at 37 °C for 24 h. Reference standard discs were prepared with gentamycin (50 µg/ml) to compare the antibacterial activity results of seagrass extracts. After drying, discs with seagrass extracts and gentamycin were placed on Muller Hinton agar plates where the bacterial culture was swabbed on the surface of the agar and incubated for 24 h at 37 °C. After the incubation, plates were examined for clear zone (growth inhibition) around each disc. The values obtained for seagrass extracts were compared with the values from normal and the difference is considered as growth inhibition activity. MIC was defined as the lowest concentration of the compound to inhibit the growth of microorganisms.

2.3.1.4. TLC bioautography assay. Seagrass extract fractions identified in thin layer chromatogram developed without iodine vapour was utilized for this study. Muller Hinton agar plates were prepared freshly and the developed thin layer chromatogram sheet was placed on the surface of the agar (the fractions facing upside). This assay shows the bioactivity of different fractions. Then the above setup was over layered with the Muller Hinton soft agar (0.8% agar content) which was premixed with 12 h, pathogen culture. The plates were incubated for 24 h at 37 °C (Weinstein & Wagman, 1978). After incubation, plates were observed for clear zone (growth inhibition) around each fraction, fraction with clear zone around was considered as most active and subject to further chemical analysis.

2.3.2. Cytotoxicity

2.3.2.1. Hatching of brine shrimp. Cysts of brine shrimp (\textit{Artemia salina}, Artemiidae) were acquired from Larval Rearing Facility, Centre of Advanced Study in Marine Biology, Faculty of Marine Science, Annamalai University, India. And were incubated for 48 h in a culture vessel (15 × 15 × 15 cm) containing saltwater (1% NaCl) prepared from nitrate, phosphate, and silicate-free sea salt and deionised water (35 g/L) at 24–28 °C under light. The saltwater with artemia cysts was aerated continuously during incubation with an aquarium air pump (AirTech-2KO). After 48 h the nauplii (larvae) were collected from the culture vessel.

2.3.2.2. Brine shrimp lethality assay. An appropriate amount of the stock solution of individual seagrass extract was mixed with 1% NaCl solution in vials to make concentrations of 10, 50, 100, 500, 1000 µg/ml. Control brine shrimp larvae were placed in 1% NaCl solution devoid of extracts. Three replicates were prepared for each dose level. The brine shrimp lethality assay (BLSA) has been used to determine the cytotoxicity of medicinal plant extracts (Meyer et al., 1982). Twenty-five nauplii of \textit{A. salina} were placed in each of the triplicate vials (thus, 75 nauplii per concentration) using a plastic pipette with a 2 mm diameter tip. The larvae were released under the surface of the solution to avoid killing them by trapping air under their carapaces. Survivors were counted under the stereomicroscope after 24 h, and the percentage of mortality at each dose and control was determined. LC$_{50}$ value of each extract was calculated at the concentration where 50% of the larval mortality was observed within 24 h of exposure.

2.3.3. Haemolytic assay

Freshly collected human red blood cells from healthy individuals were employed in this assay. Aliquots of 7 ml of blood were washed three times with sterile saline solution (0.89%, w/v, NaCl, pyrogen free) by centrifugation at 180g for 5 min. The cell suspension was prepared by finally diluting the pellet to 0.5% in saline solution. A volume of 0.5 ml of the cell suspension was mixed with 0.5 ml diluent containing 5, 10, 25, 50, 100, 250, 500 and 1000 µg/ml concentrations of individual seagrass extracts in saline solution. The mixtures were incubated for 30 min at 37 °C and centrifuged at 70g for 10 min. The free haemoglobin in the supernatants was measured spectrophotometrically at 412 nm. saline and distilled water were included as minimal and maximal haemolytic controls. The haemolytic percent developed by the saline control was subtracted from all groups. The experiment was done in triplicate and mean ± S.D. was calculated (Sun, Ye, Pan, & Pan, 2004).
2.3.4. Thin layer chromatography

Highly active seagrass extracts were fractionated using thin layer chromatography (TLC). 10 µl of a 100 mg extract/ml solution was loaded on Merck TLC F254 silica gel sheets, toluene:ethyl acetate:methanol (8:1:1) were used as mobile phase. Separated fractions were visualised under visible and ultraviolet light (254 and 336 nm, UV lamp, respectively). To locate the fractions, plates were sprayed with 5% sulphuric acid in ethanol and 0.36% vanillin in 3.6% sulphuric acid in methanol and heated for 2.5 min at 100 °C.

2.3.5. Ultraviolet spectrum

UV–Visible spectra for active fractions were recorded on a PerkinElmer Lambda 25 UV–VIS spectrophotometer (USA) equipped with 1.0 cm quartz cells. The width of excitation slits were set to 1.0 nm. The spectra collected with subsequent scanning spectra from 700 to 200 nm at 1.0 nm increments. The scanning speed used in this measurement was set as 240 nm/min for all measurements.

2.3.6. Phytochemical analysis

The qualitative test for the identification of phytochemical constituents such as alkaloids, glycosides, coumarins, flavonoids, phenols, proteins, free amino acids, quinones, saponins, sterols, sugars and terpenoids were carried out according to standard procedures (Sofowora, Olaniyi, & Oguntimehin, 1982).

2.3.7. Data analysis

Mean results of brine shrimp mortality were plotted against the logarithms of concentrations using Probit Analysis Version 1.5 developed by the US Environmental Protection Agency from which median lethal concentrations (LC50) at 95% confidence intervals (CI) were calculated, according to the method of Finney (1971).

3. Results and discussion

The highest quantity, 5.9 g (23.6% yield, w/w of plant material in dry weight) of crude bioactive compound was extracted from S. isoetifolium, and the lowest quantity of 0.96 g (9.6%) from C. serrulata (Table S1). Padmini Sreenivasa Rao, Sreenivasa Rao, and Kamarkar (1986) reported shade dried samples of Sargassum jonstonii possessed higher antibacterial properties than fresh (soon after collection), semi-fresh (stored for 2 days), sun dried and oven dried samples. They also suggested that degradation of antibiotic substances may not occur during shade drying. The solvent system chosen for the present study was aqueous methanol with the aim of extracting phenolic acids and flavonoids in the extracts. Tsao and Deng (2004) suggested that aqueous methanol between 50% and 80% has been used for the extraction of hydroxycinnamic acids, and many subgroups of flavonoids. They also added that higher water composition in the solvent can aid in extraction of glycosides of these compounds, although due to the complexity of heterosidic combinations, certain groups of flavonoids, such as flavones and flavanols, are not generally characterised as intact compounds but in the form of their aglycones (Tsao & Deng, 2004).

Among the six seagrasses examined, H. pinifolia and C. rotundata exhibited predominant growth inhibitory activity against all the human bacterial pathogens tested. Growth inhibitory activity found with H. pinifolia was in the range of 10.3 ± 1.53–14.3 ± 1.15 mm and 9.7 ± 0.58–12 ± 1.0 mm for C. rotundata. This result is in agreement with our previous study (Ragupathi Raja Kannan, Arumugam, & Anantharaman, 2012), where we reported extracts of the above seagrasses were being effective against urinary tract infection (UTI) pathogens. Next to this, moderate levels of antagonistic activity were registered by T. hemprichii and E. acoroides. T. hemprichii was active against all the bacteria tested and the activity was in the range of 9.3 ± 0.57–11.3 ± 1.15 mm. Growth inhibitory activity found with E. acoroides extracts was in the range of 6.3 ± 0.58–9.3 ± 1.53 mm. (Fig. 1). Alam et al. (1994) made preliminary screening of antibacterial and antifungal activities of E. acoroides and found that the methanolic extract was effective.
against *S. aureus*, *K. pneumoniae* and *P. aeruginosa*. Qi, Zhang, Qian, and Wang (2008) also reported that the flavonoids isolated from *E. acoroides* were active against several marine bacteria. These reports support the antibacterial activity obtained for seagrass extracts in this study.

The extracts that showed any antibacterial activity in this assay were subjected to the MIC evaluation and the results are presented in Table S2. The minimum inhibitory concentrations of seagrasses were determined by serial broth dilution method, *C. serrulata* showed the maximum inhibitory effect against *S. dysenteriae* and *S. paratyphi* with the MIC value of 130 µg/ml. *H. pinifolia* was ranked in the second place for its activity against *S. dysenteriae* (34 µg/ml), *S. paratyphi* (510 µg/ml) and *S. boydii* (510 µg/ml). The extracts of *C. rotundata* were most active against *S. dysenteriae*, *S. boydii* and *S. paratyphi* with average MIC values of 68 and 34 µg/ml.

Bioautography results (Table S3) of the TLC plates developed with the solvent system tolune: ethyl acetate: methanol showed clear zones or bands of inhibition. A compound with *Rf* value 0.92 in the extract from *H. pinifolia* extract strongly inhibited the growth of *S. aureus*. Two compounds from *C. rotundata* extract with *Rf* values 0.79 and 0.92 were active against *S. aureus*, *S. dysenteriae* and *S. paratyphi*. The clear zones were located separately on TLC suggesting that more than one compounds involved in antibacterial effects.

The percentage mortality caused by various concentrations of seagrasses was taken as log dose against nauplii of *A. salina* (Fig. 2 and Table S4). From the present data, it is revealed that *C. rotundata* showed the maximum cytotoxicity with *LC50* value of 132.469 µg/ml followed by *T. hemprichii* (154.081 µg/ml), *H. pinifolia* (175.021 µg/ml), *C. serrulata* (392.124 µg/ml), *E. acoroides* and *S. isoetifolium* recorded lesser cytotoxicity with *LC50* values of 479.575 and 699.096 µg/ml, respectively.

Most of the selected seagrasses have not yet been assessed for in vitro cytotoxicity, apart from *E. acoroides* and *S. isoetifolium* which have shown cytotoxic properties (Kontiza, Abatis, Malakate, Vagias, & Roussis, 2006; Prabhadevi et al., 1998; Qi et al., 2008). Among the six seagrasses examined, five of them showed *LC50* values lesser than 500 µg/ml, but the extracts of *C. rotundata*, *T. hemprichii* and *H. pinifolia* exhibited *LC50* lower than 200 µg/ml (Table S4). *C. rotundata* revealed maximum cytotoxicity at very low concentration with the *LC50* values of 132.469 µg/ml. In earlier reports, two new diarylheptanoids namely Cymodienol, Cymodiene (Kontiza et al., 2005) and 3-keto steroids (Kontiza et al., 2006) were isolated from *Cymodocea nodosa* as cytotoxic agents. The extract of *S. isoetifolium* showed least cytotoxicity with the *LC50* value of 699.096 µg/ml. This result is consistent with the result of Prabhadevi et al. (1998), who reported petroleum ether extract from *S. isoetifolium* did not cause *LC50* even at 500 ppm. No informations noticed in previous literatures on the cytotoxicity of *H. pinifolia*, *C. serrulata* and *C. rotundata*.

Results of haemolytic activity have shown that, extract of *C. rotundata* exhibited the maximum haemolytic activity with 5.26 ± 1.63% at 1000 µg/ml concentration and the minimum haemolytic activity of 2.07 ± 0.63% at 1000 µg/ml concentration is revealed by *H. pinifolia* extract. In general, the increase in concentration of test extracts from 5 to 1000 µg/ml has found to be increase the haemolytic activity. Haemolytic activity of selected seagrass extracts were in the following order *C. rotundata* > *T. hemprichii* > *S. isoetifolium* > *C. serrulata* > *E. acoroides* > *H. pinifolia* (Fig. 3). It is vital to test the haemolytic activity of drugs as it is an indicator for cytotoxicity. Performing haemolytic assay is important to determine whether a drug possessing antioxidant and other bioactivities can be used in pharmacological applications (Kalaivani, Rajasekaran, Suthindhiran, & Mathew, 2010). The present results on haemolytic activity of seagrasses extracts were comparatively lower than many plant extracts previously screened and suggest its suitability for pharmacological purpose.

TLC analysis of active extract revealed, 12 fractions of phenolic compounds in *H. pinifolia* and 10 fractions in *C. rotundata* (Fig. 4). The fractions with *Rf* value 0.76, 0.86 and 0.92 were more sensitive to the pathogens tested and other remaining fractions in both extracts showed only trace amount of activity. The spectral properties of fractions according to their *Rf* are as follows: *Rf* 0.76 (*λ*max = 203 nm), *Rf* 0.86 (*λ*max = 204 nm) and *Rf* 0.92 (*λ*max = 206 nm). Based on the UV–Vis spectra results obtained for standards and active fractions revealed the presence of phenolic acids in the extracts. Qi et al. (2008) reported the flavane glycosides are responsible for the antibacterial activity of *E. acoroides*.
Phytochemical analysis of aqueous methanol extracts of six seagrasses revealed the presence of coumarins, flavonoids, phenols, proteins, free amino acids, quinones, saponins, steroids, sugars and terpenoids. Alkaloids and glycosides were absent in all the six seagrasses, quinone was absent in *E. acoroides* and *S. isoetifolium*. (Table S5). There are several reports demonstrating the phytochemical constituents of seaweeds, mangroves and other marine life forms, but only limited information is available for seagrasses especially from India. The other common phytochemicals were present in all tested seagrasses based on species variability. Athiperumalsami et al. (2008) screened four seagrasses such as *Halophila ovalis*, *S. isoetifolium*, *C. serrulata* and *H. pinifolia* and reported 15 phytochemicals from benzene and petroleum ether extract of *S. isoetifolium*, which includes 12 phytochemicals reported from the present study. The results of the present investigation are consistent with the earlier report (Ragupathi Raja Kannan, Arumugam, Hemalatha, et al., 2010). Some of the existing reports stated the presence of polyphenols in seagrasses and related antioxidant activity (Gokce & Haznedaroglu, 2008; Ragupathi Raja Kannan, Arumugam, & Anantharaman, 2010; Ragupathi Raja Kannan, Arumugam, Meenakshi, et al., 2010; Ragupathi Raja Kannan, Arumugam, & Anantharaman, 2010b). Ragupathi Raja Kannan, Arumugam, and Anantharaman. (2011) reported that the toxic elements Pb, Cr and Cd were found in all six seagrasses, but their concentrations were below the permissible limits and indicated that these seagrasses may be used as a potential source for searching valuable molecules.

4. Conclusions

To the best of our knowledge, the present study is the first report on the haemolytic and cytotoxic property (except *S. isoetifolium*) of the seagrasses of Gulf of Mannar (India). The pharmacological properties extended by *H. pinifolia* and *C. rotundata* is very much appreciable for the future development of novel functional food ingredients. Further bioassays, purification and structural characterisation of these biological metabolites will yield noteworthy information about their usage in pharmaceuticals, cosmetics and food industry.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.foodchem.2012.09.006.

References


