Antimicrobial, Antioxidant, and Antifouling Activity from Extracts of Aboveground and Belowground Parts of Seagrasses *Cymodocea rotundata* and *Cymodocea serrulata*

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ABSTRACT

Seagrasses have been known to produce metabolites with significant bioactivities, which vary by plant part and age, season, location, and solvent used for extraction. This study is the first to specify the most effective parts of seagrasses and solvents for their extraction. Two seagrass species were used in the present study: *Cymodocea rotundata* (CR) and *Cymodocea serrulata* (CS). Extracts from aboveground (leaves) and belowground parts (rhizomes and roots) using three different solvents showed significantly different antimicrobial, antioxidant and antifouling activity ($p<0.05$). The maximum antimicrobial activities were found for the root-dichloromethane extracts of both CR and CS which showed broad antimicrobial activity against seven and five microorganisms, respectively, with minimum inhibition concentration (MIC) of 312.5 $\mu g \cdot mL^{-1}$ against *Staphylococcus aureus*, *Candida albicans* and *Bacillus subtilis*. The root-70% acetone extract of CS and leaf-70% acetone extract of CR possessed the most effective DPPH and ABTS radical scavenging activity, with IC50 values of 62.1 and 43.96 for CS and 125.8 and 60.60 $\mu g \cdot mL^{-1}$ for CR, respectively. The most promising extracts as antifoulants (by their inhibition of byssus production) were leaf-70% acetone extracts of CR and CS, with EC_{50} of 6.18 and 6.12 $\mu g \cdot mL^{-1}$, respectively. Phenol content was correlated with DPPH radical scavenging and inhibition of byssus production ($r = 0.468$ [$p<0.05$] and 0.577 [$p<0.05$], respectively). This study clearly demonstrates beneficial properties of extracts made from CR and CS, and the findings can guide future development of the extracts into safe antimicrobial and antioxidant products and environmentally friendly antifoulants.

Keywords: Antifouling, Antimicrobial, Antioxidant, *Cymodocea rotundata, Cymodocea serrulata*

INTRODUCTION

Seagrasses are unique submerged marine angiosperms and the only group of flowering plants to recolonize the sea by developing specialized aboveground and belowground parts. The aboveground part includes leaves with air sacs to help them float above the seabed. The belowground parts consist of rhizomes and roots for mechanical support and anchoring on the sea substratum (Lewmanomont and Ogawa, 1995). Seagrasses have long been utilized as ethnomedicine, nutritious food for humans and livestock, and also as fertilizer (Kannan *et al*., 2010b; Namadevan and Varadharaj, 2017; Kavitha *et al*., 2020).

There is evidence that seagrasses produce secondary metabolites for defense against herbivores, pathogens, fouling organisms, and competitors for space (Harrison 1982; Jensen *et al*., 1998; Ferrat

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et al., 2001). These defense metabolites suggest the potential of seagrasses as a source of bioactive compounds for application in biomedicine and industry. Seagrasses have been known to produce metabolites with significant antibacterial, antifungal, antioxidant, antifouling and cytotoxicity properties (Mayavu *et al*., 2009; Iyapparaj *et al*., 2014; Namadevan and Varadharaj, 2017; Bel Mabrouk *et al*., 2020; Kavitha *et al*., 2020). The chemical constituents from seagrasses producing these bioactivities are mainly phenolic compounds (Zapata and McMillan, 1979; Agostini *et al*., 1997). The remarkable zosteric acid from *Zostera marina*, with its potent antibacterial and antifouling properties and ability to inhibit barnacle nauplius attachment and fungal spore adhesion (Todd *et al*., 1993; Stanley *et al*., 2002), has been developed commercially as a model from the sea for environmentally friendly natural antifouling coatings (Vilas-Boas *et al*., 2017). Extracts from *Cymodocea serrulata* (CS) have been developed as eco-friendly nanoparticles for application against bacterial pathogens and cancer cells, as an antioxidant, for *in vitro* cytotoxic studies, and for curing shrimp diseases (Chanthini *et al*., 2015; Palaniappan *et al*., 2015; RathnaKumari *et al*., 2018; Rajeswaran *et al*., 2019). These studies have shown that CS is a valuable resource for developing eco-friendly products for humans and for use in the marine environment.

Cymodocea rotundata (CR) and *Cymodocea serrulata* (CS), members of the Cymodoceae family, have been used as traditional medicine and are reported to produce many bioactive metabolites with various chemical constituents (Namadevan and Varadharaj, 2017). CR and CS are dominant in seagrass meadows in Thailand and are also widely distributed along the coasts of the Indo-Pacific (Lewmanomont and Ogawa, 1995). In our previous work, CR and CS collected in Thailand possessed more potent bioactivities than other seagrasses and marine algae (Wisespongpand *et al*., 2010; 2012; 2014). Potent bioactivities have also been reported from *Cymodocea* in other regions, such as antibacterial (Kumar *et al*., 2008; Ravikumar *et al*., 2011a; 2011b; Mani *et al*., 2012; Kannan *et al*., 2013a) and antioxidant properties (Kannan *et al*., 2010b; 2013b; Santoso *et al*., 2012; Jeyapragash *et al*., 2016), antifouling by inhibiting attachment of byssus of mussels (Iyapparaj *et al*., 2014), and inhibition of biofilm settlement of marine bacteria on boat hulls (Mayavu *et al*., 2009).

Most of the previous studies on bioactivities of *Cymodocea* were conducted using the whole plant or leaves with samples from India, and using methanol or ethanol as extraction solvents (Kumar *et al*., 2008; Ravikumar *et al*., 2010; Kannan *et al*., 2012; 2013a; 2013b; Mani *et al*., 2012; Jeyapragash *et al*., 2016). The studies showed that bioactivity of metabolites from these species varied by the age and part of the plants used, by season and collection location, and by the solvent used for extraction. In this study we aimed to evaluate variation in three bioactivities (antimicrobial, antioxidant, and antifouling) of two seagrasses (CR and CS) collected from the Andaman coast of Thailand. We compared three aboveground and belowground parts and three solvents to identify the sources of bioactivities. Antimicrobial, antioxidant, and antifouling bioactive metabolites that are non-toxic and friendly to human health and the environment are needed for biomedicine, in cases of drug resistance, and for industrial purposes.

MATERIALS AND METHODS

Sample collection and extraction

Two seagrasses, CR and CS, were collected in December 2014 from a seagrass meadow at Tang Ken Bay in Phuket Province, on the Andaman coast of Thailand (12°53'56.65"N, 100°46'37.34"E). Approximately 1,000 g of each species was collected using a shovel. The specimens were washed to remove sediment, and then separated into three parts: one aboveground part comprised leaves and two belowground parts comprised rhizomes and roots. They were then shade-dried for 24-48 h and ground. The system for extraction by solvents was adapted from Hellio *et al*. (2004) and our previous work. Initially, each part of seagrass (20 g) was mixed with hexane for three hours to remove fat. Each part was then separately extracted in one of three solvents,

70% acetone, methanol, or dichloromethane, in a ratio of 1:3 (w/v). These extracts were sonicated for ten minutes and maintained for three days at ambient temperature. Then, the filtrates were concentrated under reduced pressure using a rotary evaporator (Buchi: R200), finally yielding 18 crude extracts (2 species×3 parts×3 solvents).

Antimicrobial assay

The antimicrobial assay for screening active extracts was conducted using the standard disc diffusion method against 13 microorganisms. We used six strains of human pathogenic bacteria, *Bacillus subtilis* (BS) DMST 15896, *Staphylococcus aureus* (SA) DMST 8840, *Enterococcus faecalis* (EF) DMST 4732, *Escherichia coli* (EC) DMST 4212, *Aeromonas hydrophila* (AH) DMST 21150 and *Vibrio cholerae* (VC) DMST 2873, and two strains of yeast, *Saccharomyces cerevisiae* (SC) DMST 22805 and *Candida albicans* (CA) DMST 898, obtained from the Department of Medical Sciences (Ministry of Public Health) type culture collection of Thailand (DMST). Two strains of shrimp pathogenic bacteria, *Vibrio harveyi* (VH) and *Vibrio parahaemolyticus* (VP) were obtained from the Department of Aquaculture, Kasetsart University, and three strains of plant pathogenic bacteria, *Xanthomonas oryzae* (XO), *Erwinia carotovora* (EA) and *Ralstonia solanacearum* (RS) were obtained from the Department of Plant Pathology, Kasetsart University. Broth cultures were prepared in Tryptic Soy Broth (TSB) or in TSB with 2% NaCl (for *Vibrio*).

The eighteen crude extracts were dissolved in ethanol and then dispensed onto a 9 mm filter paper disc at a concentration of 500 µg per disc, in three replications. Three extraction solvents were used along with, ethanol and Augmentin as negative and positive controls, respectively. Each test microorganisms was spread with sterile cotton buds on TSA or TSA with 2% NaCl plates at an inoculum concentration giving turbidity of 0.5 (abs), then incubated at 30-37 ºC for 18 h. The inhibition zone of active extracts was recorded by measuring the diameter after the incubation period.

The efficacy of extracts was further determined by the minimum inhibition concentration (MIC) using colorimetric microdilution broth assay with Alamar-blue dye (Kanjana-opas, 2007). Each tested microorganism was cultured in RPMI 1640 media at 37 ºC for 18 h, then adjusted to optimized OD_{570} and mixed with Alamar-blue at a ratio of 10 $\mu L \cdot mL^{-1}$ into 96-well plates. Multidiluted extracts dissolved in DMSO (0-2,000 $\mu g \cdot mL^{-1}$) were added with three replications and controls were the same as the disc diffusion method. After the incubation time of 24 h at 37 ºC, the extracts that possessed antimicrobial activity displayed blue color and the dilution factors were calculated for MIC.

Antioxidant assay

DPPH and ABTS radical scavenging assays were applied according to Chatatikun and Chiabchalard (2013). A 20 µL volume of each extract (1,000 $\mu g \cdot mL^{-1}$) was dispensed into 96-well plates with three replications. The same amounts of ascorbic acid and methanol were used as standard control and blank, respectively. For the DPPH scavenging assay, 180 µL of DPPH (0.016 mM in methanol) was mixed with the sample. For the ABTS scavenging assay, 180 µL of ABTS (0.7 mM ABTS mixed with 2.45 mM potassium persulfate and given the absorbance 0.07±0.02 at 750 nm) were mixed with the sample. The mixtures were maintained in darkness for 30 min, and then the absorbance was measured by a microplate reader (Biochrome: EZ Read 400) at 540 and 750 nm for DPPH and ABTS, respectively. DPPH and ABTS radical scavenging activity (%) of the extracts was calculated by the equation $[1-(A_{sample}-A_{sample\ blank})/A_{control}]\times 100$, where A_{sample} = absorbance of extracts with DPPH; $A_{sample\ blank}$ = absorbance of extracts and methanol; and $A_{control}$ = absorbance of DPPH without extracts.

The extracts that showed DPPH or ABTS radical scavenging activity higher than 50 % were further evaluated for antioxidant efficacy by IC_{50} value. The dilutions of active extracts (0-1,000 $\mu g \cdot mL^{-1}$) were tested with the same procedure as the screening method. The IC_{50} was analyzed through probit analysis.

Antifouling assay

The inhibition of byssus production was evaluated as an indicator for antifouling activity (Da Gama *et al*., 2003). This assay was applied according to Iyapparaj *et al*. (2014). Before being placed in plastic cups with a salinity of 30 ‰, mussels (*Perna viridis*) (1.5-2 cm) from Sriracha Fisheries Station, Kasetsart University had all byssus material cut off. One mussel was placed per 10 mL plastic cup containing seawater, and each extract dissolved in ethanol was added at the final concentration of 100 $\mu g \cdot mL^{-1}$, with five replications. Positive and negative controls were also prepared by using 0.01% $CuSO_4$ and ethanol. After 24 h the number of byssuses was counted and calculated as the % inhibition of byssus production relative to the control (with seawater only). After that, extracts that had % inhibition of byssus production higher than 50 % were evaluated for EC_{50} by serial dilution of the extracts at concentrations between 0-100 $\mu g \cdot mL^{-1}$. The EC_{50} was analyzed through probit analysis.

Brine shrimp toxicity assay

The bioassay with *Artemia* adults was conducted to determine the acute toxicity of antifouling paints according to Persoone and Castritsi-Catharios (1989). Ten brine shrimp were put in well-plates containing 40 ‰ seawater. After that, each extract dissolved in ethanol was added to make a final concentration of 150 $\mu g \cdot mL^{-1}$, with three replications. The positive control was 0.01% $CuSO_4$, while ethanol and seawater were used as negative controls. The mortality rate of brine shrimp was calculated. Then, the extracts were further evaluated for LC_{50} at concentrations between 0-300 $\mu g \cdot mL^{-1}$. The LC_{50} was analyzed through probit analysis.

Chemical constituents of CR and CS extracts

The chemical constituents of CR and CS extracts were analyzed for total phenolic and flavonoid content. Constituents including some bioactive moieties were identified by TLC. Total phenolic contents were analyzed by the Folin-Ciocalteu method according to Chatatikun and Chiabchalard (2013). In brief, 50-µL aliquots of distilled water, crude extract, Folin-Ciocalteu reagent (10%), and sodium carbonate (1 M) were dispensed and mixed into 96-well plates, with three replications. The mixtures were maintained in darkness for 60 min. The standard curve of gallic acid between 5-100 $\mu g \cdot mL^{-1}$ was also analyzed. Absorbance was measured at 750 nm by a microplate reader (Biochrome: EZ Read 400). The content of phenol was calculated by comparing with the standard curve of gallic acid and recorded as mg equivalent to gallic acid per gram of dry weight of seagrass (mg $GAE \cdot g^{-1}$).

The total flavonoid content was determined according to Chang *et al*. (2002). In brief, 10 µL of aluminum chloride (10%), 50 µL of crude extract, 50 µL of ethanol (95%) and 50 µL of sodium acetate (1 M) were mixed into 96-well plates with three replications. The mixtures were maintained in darkness for 40 min. The standard curve of quercetin between 5-100 $\mu g \cdot mL^{-1}$ was also analyzed. Absorbance was measured at 405 nm by a microplate reader. The content of flavonoid was calculated by comparing with the standard curve of quercetin and recorded as mg equivalent to quercetin per gram of dry weight of seagrass (mg $QTE \cdot g^{-1}$).

The chemical constituents of the extracts were identified by TLC. The extracts were diluted with methanol and added to 5×5 cm TLC plates (Si60, F_{254}) eluted with hexane and ethyl acetate at a ratio of 3:1. Functional moieties indicating bioactivities of terpene, alkaloid, coumarin, anthraquinone and phenol were identified under UV light at 254 and 320 nm and by vanillin-sulfuric acid, dragendorff reagent, bontrager reagent, and ferric chloride as visualizing agents (Wagner and Bladt, 1996).

Statistical analysis

All data were expressed as mean±standard deviation (n = 3-5). Differences among group means were analyzed using one-way ANOVA followed by a post-hoc test (Tukey test) at a 95 % confidence level ($p<0.05$) for all assays. The correlation (r) between chemical constituents (phenol, flavonoid) and antioxidant and antifouling

properties were evaluated with Pearson correlation. The IC_{50} and EC_{50} were analyzed through probit analysis. All statistical analyses were performed by IBM SPSS Statistic Standard V.27 software.

RESULTS AND DISCUSSION

Antimicrobial activity

The extracts from the aboveground part (leaves) and the belowground parts (rhizomes, roots) of CR and CS using three solvents showed significantly different antimicrobial activity ($p<0.05$) (Table 1). The maximum antimicrobial activity belonged to the root-dichloromethane extract of CR against seven tested bacteria and yeasts (Table 1). CS extracts, although slightly less potent than those of CR, also showed broad activity of the dichloromethane extracts of root and rhizome (high inhibition zone against 5 microorganisms). Interestingly, only three extracts inhibited XO and EA, which are gram-negative plant pathogenic bacteria. Only the root-dichloromethane extract of CR inhibited SC. No antimicrobial activity of these seagrass extracts against EC, AH, VC, VH, VP, or RS was evident. Of the 18 seagrass extracts tested, the MIC of the root-dichloromethane extract was the most effective in controlling the growth of microorganisms. Three effective antimicrobial extracts with MIC of 312.5 $\mu g \cdot mL^{-1}$ were root-dichloromethane extract of CR against both SA and CA and root-dichloromethane extract of CS against BS (Figure 1).

Table 1. Inhibition zone (mm) of extracts from *Cymodocea rotundata* and *C. serrulata* with three extraction solvents against various microorganisms.

Seagrass	Part	Solvent	Human pathogenic bacteria			Plant pathogenic bacteria bacteria		Yeast	
			BS	SA	EF	XO	EA	SC	CA
CR	Root	70% Acetone	0	0	0	0	0	0	0
		Methanol	0	0	0	0	0	0	0
		Dichloromethane	14.46±1.43^{a}	14.55±1.82^{a}	10.83±0.5^{a}	10.48±0.48^{a}	10.94±0.01^{a}	9.67±0.09^{a}	13.86±1.03^{a}
	Rhizome	70% Acetone	0	0	9.63±0.15^{b}	0	0	0	0
		Methanol	10.97±2.26^{bc}	0	0	0	0	0	0
		Dichloromethane	0	11.78±0.27^{b}	0	0	0	0	10.60±0.98^{b}
	Leaf	70% Acetone	0	0	0	0	0	0	0
		Methanol	0	0	0	0	0	0	0
		Dichloromethane	0	0	0	0	0	0	0
CS	Root	70% Acetone	10.48±0.17^{bc}	10.01±0.10^{bc}	9.74±0.14^{b}	0	0	0	0
		Methanol	9.71±0.09^{c}	0	0	0	0	0	0
		Dichloromethane	11.64± 0.1^{bc}	11.67±0.35^{b}	0	10.38±0.02^{a}	10.74±1.35^{a}	0	10.00±0.16^{b}
	Rhizome	70% Acetone	10.72±0.03^{bc}	9.27±0.05^{c}	9.44±0.28^{b}	0	0	0	0
		Methanol	9.50±0.58^{c}	10.21±0.54^{bc}	9.66±0.60^{b}	0	0	0	0
		Dichloromethane	12.99±1.10^{ab}	11.66±1.71^{b}	×	10.40±0.11^{a}	10.71±0.38^{a}	0	10.76±0.71^{b}
	Leaf	70% Acetone	0	0	0	0	0	0	0
		Methanol	0	0	0	0	0	0	0
		Dichloromethane	0	0	0	0	0	0	0
Control		Augmentin	19.78±0.2	18.35±1.02	17.22±1.2	16.29±0.75	17.85±0.78	15.5±1.11	16.24±1.31

Note: Values within a column superscripted with different lowercase letters are significantly different ($p<0.05$).

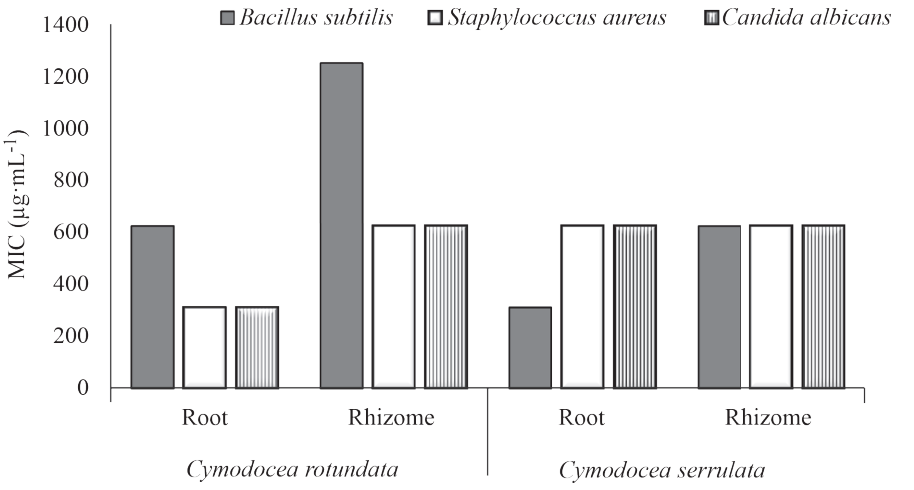

Figure 1. Minimum inhibition concentration (MIC) of active extracts from roots and rhizomes of *Cymodocea rotundata* and *C. serrulata* in dichloromethane.

There have been many reports demonstrating that seagrasses represent rich sources of antimicrobial metabolites (Namadevan and Varadharaj, 2017; Kavitha *et al*., 2020). Ten seagrasses collected from Thailand in our previous work possessed some degreee of antimicrobial activity; of these, CR and CS showed broad antimicrobial activity (Wisespongpand *et al*., 2005). Our present findings are consistent with earlier reports showing that CR and CS are important sources for antimicrobial activity (Kumar *et al*., 2008; Mayavu *et al*., 2009; Ravikumar *et al*., 2011a; 2011b; Kannan *et al*., 2012; 2013a; Mani *et al*., 2012).

In this study, CR extracts had stronger antimicrobial activity than CS, which concurred with the report of Kannan *et al*. (2012). Our study revealed more specific details regarding the most appropriate plant parts and solvents than the previous reports, which mostly used the whole plants or leaves. We found that the extracts from the roots of CR and CS possess potent antimicrobial activity, and that dichloromethane is an effective solvent for extraction. The collection of the roots of seagrass takes more effort and yields smaller samples than gathering leaves and rhizomes. This may explain why only limited studies have used the roots of seagrass for extraction (Ravikumar *et al*., 2011b). Several studies which used leaves or whole plants of CS showed various antibacterial activities. Mani *et al*. (2012) reported broad-spectrum activity against 10 human pathogenic bacteria from leaf-methanol extracts of CR, whereas our results show variation of antimicrobial activity by plant part and solvent. There was no antibacterial activity against shrimp pathogenic bacteria in our study, whereas the extracts from roots in 70% acetone of CS have been shown to have strong antimicrobial activity against fish pathogenic bacteria including AH (Ravikumar *et al*., 2011a).

The root-dichloromethane extract of CR and root-and rhizome-dichloromethane extracts of CS have potential application in agriculture because these three extracts showed activity against gram-negative plant pathogenic bacteria (XO and EA) (Table 1). The numbers of metabolites that inhibit gram-negative bacteria from natural products are less than for gram-positive bacteria because the susceptibility of the microorganisms towards an antibiotic depends upon the mechanism of action of the compound and due to the thicker cell wall of gram-negative bacteria (Mani *et al*., 2012). The utilization in agriculture of CS extracts was also reported by Ravikumar *et al*. (2011a; 2011b), who suggested that CS extracts can be effectively used as an alternative fish and poultry medicine to avoid some adverse side effects of antibiotics.

In this study, the extracts which showed potent antimicrobial activity were mostly belowground parts extracted in dichloromethane. The rhizospheres below the ground of seagrasses are a complex community of microorganisms from which many bacteria and fungi are known to possess antimicrobial activity (Ismet *et al*., 2020). The endo-and epiphytic bacteria in two seagrass species, *Syringodium isoetifolium* and CS, showed inhibitory activity against human bacterial pathogens (Ravikumar *et al*., 2010). The endophytic fungi from CS, *Thalassia hemprichii*, and *Halophila ovalis* were reported to have antimicrobial activity (Supaphon *et al*., 2013). The marine-derived fungi isolated from *Enhalus acoriodes* also showed active antibacterial activity (Notarte *et al*., 2018). Our supposition in this study was that the bacteria associated with CR and CS, especially in root and soil, may produce some antimicrobial metabolites which are absorbed and accumulated in seagrasses.

The extracts of both CR and CS in dichloromethane showed more potent antimicrobial activity than 70% acetone and methanol, which might be due to non-polar metabolites. In another study, chlorophyll *a* and a mixture of β-sitosterol and stigmasterol were isolated from dichloromethane extract of CR and were suggested to exhibit antibacterial activity (Perez *et al.*, 2018). The variation we observed in antimicrobial activity with different extraction solvents is in agreement with several reports (Kumar *et al*., 2008; Kannan *et al*., 2010a; Mani *et al*., 2012; Bel Mabrouk *et al*., 2020). Kumar *et al*. (2008) used six different solvents for the extraction of the leaves of three seagrasses. Among those solvents tested, methanol and chloroform extracts inhibited the growth of all the pathogens in the study, with methanol extracts being more active than chloroform. Many factors could affect the antimicrobial activity such as the age of the plant, duration of storage, temperature, preparation of the media, pH, geographical and seasonal variation, or even the different culture conditions of tested strains (Hellio *et al*., 2004). The antimicrobial activity from the root parts of CR and CS in dichloromethane as well as the rhizomes of CS in dichloromethane could have promising applications for the treatment of drug-resistant bacteria as well as for agricultural purposes.

Recent research showed the value of the bioactive metabolites from CS in potential applications in eco-friendly nanoscale drug devices. These nanoscale devices can easily enter cells and interact with DNA, proteins, enzymes, and cell receptors (Rajeswaran *et al*., 2019). Among applications of CS for medical purposes, nanoparticles coated with CS extract were found to have potential against bacterial pathogens and cancer cells, and in curing shrimp diseases (Palaniappan *et al*., 2015; RathnaKumari *et al*., 2018; Rajeswaran *et al*., 2019). Interestingly, the utilization of CS in a nanoscale device was in the form of crude extracts; our findings similarly ascertained that CR and CS extracts can be developed into products without further separation and purification. The broad and potent antimicrobial activity of CS as well as CR, now documented in this study, confirms their suitability for use in effective, low-cost, and eco-friendly nanoparticles in future applications. The antimicrobial activity of seagrasses also plays an important role in the marine ecosystem. There are many reports that seagrasses produce secondary metabolites for defense against herbivores, pathogens, fouling organisms, and competitors for space (Harrison, 1982; Bernard and Pesando, 1989; Jensen *et al*., 1998; Ferrat *et al*., 2001; Papazian *et al*., 2019).

Antioxidant activity

The extracts from the aboveground (leaves) and belowground parts (rhizomes, roots) of CR and CS obtained with three different solvents showed significantly different DPPH scavenging activity ($p<0.05$) at the concentration of 1,000 $\mu g \cdot mL^{-1}$ (Figure 2). The highest DPPH radical scavenging activity of CR belonged to root, rhizome and leaf extracted with 70% acetone and root and rhizome extracted with methanol, while highest DPPH of CS was from root and leaf extracted with 70% acetone and root extracted with methanol (Figure 2). The extracts from all parts of CR and CS in dichloromethane showed very low DPPH scavenging activity (19-33 %). The most active extract was the root-70% acetone extract of CS and leaf-70% acetone extract of CR, with IC_{50} of 62.1 and 125.8 $\mu g \cdot mL^{-1}$, respectively (Table 2).

Table 2. IC_{50} values ($\mu g \cdot mL^{-1}$) for DPPH and ABTS scavenging activity of extracts from *Cymodocea rotundata* and *C. serrulata* with three extraction solvents.

Seagrass	Part	Solvent	DPPH	ABTS
CR	Root	70% Acetone	167.1±6.41	146.6±5.32
		Methanol	136.2±6.71	120.9±6.63
		Dichloromethane	nt	nt
	Rhizome	70% Acetone	259.3±8.15	226.7±6.91
		Methanol	194.8±6.64	170.9±5.23
		Dichloromethane	nt	nt
	Leaf	70% Acetone	125.8±6.89	60.60±3.92
		Methanol	196.8±8.72	197.5±5.31
		Dichloromethane	nt	nt
CS	Root	70% Acetone	62.1±4.01	43.96±4.28
		Methanol	243.7±6.44	174.9±7.38
		Dichloromethane	nt	nt
	Rhizome	70% Acetone	335.4±10.16	430.4±10.24
		Methanol	nt	nt
		Dichloromethane	nt	nt
	Leaf	70% Acetone	112.9±6.68	65.60±6.18
		Methanol	nt	nt
		Dichloromethane	nt	nt
Ascorbic acid			56.08±4.95	50.12±3.95
BHT			105.6±3.39	98.65±4.12

Note: nt = not tested (% scavenging activity <50 %)

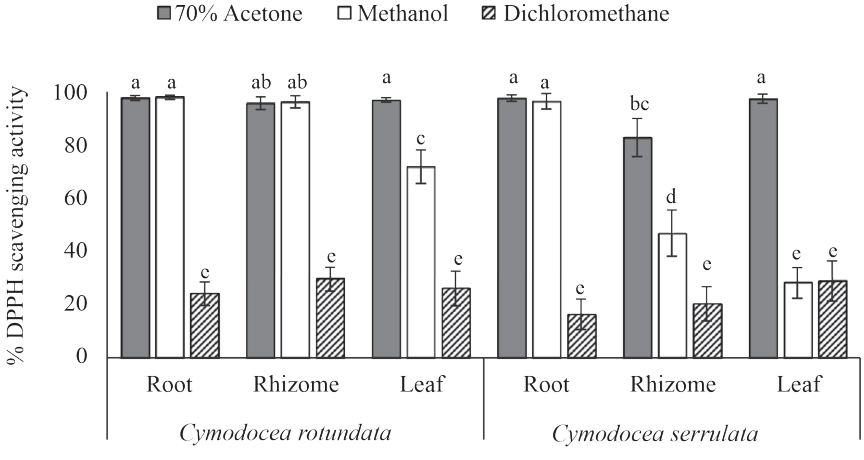

Figure 2. Histograms representing mean percentage of DPPH scavenging activity of extracts from *Cymodocea rotundata* and *C. serrulata* with three extraction solvents; Error bars represent ±SD.

Note: Different lowercase letters above bars indicate significant difference ($p<0.05$).

The 18 extracts also had significantly different ABTS scavenging activity ($p<0.05$) (Figure 3). The maximum activity belonged to root, rhizome and leaf extracted with 70% acetone and root and rhizome extracted with methanol, while maximum activity of CS was found for root and leaf extracted with 70% acetone (Figure 3). The most effective extracts for ABTS scavenging activity were root-70% acetone extract of CS and leaf-70% acetone extract of CR, with IC_{50} of 43.96 and 60.60 $\mu g \cdot mL^{-1}$, respectively (Table 2). The extracts that have high potential for development as natural antioxidants, possessing both effective DPPH and ABTS scavenging activity, are leaf-70% acetone extract of CR and root-70% acetone extract of CS. Interestingly, potent DPPH and ABTS scavenging activity was found in extracts from both aboveground and belowground parts. We observed very weak activity from dichloromethane extracts, which was a notable contrast from antimicrobial activity, and suggests that different compounds presented in the extracts are responsible for the different bioactivities.

Natural antioxidants are in demand for promoting human health. They are seen as a potential replacement of chemical antioxidants, which may have adverse impacts on health and the environment (Sureda *et al*., 2008; Kannan *et al*., 2010b; 2013b; Santoso *et al*., 2012). Among the various species of seagrass examined, CR extracts have shown higher DPPH scavenging activity than other species (Kannan *et al*., 2013b; Jeyapragash *et al*., 2016). Our study reinforced the conclusion that CR and CS are suitable seagrass sources for the development of alternative antioxidants of natural origin. Chanthini *et al*. (2015) prepared silver nanoparticles (AgNPs) from CS aqueous extract, which possessed both antioxidant and in vitro cytotoxic activity.

The variation of antioxidant activity of extracts depends on many parameters such as plant parts, sample location, and the solvents used in the extraction process (Santoso *et al*., 2012; Kannan *et al*., 2013b; Jeyapragash *et al*., 2016). The separated parts and solvents for extraction in this study showed more potent antioxidant activity than previous reports that mostly used only the leaves. The DPPH and ABTS radical scavenging activity in this study was substantially higher (>90 %) than leaf-methanol extract of CR (78.84 %) (Jeyapragash *et al*., 2016), leaf-aqueous methanol extract of CR (70.30 %) (Kannan *et al*., 2013b), and leaf-ethanol extract of *Enhalus acoroides* (25.76 %) (Kannan *et al*., 2010b). In contrast, methanol extracts of whole plants of CR showed high DPPH scavenging activity, with IC_{50} of 123.72 ppm, which was similar

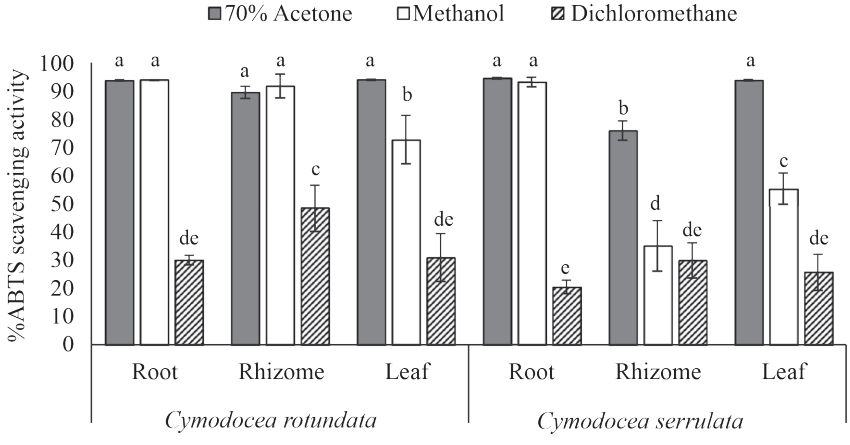

Figure 3. Histograms representing mean percentage of ABTS scavenging activity of extracts from *Cymodocea rotundata* and *C. serrulata* with three extraction solvents; Error bars represent ±SD.

Note: Different lowercase letters above bars indicate significant difference ($p<0.05$).

to this study (Santoso *et al*., 2012). An antagonistic effect might be involved from using the whole plants or using separate parts of the seagrasses, leading to differences in antioxidant activity. The antioxidant activity produced by CR and CS suggests an important role in chemical defense, considering the report that *Posidonia oceanica* was induced to produce higher amounts of antioxidant metabolites when they were fouled by epiphytes (Sureda *et al*., 2008). Loucks *et al*. (2013) reported that *Thalassia testudinum* produced lipopolysaccharide as an oxidative burst for its defense response system.

Antifouling activity and brine shrimp toxicity

Presently, preventive measures for biofouling are based mainly on chemical-based paints with high toxicity, so alternative natural-based products that are safe and environmentally friendly are in need (Vilas-Boas *et al*., 2017). The inhibition of byssus attachment assay is a reliable and cost-saving method for screening candidates during antifoulant development (Da Gama *et al*., 2003). The 18 extracts in our study had significantly different % inhibition of byssus production and attachment ($p<0.05$) (Figure 4). The maximum inhibition byssus productions (100±0.00 %) was found for root-70% acetone and leaf-70% acetone extracts of CS. The same two extracts from CR also inhibited byssus production at high rates of 90.07±3.44 and 94.07±1.15 % (Figure 4). The highest efficacy for byssus production inhibition expressed as ED_{50} was for leaf-70% acetone extracts of both CR and CS, with the values of 6.18 and 6.12 $\mu g \cdot mL^{-1}$, respectively (Table 3).

Interestingly, the only potent antifouling extracts were those from leaves in 70% acetone. The leaves of CR and CS had more surface area than the rhizomes and roots, and also were growing aboveground, which is the most suitable substrate for fouling organisms. The leaf parts of CR and CS likely provide protection from fouling organisms by producing antifouling metabolites. Moreover, we also found that the root-70% acetone extracts of CR and CS inhibited byssus production. The roots of seagrasses might release antifouling metabolites against epiphytes and pathogenic bacteria.

There was a report that leaf-methanol extracts of CS possessed interesting antifouling properties, such as antimicrofouling, inhibiting settlement of limpets, inhibiting byssus production and inhibiting attachment of mussels (*Perna indica*), with ED_{50} of 17.82±1.07 $\mu g \cdot mL^{-1}$ (Iyapparaj *et al*., 2014). We also found the leaves to be the most active part for antifouling activity, but the most suitable extraction solvent in our study was 70% acetone.

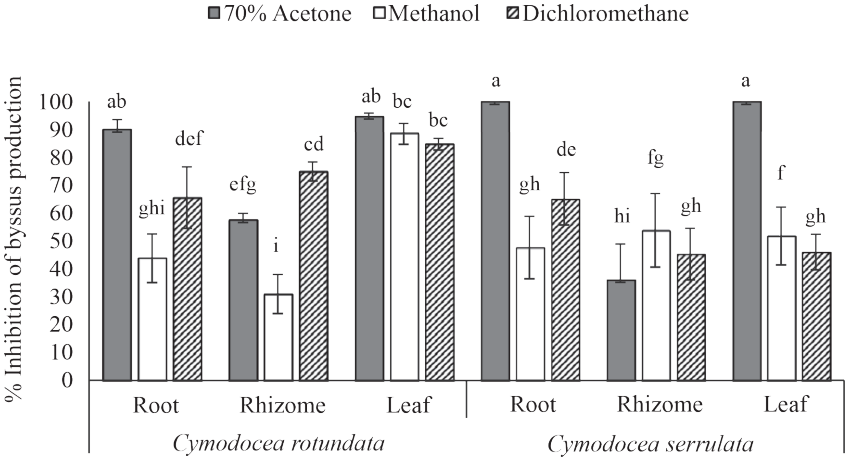

Figure 4. Histograms representing mean percentage of inhibition of byssus production of extracts from *Cymodocea rotundata* and *C. serrulata* with three extraction solvents; Error bars represent ±SD.

Note: Different lowercase letters above bars indicate significant difference ($p<0.05$).

Table 3. ED_{50} values ($\mu g \cdot mL^{-1}$) for inhibition of byssus production, % mortality and LC_{50} ($\mu g \cdot mL^{-1}$) of brine shrimp treated with extracts from *Cymodocea rotundata* and *C. serrulata* with three extraction solvents.

Seagrass	Part	Solvent	Inhibition of byssus production	Toxicity to brine shrimp	
			(ED_{50})	% mortality	LC_{50}
CR	Root	70% Acetone	9.52±4.16	20.00±10.00^{cd}	189±5.92
		Methanol	nt	0.00±0.00^{d}	nt
		Dichloromethane	nt	6.67±1.71^{cd}	nt
	Rhizome	70% Acetone	nt	16.67±5.77^{cd}	nt
		Methanol	nt	59.00±0.00^{a}	106±6.24
		Dichloromethane	18.56±6.35	26.67±5.77^{bc}	169±7.57
	Leaf	70% Acetone	6.18±3.51	16.67±5.77^{cd}	nt
		Methanol	6.55±3.05	0.00±0.00^{cd}	nt
		Dichloromethane	12.93±3.21	23.33±5.77^{cd}	169±3.29
CS	Root	70% Acetone	7.12±2.73	6.67±1.43^{cd}	nt
		Methanol	nt	50.00±10.00^{ab}	140.3±8.81
		Dichloromethane	nt	6.67±5.77^{cd}	nt
	Rhizome	70% Acetone	nt	23.33±5.77^{cd}	191.7±4.84
		Methanol	nt	56.67±11.55^{a}	125.5±14.85
		Dichloromethane	nt	3.33±1.71^{cd}	nt
	Leaf	70% Acetone	6.12±2.77	16.67±5.28^{cd}	nt
		Methanol	nt	10.00±4.43^{cd}	nt
		Dichloromethane	nt	13.33±8.71^{cd}	nt

Note: nt = not tested (the % inhibition byssus production <50 % and % mortality <20 %); Values within a column superscripted with different lowercase letters are significantly different ($p<0.05$).

Therefore, both the seagrass part and extraction solvent affects the antifouling activity of CR and CS. CS extracts have been reported as potential antifoulants due to their inhibition of marine biofilm bacteria (Mayavu *et al*., 2009). Furthermore, Iyapparaj *et al*. (2013) reported that the toxicity of methanolic extract of *Syringodium isoetifolium* against mussels was lower than the most toxic approved chemical, tributyltin (TBT) demonstrating the safety of seagrass extracts as natural antifoulants. In the seagrass ecosystem, the seagrass *Thalassia testudinum* produced a new antibiotic, sulfated flavone glycoside, which provided defense against fouling microorganisms (Jensen *et al*., 1998). *Zostera marina*, also known to produce metabolites, alters the composition of the epiphytic community (Harrison, 1982). Thus, the inhibition of byssus production by CS and CR extracts provides evidence supporting their use against fouling organisms.

The toxicity of antifouling extracts must be evaluated before the development of these extracts for safe utilization in the marine environment. The CR and CS extracts with high potency in controlling bacteria and yeasts (root-dichloromethane extract of both species and rhizome-dichloromethane extract of CS) showed the lowest toxicity to brine shrimp (% mortality between 3.33±1.71 and 6.67±5.77) (Table 3). The efficacy of inhibition of byssus production and lower toxicity to *Artemia* of leaf-70% acetone extracts of both CS and CR suggested that they can be developed as environmentally friendly natural antifoulants.

Our results are consistent with Kannan *et al*. (2013b), who found low toxicity of extract from CR against brine shrimp nauplii. Another study on toxicity of extracts of *Syringodium isoetifolium* and CS to brine shrimp reported LC_{50} values of 732.14±9.21 and 394.16±5.16 $\mu g \cdot mL^{-1}$, respectively, which indicates very low toxicity to the marine environment (Iyapparaj *et al*., 2014).

Chemical constituents of CR and CS

Seagrasses have been reported to possess various classes of bioactive constituents (Namadevan and Varadharaj, 2017; Kavitha *et al*., 2020), with phenolic compounds (such as phenolic acid) being the major active constituents of seagrasses (Zapata and McMillan, 1979; Todd *et al*., 1993; Agostini *et al*., 1997). The antimicrobial, antioxidant, and antifouling activities from the extracts of CR and CS in this study might be due to the presence of various compounds, especially phenolic compounds. Phenolics (tannin and phenol) have been implicated as antioxidants in algae and terrestrial plants. The phenol and flavonoid content of the 18 extracts in this study were significantly different ($p<0.05$) (Table 4). The highest amounts of phenol in CR and CS were observed in leaf-70% acetone and root-70% acetone extracts (at 5.47 and 5.02 mg $GAE \cdot g^{-1}$, respectively) (Table 4). This is consistent with the report by Kannan *et al*. (2010b), wherein leaf extracts of *Enhalus acoroides* were found to have high levels of phenolic compounds. The amount of phenol in extracts of both CR and CS in this study correlated well with % inhibition of byssus production ($r = 0.557$). We found a weaker correlation between phenol and DPPH and ABTS scavenging activity ($r = 0.468$ and 0.381); this was also in agreement with previous results for ethanol extracts of *E. acoroides* (Kannan *et al*., 2010b). There was also a report that the antibacterial activity of crude extracts of leaves of *Halodule pinifolia* and CR correlated well with their total phenolic content (Kannan *et al*., 2012).

Table 4. Phenol (mg $GAE \cdot g^{-1}$) and Flavonoid (mg $QTE \cdot g^{-1}$) content of extracts from *Cymodocea rotundata* and *C. serrulata* with three extraction solvents.

Seagrass	Part	Solvent	Phenol	Flavonoid
CR	Root	70% Acetone	4.01 ± 0.05^{ef}	0.07 ± 0.04^{hi}
		Methanol	3.73 ± 0.01^{gh}	0.17 ± 0.01^{ghi}
		Dichloromethane	3.72 ± 0.12^{h}	0.17 ± 0.01^{ghi}
	Rhizome	70% Acetone	5.16 ± 0.14^{b}	0.26 ± 0.05^{gh}
		Methanol	$3.82\pm0.0.3^{fgh}$	0.19 ± 0.02^{ghi}
		Dichloromethane	4.12 ± 0.04^{e}	0.21 ± 0.04^{ghi}
	Leaf	70% Acetone	5.47 ± 0.01^{a}	4.28 ± 0.09^{c}
		Methanol	4.61 ± 0.01^{cd}	1.15 ± 0.04^{e}
		Dichloromethane	3.93 ± 0.06^{efg}	6.82 ± 0.09^{a}
CS	Root	70% Acetone	5.02 ± 0.06^{b}	0.58 ± 0.01^{f}
		Methanol	3.21 ± 0.02^{j}	0.25 ± 0.06^{gh}
		Dichloromethane	3.45 ± 0.12^{i}	0.27 ± 0.07^{g}
	Rhizome	70% Acetone	3.29 ± 0.06^{ij}	0.01 ± 0.005^{i}
		Methanol	4.51 ± 0.09^{d}	0.00 ± 0.00^{i}
		Dichloromethane	3.88 ± 0.05^{fgh}	0.61 ± 0.02^{f}
	Leaf	70% Acetone	4.74 ± 0.02^{c}	2.79 ± 0.05^{d}
		Methanol	2.06 ± 0.03^{k}	0.68 ± 0.04^{f}
		Dichloromethane	3.12 ± 0.06^{j}	5.46 ± 0.20^{b}

Note: Values within a column superscripted with different lowercase letters are significantly different ($p<0.05$).

Some phenolic compounds in seagrasses have been suggested to act in chemical defense and be involved with the adaptation of seagrass. *Zostera marina* leaf surface-associated phenolics and fatty acids were suggested to modulate microbial biofoulers and provide chemical defenses and structural protection to eelgrass in its marine environment (Papazian *et al*., 2019). Further, the total phenolic compounds in *Z. marina* have been shown to increase in response to infection by *Labyrinthula zostera* (Verger and Develi, 1996), to deter the feeding of amphipods, to impact the food web in seagrass meadows, and to alter the composition of the epiphytic community (Harrison, 1982). *Posidonia oceanica* was shown to produce phenolic compounds for space competition with the seaweed *Caulerpa taxifolia* (Ferrat *et al*., 2001). The dissolved phenol from decomposed seagrasses reportedly affects other marine organisms and changed the nitrogen cycle in the marine environment (Zapata and McMillan, 1979). Recently, the secondary metabolites (especially phenolic compounds) in *P. oceanica* were found to increase when seagrasses were stressed from human impacts and climate change (Mannino and Micheli, 2020).

In plants, flavonoids serve many functions and also modulate interactions with other organisms (Harborne and Williams, 2000). The flavonoid content of CR and CS extracts was less than their phenol content. The maximum flavonoid content was found in leaf-dichloromethane extracts from both CR and CS (6.82 and 5.46 mg QT·g^{-1}, respectively), similar to CS collected in India (5.12 mg·g^{-1}) (Kannan *et al*., 2013b). The correlation between flavonoid and % DPPH scavenging activity, % ABTS scavenging activity, and % inhibition byssus production was quite low (r = -0.169, -0.227, and 0.307, respectively). Mani *et al*. (2012) reported on the antibacterial activity of CR, with several of its phytoconstituents and flavonoids showing negative activity. Gavin and Durako (2011) reported that *Halophila johnsonii* produced flavonoids with sunscreen and antioxidant functions.

The chemical constituents of the 18 CS and CR extracts differed based on TLC. The moieties that appeared on the chromatogram were terpene, phenol, coumarin, anthraquinone, and anthrone. The terpene and anthraquinone moieties appeared in root-dichloromethane extracts of both CR and CS, and have been suggested to produce antibacterial activity. The blue spot indicating the coumarin moiety appeared in the leaf-methanol and leaf-70% acetone extracts of both CR and CS; this moiety is suggested to act as an antioxidant. Kannan *et al*. (2013b) reported the presence of p-coumaric acid in the aqueous methanolic extracts of CR and suggested it was acting as an antioxidant. Jeyapragash *et al*. (2016) reported that caffeic acid and p-coumaric acid from CR extracts produced antioxidant activity. Moreover, the chromatogram in our study revealed a red spot indicating polyphenol in root and rhizome parts of CR and CS in 70% acetone. We also found a yellow spot produced by the anthrone moiety in extracts of roots and rhizomes in both 70% acetone and methanol of CR and CS. These data reveal the varied chemical constituents in the aboveground and belowground parts of these two species extracted with different solvents, and can help explain the differences in bioactivities. Mani *et al*. (2012) reported the antibacterial activity of CR and described several of its phytoconstituents, including tannins, saponins, terpenoids, cardiac glycosides and alkaloids. For CS, various constituents have been reported: alkaloids, carboxylic acid, coumarins, flavonoids, phenols, saponin, xanthoprotein, protein, steroids, tannins, and sugars (Ravikumar *et al*., 2011b). Iyapparaj *et al*. (2014) reported the fatty acids (C16 to C24) as the major components responsible for the antifouling properties of CS and *Syringodium isoetifolium*. Pushpabharathi *et al*. (2018) revealed bioactive compounds from CS including hexadecanoic acid methyl ester, tetradecanoic acid, cholesta 4, 6 dien 3 ol, and stigmasterol that were responsible for antioxidant activity. Other compounds from seagrasses have been proposed to have bioactive properties. Bel Mabrouk *et al*. (2020) reported on many compounds from *Halophila stipulacea* and their various activities, such as polyphenols (cytotoxicity), amino acids (metaboloic diseases), and fatty acids (antifouling activity).

CONCLUSION

To our knowledge, the present research is a pioneering report on the different bioactivities of CR and CS extracts and their variation among aboveground (leaves) and belowground parts (rhizomes, roots). We were able to identify the plant parts and extraction solvents that produced the most potent antimicrobial, antioxidant, and antifouling activities. We confirmed that CR and CS extracts are valuable resources for antimicrobial, antioxidant, and antifouling applications. These extracts showed a high potential to be used in the development of safe, environmentally acceptable, and effective biomedicines, cosmetics and antifoulants, and for industrial purposes. Further studies should address the separation, purification, and structure elucidation of the bioactive compounds from extracts of CR and CS.

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