Evaluation of *In-vitro* Antioxidant Activity of Seagrasses: Signals for Potential Alternate Source

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ABSTRACT

Background and Aim: Natural antioxidants have gained unique attention in recent years. Because of the carcinogenicity of synthetic compounds, there is a dearth for antioxidants from natural origin. Currently, seagrasses, the marine plants have gained attention for their secondary metabolites. Hence, the present study aims to examine *in vitro* antioxidant activity of both leaf and rhizome extracts of six seagrass species and has not yet been investigated. **Methods:** Crude methanolic extracts of leaf and rhizome obtained, were evaluated for total phenolic contents using Folin-Ciocalteaus method. Antioxidant potential of seagrass extracts were evaluated using total antioxidant activity, DPPH, FRAP, ABTS assay, H_2O_2 and NO_2 scavenging assay and the phenolic compounds present in potent extracts were profiled by HPLC. **Results:** Maximum phenolic content and antioxidant activity was exhibited by leaf and rhizome extracts of *C. rotundata* followed by *H. uninervis*. Higher DPPH radical scavenging activity was found in leaf (78.84 ± 0.87) and rhizome extracts (75.480 ± 0.97) of *C. rotundata* and the lowest scavenging activity was found in the leaf and rhizome extracts of *H. ovata* (12.01 ± 0.63 and 5.769 ± 1.14). Among six species, *C. rotundata* exhibited higher radical scavenging activity containing the potential phenolic compounds. Conclusion: Present study portrays that leaf and rhizome extracts of *C. rotundata* acts as a potential source of antioxidant compounds with predominant presence of caffeic acid and ρ-coumaric acid that paves a way for the application of these compounds in both food and pharmaceutical industries as a multipotent antioxidant.

Key words: Caffeic acid, DPPH, Free radicals, Phenolic compounds, Reactive oxygen species, Radical scavenging activity.

INTRODUCTION

Natural antioxidants and their association with health benefits have gained unprecedented attention in recent years. They have multiple functions in biological systems and mainly defense against oxidation that produce free radicals in food, chemicals and in living systems.[1] During normal cellular activities, various processes produce reactive oxygen species (ROS) inside the cell, which can damage the cellular components such as lipids, proteins, and DNA, when produced at high rates.[2] The major action of antioxidants in cells is to prevent the damage caused by the action of reactive oxygen species. Several synthetic antioxidants, such as Butylated hydroxyl anisol (BHA), Butylated hydroxytolune (BHT) and Tetra butyl hydroquinone (TBHQ) are commercially available and are currently in use.[3] Because of carcinogenicity of synthetic antioxidants, there is dearth for antioxidants from natural origin.[4] Natural antioxidants play a vital role in antioxidant defense mechanism in the biological system and acts as free radical scavenger.

Currently, research on marine plants has brought to limelight bioactive natural products produced by them in response to physical, chemical and biological changes in the environment. In folk medicine, seagrasses have been used for a variety of remedial purpose, eg: for the treatment of fever and skin

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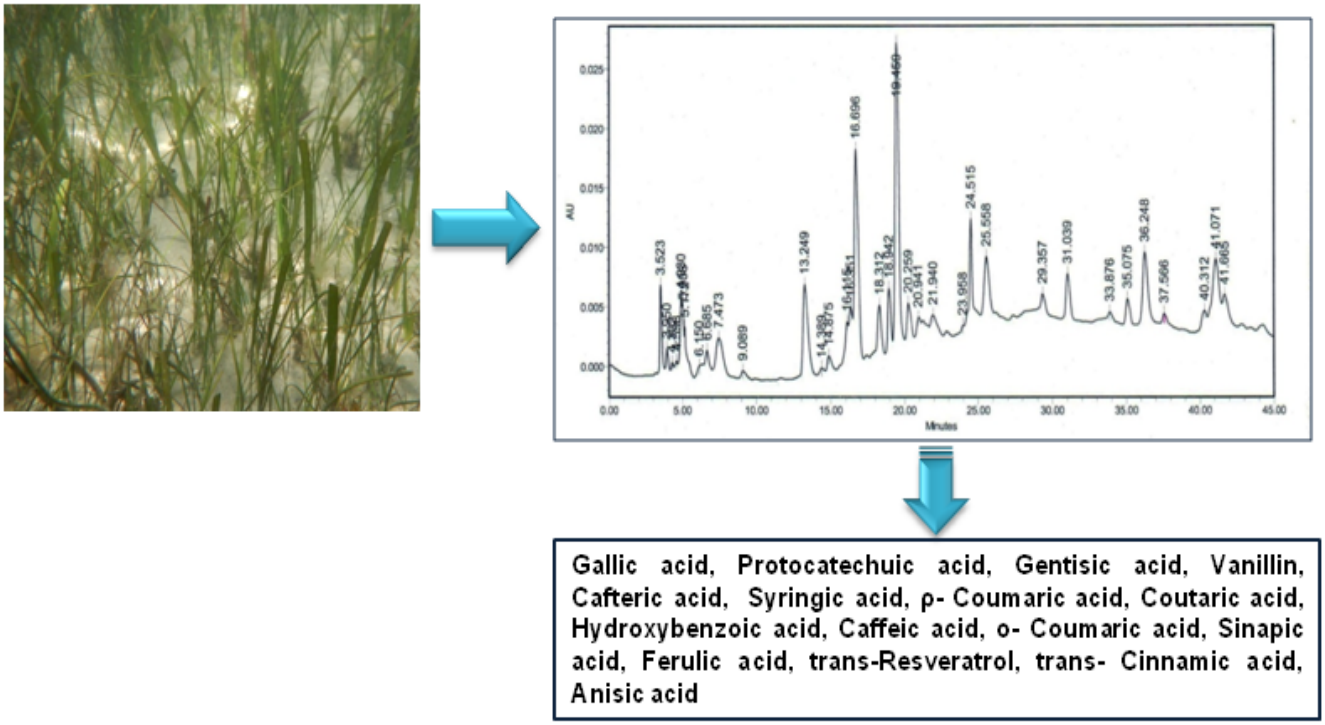

Graphical Abstract

disease, muscle pain, wounds and stomach problems, remedy against stings of different kind of rays, tranquillizer for babies.[5] Seagrasses are known to produce secondary metabolites as defence mechanism under stress conditions and[2] these compounds are found to be anti-oxidative in nature.

These bioactive natural products have been proved to have unique pharmacological properties.[6-8] Hence, the growing interest to find cheap, renewable and abundant sources of antioxidants has fostered research on marine plants. Many of the biological functions of seagrasses such as antioxidant property, antiviral, anti-diabetic and vaso protective; insecticidal and larvicidal activity have been attributed by the higher phenolic content of seagrass tissue. However, research on the antioxidant activity of seagrasses has not been much carried out compared to the seaweeds, and initiated only recently.[9-10] Hence, the present study aims to examine the antioxidant capacity of both leaf and rhizome extracts of six seagrass species for their in vitro antioxidant activity and to interpret the results with phenolic compounds of the potent extract.

MATERIALS AND METHODS

Chemicals and reagents

Chemicals such as 2, 2-diphenyl-1-picrylhydrazyl (DPPH), 2, 2'-azinobis-3-ethylbenzothizoline-6-sulphonic acid (ABTS), 2,4,6-tripyridyl-s-triazine (TPTZ) and trolox were obtained from Hi-media Laboratories Ltd., Mumbai. Other chemicals and reagents used in the present study were procured from Merck Ltd., Mumbai.

Sample collection

Fresh leaves and rhizome of *Halodule uninervis* (Forsk.) Asch., *Syringodium isoetifolium* (Asch.) Dandy, *Cymodocea rotundata* Ehrenb. & Hempr. Ex Asch., *Thalassia hemprichii* (Ehrenb.) Asch., *Enhalus acoroides* (L.F.) Royle and *Halophila ovata* Gaud. were collected during December, 2012 from Palk Bay, India. Seagrass samples were identified following the field key and[11] confirmed later in accordance with Ramamurthy *et al*.[12] Samples were washed immediately using native seawater to remove epiphytes and sand particles, and then rinsed several times using distilled water. Both leaves and rhizomes were segregated and blotted using filterpaper, shade dried to constant weight and stored for further analysis.

Extraction of seagrass polyphenolic compounds

For the extraction of polyphenolic compounds, 1g of the sample (both leaves and rhizome) was suspended in 50 mL of 50% aqueous ethanol (v/v) separately and left for 24 hrs at room temperature. Samples were then homogenized using a pestle and mortar. The mixture was heated in a water bath at 60°C for 3 hours and centrifuged (5000 rpm for 10 minutes). The supernatant was then acidified using 0.5 ml of 1N HCL and incubated at 60°C for overnight. The residue was filtered using Whatman No.1 filter paper and extracted using equal volume of methanol and evaporated to dryness under reduced pressure

in a desiccator. The dried powder was then re-dissolved in 1ml of methanol (HPLC grade) and treated as the stock solution.

Total Phenolic content

The concentration of total phenols was determined by the Folin-Ciocalteu method[13] using a Shimadzu UV-VIS spectrophotometer (UV-2450). The total phenolic content was expressed as gallic acid equivalent (GAE) in milligram per gram of extract.

Total antioxidant activity

The total antioxidant activity of the crude methanolic extracts was evaluated by phosphomolybdenum method and[14] the values are expressed as ascorbic acid equivalents per gram extract (mg AE/g extract).

DPPH free radical scavenging activity

Free radical scavenging activity was evaluated following the method[15] with slight modifications, with 0.1 mL of sample solution; 2.9 mL of DPPH solution (60 μM) was added. The reaction mixture was left to stand in the dark for 30 minutes at room temperature and the absorbance was measured at 517 nm. Butylated hydroxytolune (BHT, 20-100 μg/ml) was used as positive control. The scavenging effect of DPPH radicals was calculated using the following equation,

$$DPPH\ Scavenging\ effect\ (\%) = A0 - A1/\ A0 * 100$$

Where A0 is the absorbance of the control, A1 is the absorbance of the sample.

ABTS radical scavenging activity

An improved ABTS decolorisation assay[16] was carried out that involves the generation of $ABTS^{+}$ chromophore by the oxidation of ABTS with potassium persulfate. Scavenging capacity of the extract was expressed with that of Trolox equivalent antioxidant activity, the water soluble analogue of vitamin E as reference standard.

Ferric reducing antioxidant power (FRAP) assay

A modified method[17] was adopted for the FRAP assay. To 1.5 ml of freshly prepared FRAP reagent (25 mL of 300 mM acetate buffer, pH 3.6, 2.85 ml of 10 mM TPTZ in 40 mMHCl and 20 mMFecl3. $6H_2O$), 0.15 mL of sample (100 μg/ml) was added and let to stand for 30 min in the dark condition. Absorbance of the colored product (Ferrous tripyridyltriazine complex) was taken at 593 nm. ΔA is proportional to the combined ferric reducing/ antioxidant power (FRAP) value of the antioxidants in the sample. The relative activity of the sample was compared with standard ascorbic acid (20-100 μg/ml).

Total reducing power

Total reducing power of the extracts was determined by the method[18] with slight modification. Sample (100 μg/ml, 0.5 mL) was mixed with phosphate buffer (0.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide (0.5 ml, 1%). The mixture was incubated at 50°C for 20 min. Then, 0.5 ml of trichloroacetic acid (10%) was added to mixture, which was then centrifuged for 10 min at 3000 rpm. To the supernatant (1 ml), $FeCl_3$ (0.5 ml, 0.1%) was added and made upto 4 ml using distilled water. After 10 minutes, the absorbance was measured at 700 nm. The higher the absorbance of the reaction mixture the greater is the reducing power. Ascorbic acid (20-100 μg/ml) was used as positive control.

H_2O_2 scavenging assay

H_2O_2 scavenging activity was determined by following the method.[19] Absorbance of H_2O_2 at 230 nm was determined after 10 minutes against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of H_2O_2 scavenging was calculated using the following equation,

$$H_2O_2\ scavenging\ activity = A0\text{-}A1/\ A0 * 100$$

Where A0 was the absorbance of the control, and A1 was the absorbance in the presence of the sample.

NO_2 scavenging assay

Nitric oxide scavenging activity was evaluated by a combined method[20,21] with slight modification. Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions which were measured by Griess reaction. Reaction mixture (2.5 ml) containing sodium nitroprusside (10 mM) in phosphate buffer (pH 7.4), 0.5 ml of the sample extract was added and incubated at 25°C for 30 min. After incubation, 0.5 ml of the Griess reagent (1% sulphanilamide, 2% orthophosphoric acid and 0.1% Napthyl ethylinediamine hydrochloride) was added. The absorbance of the chromophore formed was read at 546 nm. BHT was used as the positive control (50–250 μg/ ml) and the NO scavenging activity was reported as % inhibition and calculated as follows,

$$NO_2\ scavenging\ activity = A0\text{-}A1/\ A0 * 100$$

Where A0 was the absorbance of the control, and A1 was the absorbance in the presence of the sample.

Statistical analysis

All experiments were conducted in triplicate and the values were reported as mean ± SD. The statistical significance

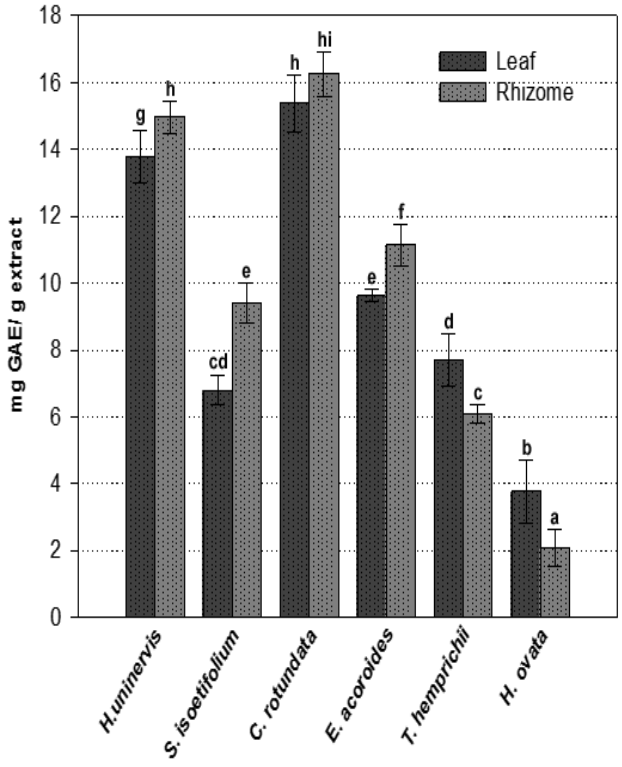

Figure 1: Total phenolic content recorded for the test seagrasses (Bars sharing the same alphabets are not significantly different, P<0.05)

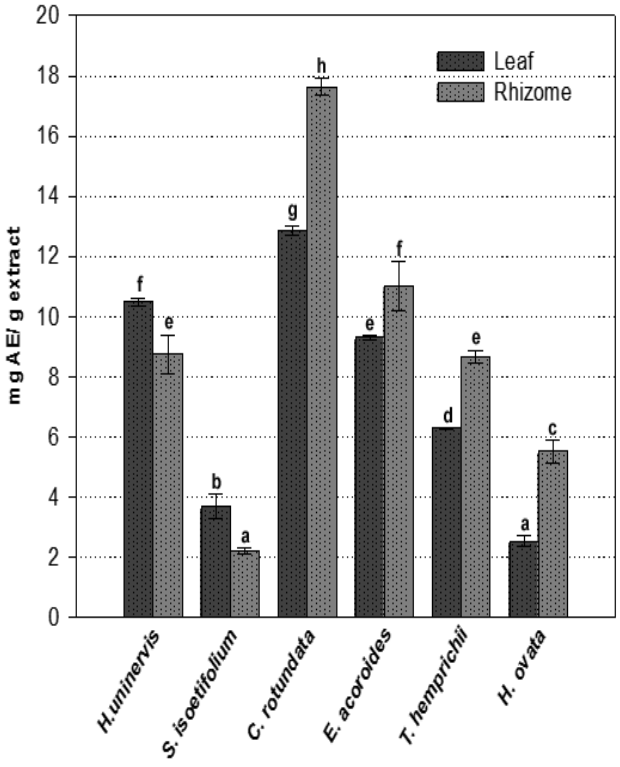

Figure 2: Total antioxidant activity recorded for the test seagrasses (Bars sharing the same alphabets are not significantly different, P<0.05)

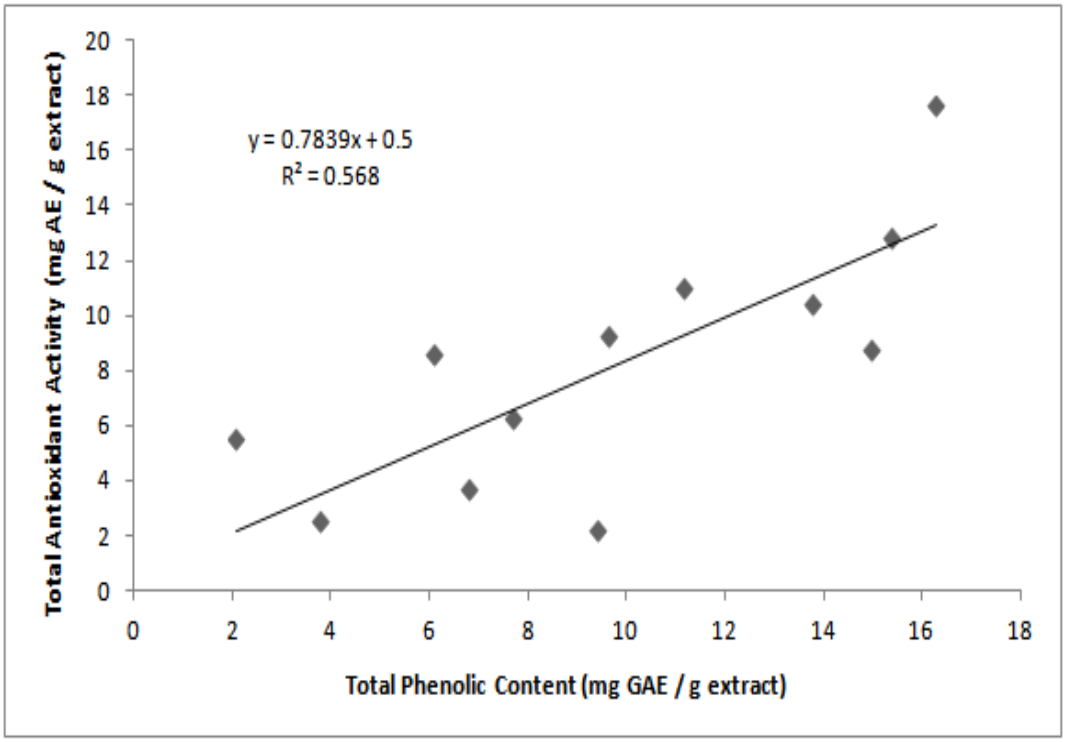

Figure 3 : Linear regression recorded between total phenolic content and total antioxidant activity of seagrassses

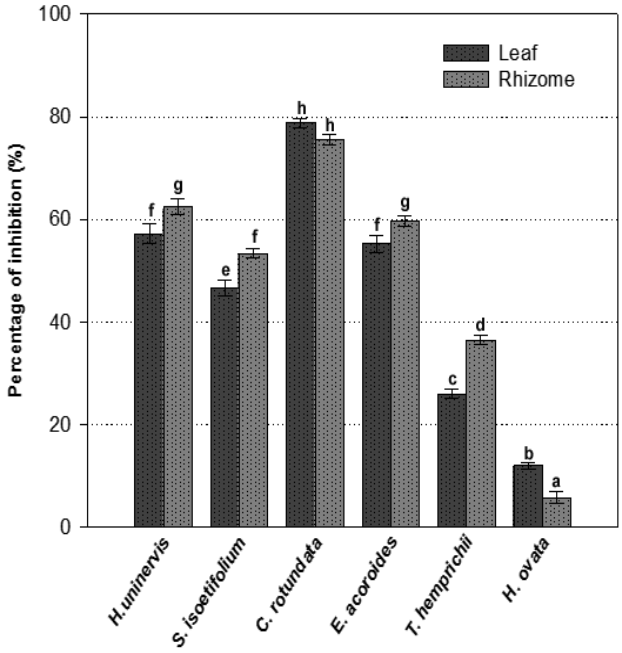

Figure 4: DPPH radical scavenging activity recorded for the test seagrasses(Bars sharing the same alphabets are not significantly different, P<0.05)

between antioxidant activity of the extracts was evaluated with one way ANOVA between the groups followed by Duncans multiple range test (P<0.05). All computations were done by employing the statistical software SPSS, version 11.5.

High Performance Liquid Chromatography (HPLC)

The analysis of phenolic compounds was carried out following the method.[22] The chromatographic system consisted of Waters 515 binary pump and Waters 2996 photodiode array (PDA) detector (Waters, Milford, MA, USA). RP-C18 column (Sun Fire™ 5 µm, 4.6 mm x 150 mm, Waters, USA) was used for separation. Water containing 0.1% formic acid (A) and methanol (B) were used as chromatographic eluents working on the gradient mode. Sample injection volume was 10 µl and the flow rate was set as 1 ml/min. The solvent gradient in volume ratios was as follows: 0–13 min, 15–33% B; 13–21 min, 33–39% B; 21–23 min, 39–45% B; 23–25 min, 45% B; 25–27 min, 45–15% B; 27–33 min, 15% B. The PDA detector was operated between 210 and 400 nm, with a resolution of 1.2 nm. The identification of phenolic compounds in the potential seagrass extract was performed by matching the HPLC retention time with those of compounds reported earlier.[23-25]

RESULTS

Prior to the polyphenolic estimation, all the extracts of seagrasses were subjected to spectroscopic scanning between 200–800 nm that exhibited absorption bands between 200–400 nm confirming the characteristic of polyphenol absorption.

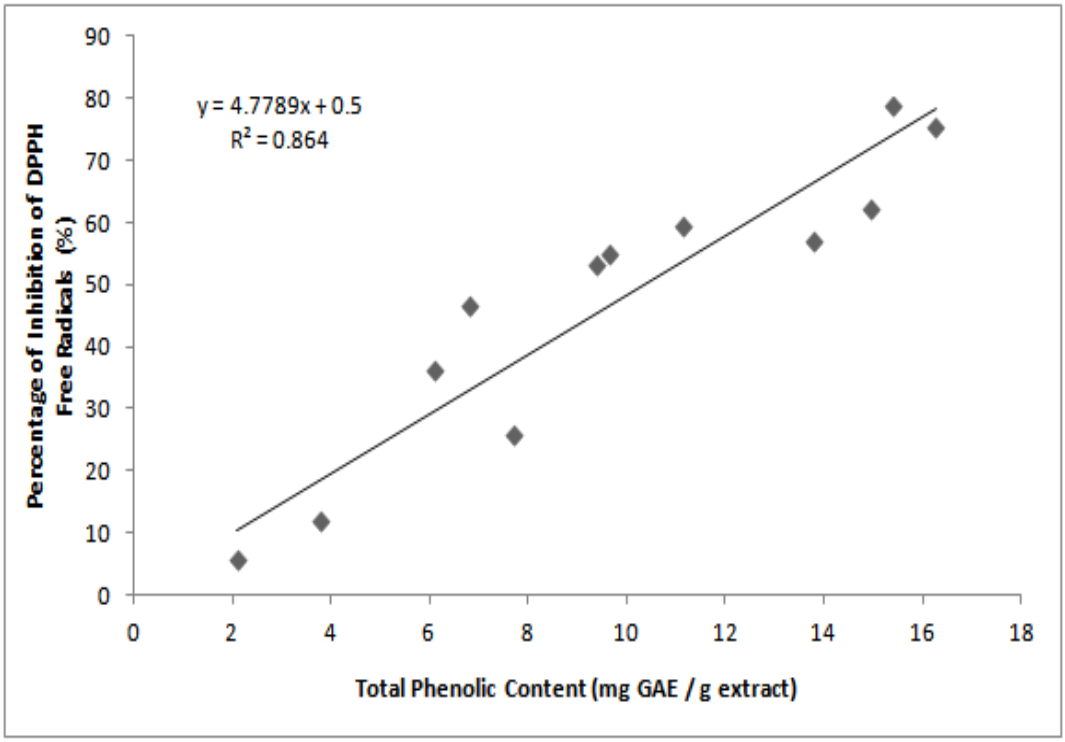

Figure 5 : Linear relationship recorded between total phenolic content and DPPH radical scavenging activity of seagrasses

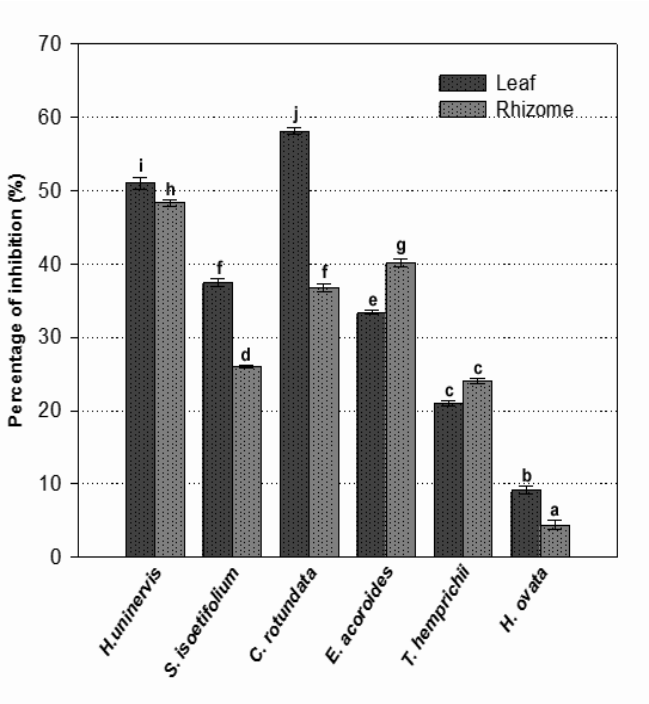

Figure 6: ABTS$^+$ radical scavenging activity recorded for the testseagrasses (Bars sharing the same alphabets are not significantly different, P<0.05)

Total phenolic content

All seagrass extracts contained a considerable amount of phenolic content. Rhizome and leaf extracts of *C. rotundata* (16.26 ± 0.67 and 15.38 ± 0.85 mg GAE/ g extract) and *H. uninervis* (14.96 ± 0.47 and 13.77 ± 0.79 mg GAE/ g extract) were found to have significantly (P<0.05) higher phenolic content than other seagrass extracts (Figure 1). Nevertheless, leaf and rhizome extracts of *H. ovate* showed the lowest phenolic content (3.77 ± 0.93 and 2.082 ± 0.557 mg GAE/ g extract).

Total antioxidant activity

The higher antioxidant capacity was observed in rhizome (17.639 ± 0.28 mg AE/ mg extract) and leaf (12.86 ± 0.14 mg AE/ mg extract) extracts of *C. rotundata* (Figure 2). The leaf extracts of *H. ovata* (2.54 ± 0.17 mg AE/ mg extract) and the rhizome extracts of *S. isoetifolium* (2.209 ± 0.119 mg AE/ mg extract) showed the lowest antioxidant activity. The results of the present study reveal that there is a significant linear relationship between the total phenolic content and total antioxidant activity with R^2 value=0.568 (Figure 3).

DPPH radical scavenging assay

Methanolic extracts of seagrasses (both leaf and rhizome) have stronger ability to scavenge DPPH radical. Leaf (78.84 ± 0.87%) and rhizome (75.480 ± 0.97%) extracts of *C. rotundata* were found have stronger ability to scavenge DPPH (Figure 4) and the lower scavenging activity was found in the leaf and rhizome extracts of *H. ovata* (12.01 ± 0.63% and 5.769 ± 1.14%). Leaf and rhizome extracts of *H. uninervis* and *E.acoroides* not showed any significance difference (P<0.05) depicting their similarity in DPPH

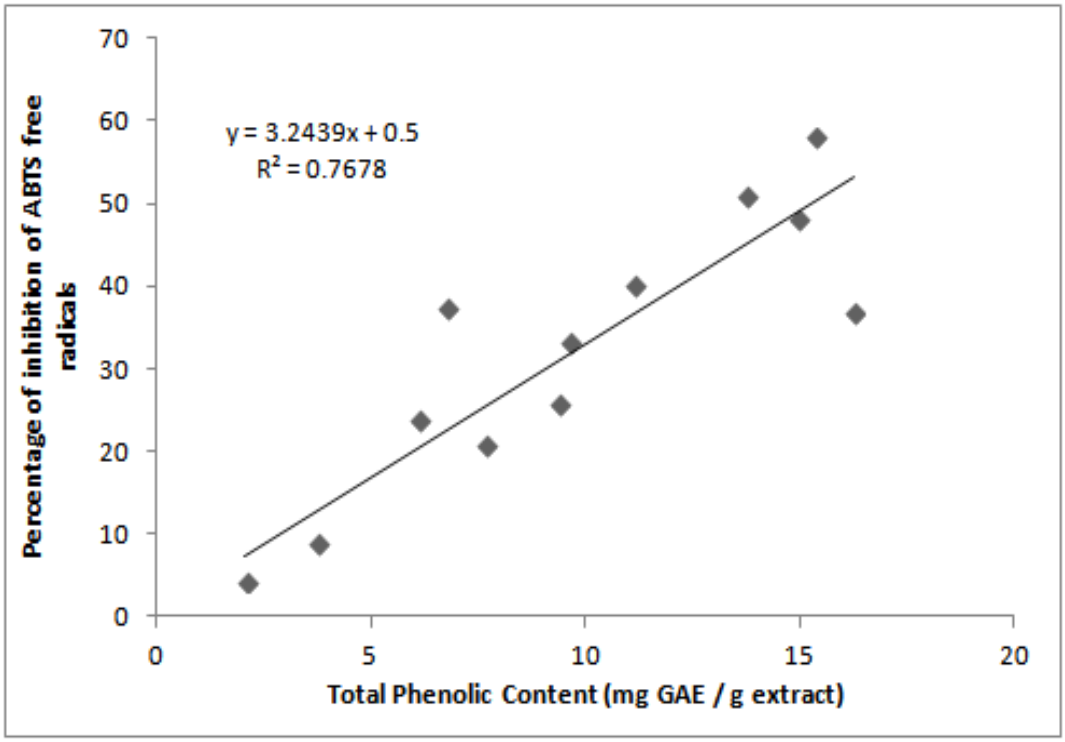

Figure 7 : Linear regression recorded between total phenolic content and ABTS$^+$ free radical scavenging activity

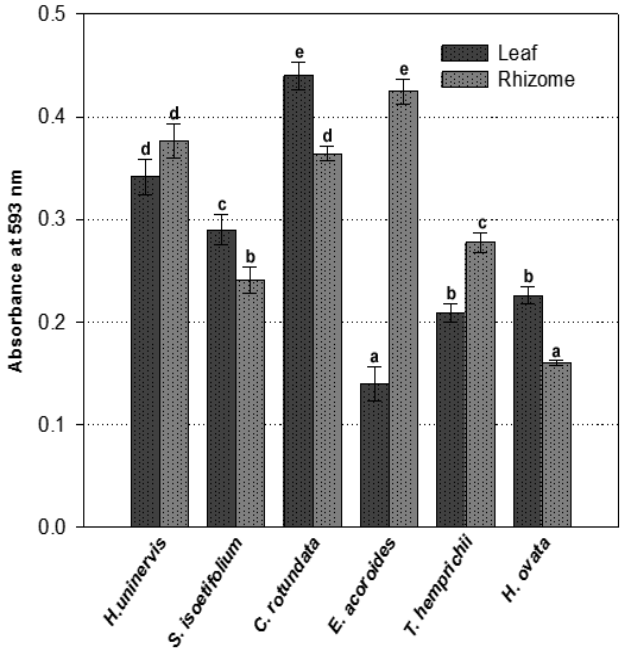

Figure 8: Ferric reducing antioxidant power recorded for the test seagrasses (Bars sharing the same alphabets are not significantly different, P<0.05)

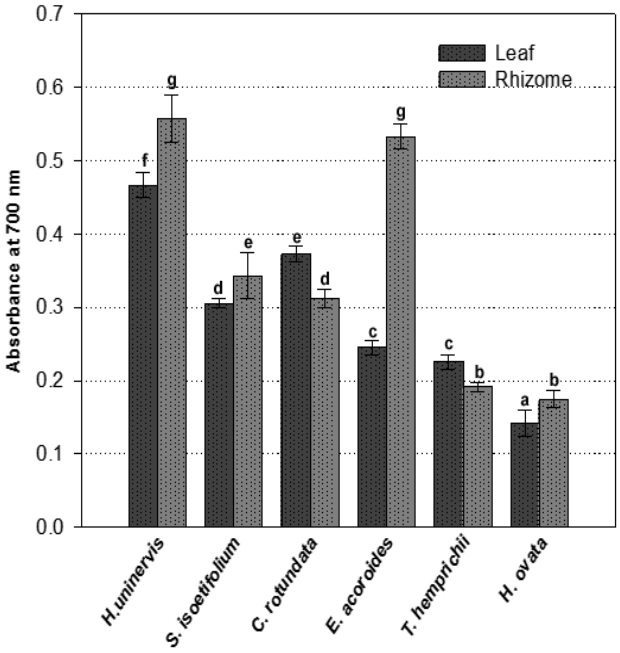

Figure 9: Total reducing power recorded for the test seagrasses (Bars sharing the same alphabets are not significantly different, P<0.05)

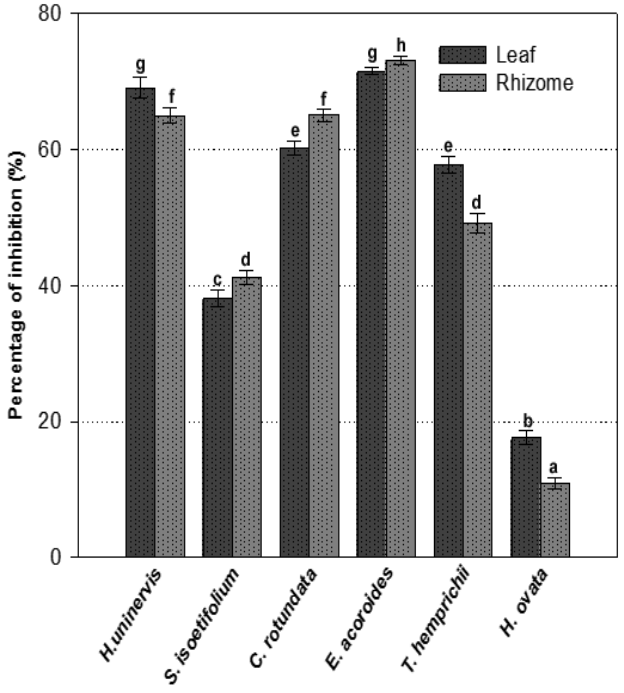

Figure 10: H_2O_2 Scavenging activity recorded for the test seagrasses (Bars sharing the same alphabets are not significantly different, P<0.05)

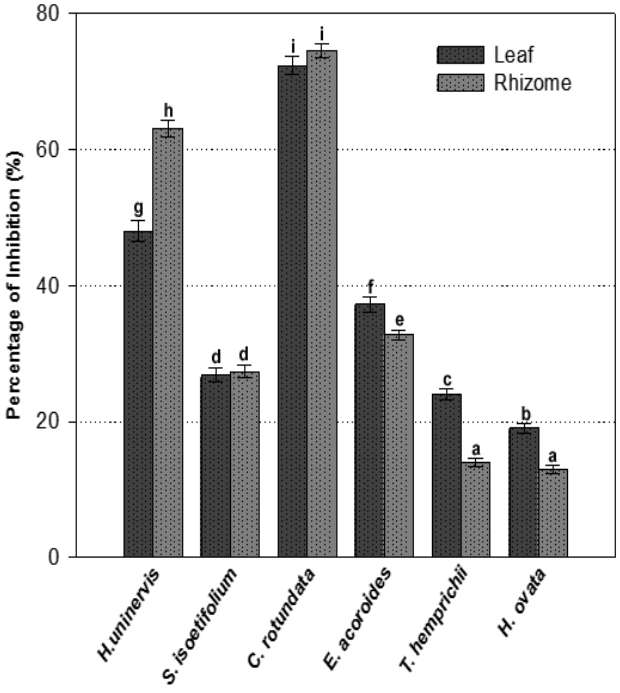

Figure 11: NO_2 scavenging activity recorded for the test seagrasses (Bars sharing the same alphabets are not significantly different, P<0.05)

Table 1: Phenolic acids detected in *C. rotundata* leaf extract based on the retention time

Retension time (min)	Phenolic compounds
4.598	Gallic acid
9.089	Protocatechuic acid
13.249	Gentisic acid
14.875	Vanillin
16.115	Cafteric acid
16.351	Syringic acid
16.696	ρ- Coumaric acid
18.312	Coutaric acid
18.942	Hydroxybenzoic acid
19.459	Caffeic acid
20.259	o- Coumaric acid
20.941	Sinapic acid
21.940	Ferulic acid
23.958	trans-Resveratrol
24.515	trans- Cinnamic acid
25.558	Anisic acid
29.357	Chicoric acid
31.039	Di-ferulolyl tartaric acid
35.075	Cinnamic acid

radical scavenging activity. A strong linear relationship (Figure 5) was found between the DPPH free radical scavenging activity with total phenolic content (R^2=0.864).

ABTS scavenging assay

The ability of the seagrass extracts to scavenge ABTS+ was expressed as trolox equivalent and the maximum scavenging activity was found in the leaf extracts of *C. rotundata* (58.10 ± 0.42), followed by *H. uninervis* (51.01 ± 0.84). *H. ovata* showed lowest scavenging activity for both leaf (9.12 ± 0.52) and rhizome (4.39 ± 0.65) extracts respectively (Figure 6). ABTS radical scavenging activity showed strong linear relationship with total phenolic content (R^2=0.767) respectively (Figure 7).

Ferric reducing antioxidant power assay

Antioxidant potential of seagrass extracts was ascertained from their ferric reducing antioxidant power and the maximum reducing ability was exhibited by the leaf extracts of *C. rotundata* (0.44 ± 0.013) and rhizome extracts of E. acoroides (0.42 ± 0.012). Minimum activity was found in the leaf (0.226 ± 0.008) and rhizome (0.16 ± 0.06) extracts of *H. ovata* (Figure 8).

Total reducing power

Total reducing power of the seagrass extracts was found to be maximum in the leaf (0.467 ± 0.017) and rhizome (0.557 ± 0.003) extracts of *H. uninervis*, followed by *C. rotundata*, whereas lower reducing ability were exhibited by *H. ovata* leaf (0.142 ± 0.017) and rhizome (0.175 ± 0.011) extracts (Figure 9).

H_2O_2 scavenging activity

The highest scavenging activity were exhibited by the rhizome extracts (73.10 ± 0.67%), followed by the leaf extract (71.57 ± 0.56%) of *E. acoroides*, whereas, *H. ovata* showed the lowest scavenging activity in both rhizome and leaf extracts (10.92 ± 0.86% and 17.59 ± 1.0%) respectively (Figure 10).

NO_2 scavenging activity

C. rotundata showed the higher NO_2 scavenging activity in both rhizome and leaf extracts (74.52 ± 1.02% and 72.34 ± 1.32%) while, the lower scavenging activity was found in leaf and rhizome extracts of *H. ovata* (18.99 ± 0.68% and 12.95 ± 0.68%) (Figure 11).

High performance liquid chromatography

By considering the overall performance of *C. rotundata* in exhibiting better antioxidant properties than the other seagrasses, the phenolic extract of the species was subjected for HPLC analysis. Chromatograms were recorded at 254 nm and the examination of the chromatogram revealed the presence of several phenolic compounds in the leaf extract of *C. rotundata* (Figure 12). The phenolic compounds were identified by comparing their retention time with similar compounds that have been previously studied under similar conditions (Table 1). Totally, nineteen compounds were identified, among which only two compounds namely caffeic acid (R_t= 19.459) and ρ-Coumaric acid (R_t=16.696) formed predominant peaks.

DISCUSSION

The present study was attempted to evaluate the antioxidant capacity of phenolic extract of both leaf and rhizomes of six seagrasses, collected from Palk Bay region. Though reports prevail on the antioxidant activity of seagrasses,[7,8,10] no studies have been yet made on comparative determination of anti-oxidative capacity of seagrass leaves and rhizomes, since both leaf and rhizome differ in their phenolic concentration. It is well known that phenolic compounds are soluble in polar solvents.[26] However, the solubility of phenolic compounds may differ in each type of solvent and the source of material. Earlier reports on antioxidant determination revealed that methanol was able to extract the phenols in high quantities showing highest scavenging activity from seagrasses.[8] So, the phenolic compounds in seagrasses may tend to be soluble in semi-polar solvent (methanol) and hence, the extraction of phenolic compounds from leaves and rhizomes of seagrasses was done using methanol.

The total phenol content was evaluated by using Folin-Ciocalteu assay, which is fast and simple method to determine the amount of phenolic content in the desired

Table 2: Total phenolic content and antioxidant capacity of different seagrasses

Seagrasses	Total Phenolic Content (mg GAE/ g extract)	Total Antioxidant Activity (mg AE/ g extract)	DPPH (% of inhibition)	ABTS (% of inhibition)	REFERENCES
C. rotundata	0.226 ± 0.006				
S. isoetifolium	0.209 ± 0.004	-	-	-	Athiperumalsami *et al*. (2008)
H. ovalis	0.070 ± 0.001				
H. pinifolia	0.183 ± 0.005				
E. acoroides					
Leaf	0.323 ± 0.028	11.770 ± 0.026	25.760 ± 0.040	-	
Root	0.258 ± 0.036	11.770 ± 0.026	20.250 ± 0.020		Kannan *et al*. (2010a)
Rhizome	0.103 ± 0.010	11.532 ± 0.003	19.750 ± 0.035		
E. acoroides	0.315 ± 0.020				
T. hemprichii	0.4187 ± 0.007	-	-	-	Kannan *et al*. (2010b)
H. pinifolia	1.0807 ± 0.039				
S. isoetifolium	0.398 ± 0.000				
H. johnsonii	-	-	-	224.2 ± 100.9	Gavin and Durako (2011)
H. decipiens				21.0 ± 7.3	
T. hemprichii	1022.58 ± 193.28				
S. isoetifolium	94.36 ± 6.71	-	-	-	Sanatoso *et al*.(2012)
C. rotundata	335.58 ± 14.92				
E. acoroides	542.56 ± 14.90				
E. acoroides	1.62 ± 0.080	8.37 ± 0.018	35.80 ± 0.238		
T. hemprichii	2.76 ± 0.170	3.19 ± 0.037	38.62 ± 0.62		
H. pinifolia	21.64 ± 1.845	15.75 ± 0.61	58.62 ± 2.43	-	Kannan *et al*.(2013)
S. isoetifolium	3.94 ± 0.265	2.57 ± 0.06	51.56 ± 1.67		
C. serrulata	13.27 ± 0.152	2.76 ± 0.044	41.28 ± 1.53		
C. rotundata	12.64 ± 0.275	6.65 ± 0.43	70.30 ± 2.30		

extracts.[27] *T. hemprichii* had lesser phenolic content when compared to *C. rotundata***.** This was found similar to that of Athiperumalsami *et al*.[28] whereas the concentration of phenolic compound recorded was far greater than that reported earlier (0.002 ±0.006). In contrast, Kannan *et al*., reported that phenolic content was found to be higher in *T. hemprichii* than *C. rotundata*.[8] However, the presence of phenolic contents was traditionally speculated with the environmental stress experienced by the plant during the time of sampling. Evaluation of antioxidant properties of plants cannot be carried out accurately by a single universal method,[27] instead a set of assays could able to provide a clue about the activity. Total antioxidant capacity was reported as ascorbic acid equivalents and the method is mainly used for the spectrophotometric quantification of total antioxidant capacity that employs cost effective reagents.[14] The result obtained from total antioxidant activity of *C. rotundata* rhizome (17.639 ± 0.28 mg AE/ mg extract) and leaf (12.86 ± 0.14 mg AE/ mg extract) extracts was found higher than that of whole plant extract of *C. rotundata* (6.65 ± 0.43 mg AE/ g extract),[8] whereas the rhizome extracts of *E. acoroides* (11.029 ± 0.813) was found similar to its ethanol extract (11.532 ± 0.003).[3] Present results reveal that there is a significant linear relationship between the total phenolic content and total antioxidant activity and thus it is evident that antioxidant properties of phenolic compounds the resultants of its reducing agents, hydrogen donors, and free radical quenchers.[32]

1, 1- Diphenyl-2-picrylhydrazyl (DPPH) is a stable free radical which can be effectively scavenged by the antioxidants in the substrate and convert it into 1, 1-Diphenyl-2-picrylhydrazine.[8] Maximum scavenging activity was exhibited by the leaf of *C. rotundata* (78.84 ± 0.87), followed by the rhizome (75.48 ± 0.97). Radical scavenging activity of all the seagrasses exhibited comparatively higher activity than the earlier reports.[8,10] ABTS radical scavenging activity (TEAC) assay is considered a method that only measure the redox power of the antioxidant mixture in relation to the radical cation ABTS and[29] considered as an excellent tool for determining the antioxidant activity of hydrogen donating antioxidants and of chain breaking antioxidants. [30] The present study found that leaf extract of *C. rotundata* (58.10 ± 0.42) with highest scavenging activity of ABTS radicals, followed by *H. uninervis, T. hemprichii, E. acoroides* and *S. isoetifolium*. ABTS radical scavenging activity of H. ovata was found lower than that of *H. decipiens*.[31] A strong linear relationship was found between the DPPH free radical scavenging activity and ABTS radical scavenging activity with total phenolic content and clearly confirms that free radical scavenging activity is attributed mainly by the polyphenolic compounds

Table 3: IC_{50} and EC_{50} values recorded for free radical scavenging and reducing power of seagrasses

Seagrasses	DPPH Scavenging activity IC_{50}	ABTS Scavenging activity IC_{50}	Ferric reducing antioxidant power EC_{50}	Total Reducing Power EC_{50}	H_2O_2 Scavenging activity IC_{50}	NO_2 Scavenging activity IC_{50}
H. uninervis						
Leaf	19.25 ± 0.75^{c}	29.5 ±2.15^{d}	3.65 ± 0.84^{ab}	1.5 ± 0.25^{a}	136.25 ± 3.5^{c}	28.4 ± 0.35^{ef}
Rhizome	14.25 ± 1.07^{b}	28.25 ± 1.75^{d}	1.56 ± 0.55^{a}	1.75 ± 0.7^{ab}	91 ± 5.8^{h}	82 ± 2.65^{b}
S. isoetifolium						
Leaf	24.75 ± 1.15^{d}	24 ± 0.750^{c}	16.78 ± 0.75^{ab}	3.25 ± 0.8^{d}	133.75 ± 7.5^{a}	9.25 ± 1.25^{e}
Rhizome	20 ± 0.825^{c}	18.75 ± 1.62^{b}	18.54 ± 1.15^{ac}	3.5 ± 1.05^{d}	231.25 ± 6.3^{d}	41.25 ± 1.645^{j}
C. rotundata						
Leaf	6.25 ± 0.655^{a}	32.5 ± 2.54^{e}	2.58 ± 0.112^{a}	3.75 ± 0.52^{e}	86.25 ± 4.2^{f}	56.2 ± 2.25^{a}
Rhizome	8 ± 0.745^{a}	23.25 ± 2.10^{c}	1.39 ± 0.32^{bc}	3.25 ± 0.46^{d}	131.25 ± 3.5i	91.15 ± 1.75^{d}
T. hemprichii						
Leaf	19 ± 1.25^{f}	22.25 ± 0.855^{a}	28.5 ± 2.25^{cd}	2.75 ± 0.25^{c}	117.5 ± 2.8^{b}	39.3 ± 2.45^{h}
Rhizome	16.25 ± 0.925^{e}	25 ± 0.655^{a}	32.52 ± 2.18^{cd}	5.75 ± 0.84^{b}	164.5 ± 4.6^{f}	43.75 ± 1.32^{f}
E. acoroides						
Leaf	39.5 ± 1.105^{c}	14.5 ± 0.635^{c}	35.6 ± 1.28^{bc}	2.4 ±0.12^{d}	209.3 ± 5.5^{d}	22.5 ± 1.10^{c}
Rhizome	32.5 ± 0.845^{b}	16.5 ± 0.445^{c}	42.5 ± 1.55^{cd}	2 ± 0.61^{f}	137.5 ± 2.35^{e}	57.5 ± 2.42^{g}
H. ovata						
Leaf	49 ± 1.50^{g}	86.25 ± 0.350^{f}	65.4 ± 2.35^{d}	5.5 ± 0.11^{f}	334.05 ± 12.4^{g}	72.5 ± 1.15^{j}
Rhizome	53 ± 0.25^{h}	72.75 ± 0.275^{g}	54.48 ± 1.85^{cd}	7.8 ± 0.23^{g}	248.5 ± 10.24^{j}	111.25 ± 2.45^{j}
BHT	3.25 ± 0.1	-	0.05 ± 0.002	0.02 ± 0.001	-	-
Ascorbic acid	-	14.2 ± 0.7	0.95 ± 0.05	-	-	-

Column wise value sharing same superscripts are not significantly (P < 0.05) different

of seagrasses. Such positive relationship between the total phenolic content and scavenging activity of the free radicals DPPH and ABTS has been reported earlier from seaweeds[32] and *E. acoroides*.[3]

The antioxidant activity of seagrass extracts is evaluated from FRAP assay based on their ability to reduce TPTZ-Fe (III) complex to TPTZ-Fe (II). The reducing ability of a compound greatly depends on the presence of reductones, which exhibit anti-oxidative potential by breaking the free radical chain by donating a hydrogen atom.[33] Since the antioxidant activity of a substance is usually correlated directly to its reducing capacity, the FRAP assay provides a reliable method to study the antioxidant activity of various compounds.[17] The ferric reducing antioxidant power of seagrass extracts (both leaf and rhizome) was in the order of *C. rotundata* > *H. uninervis* > *S. isoetifolium* > *T. hemprichii* > *H. ovata* > *E. acoroides*. It was interesting to note that, even though rhizome of *E. acoroides* (0.42 ± 0.012) exhibited higher reducing power, their leaf extract exhibited very low reducing power (0.14 ± 0.016), which was in contrast to Kannan *et al.* (Table 2).[3] The decrease in the reducing power of *Enhalus* leaf extract might be due to collection of young leaves to avoid epiphytic cover, inturn young leaves are known for their minimum phenolic content.

Reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity.[34] In this assay, the yellow colour of the test solution changes to various shades of green and blue depending on the reducing power of each extract. Presence of reducers in the substrates causes the conversion of the Fe3+/ ferricyanide complex. By measuring the formation of Pearl's Prussian blue at 700 nm, it is possible to determine the concentration of Fe3+ ion. No earlier reports are found on the reducing power of seagrasses and hence, the seagrass extracts were subjected for the determination of total reducing power. Results showed that rhizome of *H. uninervis* (0.557 ± 0.03) had the highest reducing power (Figure 9), followed by *E. acoroides* rhizome (0.532 ± 0.009) and the result was in accordance to ferric ion reducing power. It might be due to that the compound present in *H. uninervis* has the unique ability to convert the ferrous ion radical into a reduced form.

The scavenging of H_2O_2 may be attributed to their phenolics, which could donate electrons to H_2O_2, thus neutralizing it to water. H_2O_2 scavenging capacity of an extract may be attributed to the structural features of their active components, which determine their electron donating abilities.[35] Nitric oxide (NO) is an important chemical mediator generated by endothelial cells, macrophages, neurons, etc. which is involved in the regulation of various physiological processes. Excess concentration of NO is associated with several diseases. Oxygen reacts with the excess nitric oxide to generate nitrite and peroxynitrite

anions, which act as free radicals.[36] Hence, plant acquires protection from free radical generation due to the presence of antioxidant molecules and the secondary metabolites such as phenolics, flavonoids and polypropanoids have the capacity to scavenge free radicals by donating protons.[37] Both H_2O_2 and NO_2 scavenging activity resulted with maximum activity by *E.acoroides* and *C. rotundata* extracts respectively. The lower activity exhibited by *H. ovata* was found in contrast[7] with the *Halophila ovalis* exhibiting the higher H_2O_2 and NO_2 scavenging effect. This might be due to the change in phenolic concentration among different seagrass species and also due to seasonal and spatial variabilities.

IC_{50} values were calculated from the calibration curve for the test extracts and the variance among all the test extracts was assessed using Duncans multiple range test. IC_{50} refers to the concentration of the substrate that inhibits 50% of activity free radicals. Based on IC_{50} values, the antioxidant compounds can be classified as follows: very powerful antioxidants when the IC_{50} values less than 0.05 mg/ml, strong antioxidant if the value of IC_{50} between 0.05 to 0.10 mg/ml, intermediate and weak when the IC_{50} values ranged from 0.10 to 0.15 mg/ml and from 0.15 to 0.20 mg/ml, respectively.[38] The lower the IC_{50} value reflects to higher antioxidant activity of plant extracts and vice versa. Lower values obtained for *C. rotundata* and *H. uninervis* extracts (Table 3) depict them as potential antioxidant sources. IC_{50} value of both leaf and rhizome of *C. rotundata* for DPPH scavenging activity and FRAP were found comparatively closer to the IC_{50} value of BHT, the synthetic antioxidant, whereas, IC_{50} value of *H. uninervis* extracts were closer to that of BHT for total reducing power. This reveals that the phenolic compounds of *H. uninervis* are more potential in reducing the free radical ions than *C. rotundata*. However, *C. rotundata* extracts exhibited the highest ferric reducing antioxidant power. It is quite interesting to observe *E. acoroides* extracts with lower IC_{50} values for ABTS scavenging assay, comparatively closer to the IC_{50} values of ascorbic acid. This proves *E. acoroides* also acts as a potent source of antioxidants. IC_{50} values obtained for these seagrasses were comparatively lower than those obtained for common vegetables[39] depicting seagrasses as the better natural antioxidants.

Presence of caffeic acid in different seagrasses and[40-42] ρ-Coumaric acid in *H. pinifolia* and *C. rotundata*[8] has been illustrated earlier. Other than these two predominant compounds, compounds such as gallic acid, protocatechuic acid, gentisic acid, vanillin, cafteric acid, syringic acid, coutaric acid, hydroxybenzoic acid, *o*-Coumaric acid, synapic acid, ferulic acid, trans-resveratrol, trans-cinnamic acid, anisicacid, chicoric acid, di-ferulolyl tartaric acid and cinnamic acid were identified at moderate to trace levels. Among these phenolic compounds, chicoric acid and cafteric acid have been reported earlier from *S. filiforme* and[42] other compounds like ferulic acid, protocatechuic acid, p-Hydroxy benzoic acid, gallic acid and vanillic acid are found prevalent among different seagrass species.[28,40]

CONCLUSION

Overall, both the leaf and rhizome extracts of *C. rotundata* exhibited higher antioxidant capacity, which is interpreted with that of varied phenolic compounds identified from the extract. Linear relationship obtained between total phenolic content and scavenging activity depicts that antioxidant potential of seagrasses are largely mediated by its phenolic compounds and studies on other compounds and specific phenolic aids will guide us to the chemical leads with antioxidant activity. The effectiveness of these phenolic compounds as scavengers of free radicals and their reductive capacity altogether paves way for the application of these compounds together as a multipotent antioxidant leads. Therefore, *C. rotundata* can be used as an accessible source of natural antioxidants and a possible food supplement, with potential application in the pharmaceutical industry.

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CONFLICT OF INTEREST

There is no conflict of interest.

ABBREVIATION

DPPH : 2-diphenyl-1-picrylhydrazyl
ABTS : 2, 2′azinobis-3-ethylbenzothizoline-6-sulphonic acid
FRAP : Ferric reducing antioxidant power
BHT : Butylated hydroxytolune
GAE : Gallic acid equivalents
ROS : Reactive oxygen species
TEAC : Trolox equivalent antioxidant capacity
HPLC : High performance liquid chromatography

Highlights of the Paper

- Seagrass leaf and rhizome extracts showed moderate to higher antioxidant activity.
- *C. rotundata* extract showed the potential scavenging activity than the other species used for the study.
- HPLC analysis of *C. rotundata* leaf extracts revealed the presence of nineteen phenolic compounds with the dominance of caffeic acid and p- Coumaric acids.
- Present result paves way for the application of *C. rotundata* extract for food and pharmaceutical application as antioxidant.

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