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In vitro antioxidant activities of ethanol extract from *Enhalus acoroides* (L.F.) Royle

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ABSTRACT

Objective: To search for new sources of safe and inexpensive antioxidants, the leaf, root and rhizome of *Enhalus acoroides (E. acoroides)* were screened for the first time for their antioxidant activities. **Methods:** Samples of leaf, root and rhizome from *E. acoroides* were tested for total phenolic content, proanthocyanidins and *in vitro* antioxidant activity in terms of total antioxidant assay, DPPH assay and FRAP assay. **Results:** The leaf sample was found to have high levels of phenolic (0.323±0.028 mg TAE/g) and proanthocyanidins (0.570 0±0.000 3 mg TAE/g) when compared to root and rhizome. The leaf samples exhibited higher total antioxidant activity (11.770±0.026 mg Ascorbic acid equivalent/g), higher percentage of DPPH radical scavenging activity (25.76±0.04) and higher reducing power (18.060±0.073) in terms of mg GAE/g. In addition, there was a significant correlation between total phenolic content and total antioxidant activity (R^2=0.923), DPPH assay (R^2=0.509), FRAP assay (R^2=0.994). Similarly, significant correlation was found between the proanthocyanidin and total antioxidant activity (R^2=0.977), DPPH assay (R^2=0.464) and the FRAP assay (R^2=0.998). **Conclusions:** These results suggested that *E. acoroides* have strong antioxidant potential. Further study is necessary for isolation and characterization of the active antioxidant agents, which can be used to treat various oxidative stress-related diseases.

1. Introduction

Antioxidants in biological systems have multiple functions which include protection from oxidative damage and in the major signaling pathways of cells. The major action of antioxidants in cells is to prevent damage caused by the action of reactive oxygen species (ROS). ROS, such as, superoxide radicals ($O_2^{\bullet}$), hydroxyl ($OH^{\bullet}$), peroxide ($ROO^{\bullet}$) and nitric acid radicals are generated in living organisms during excessive metabolism[1] and they cause extensive oxidative damage to cells leading to age related degenerative diseases, cancer and a wide range of other human diseases[2].

Several synthetic antioxidants, such as butylated hydroxyanisol (BHA), butylated hydroxytoluene (BHT) and tert-butylhydroquinone (TBHQ) are commercially available and are currently in use. However, their use is now restricted due to their side effects. It has been shown that they promote the development of cancerous cells in rats. These findings have reinforced the efforts for the development of alternative antioxidants of natural origin[3]. In this regard many natural antioxidants have already been isolated from different kinds of natural resource, such as oilseeds, cereal crops, vegetables, spices and herbs[4-5]. *E. acoroides* (L.f.) Royle belonging to the monotypic marine genus *Enhalus* in the family Hydrocharitaceae is widely distributed along the coast of the India Southeast and the tropical part of the western pacific[6]. The seeds of the tropical seagrass *E. acoroides* have been traditionally eaten in the Philippines. The raw seeds are described as crunchy and sweet, while boiled seeds contain more starch and taste like cooked sweet potato[7]. In addition to being edible, the tropical seagrass *E. acoroides* was used as remedy against stings of different kinds of rays, and stone, lion and scorpion fish (Scorpaenidae), as well as for diverse rabbit fish (Siganidae). The species is also good for muscle pains, wounds and stomach problems. It is also used in the form of 'mafusho' against fever. 'Mafusho' is the smoke produced from a mixture of plants and herbs when burned. It can also be vapourized with water or prepared as incense. The patient inhales the vapours in order to lower body temperature[8]. Ragupathi *et al*[9] made preliminary screening of antioxidant activities of *E. acoroides* collected

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from Gulf of Mannar Biosphere Reserve, India. Gillan *et al*[10] listed the major sterol and fatty acid component of fresh leaves of *E. acoroides*. Qi *et al*[11] investigated the chemical constituents and antifeedant, antibacterial and the antilarval activities of ethanol extracts of *E. acoroides* from South China and recorded eleven pure compounds including four flavonoids and five sterols. More recently, reports have revealed that seagrasses are rich sources of antioxidant compounds[12-15].

In this paper we report the antioxidative potential of leaves, root and rhizome of *E. acoroides* from the coasts of Gulf of Mannar (India) by measuring the total antioxidant activity, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, FRAP assay, total phenolic content and proanthocyanidins in ethanolic extracts.

2. Materials and methods

2.1. Chemicals and reagents

2, 2-Diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich. Gallic acid, Tannic acid, Ascorbic acid, $FeCl_3$, TCA, Potassium ferricyanide, Vanillin, Folin-Ciocalteus's phenol reagent, Sodium carbonate was from Merck (Mumbai, India). All the chemicals were of analytical grade.

2.2. Plant material

Fresh leaves, root and rhizome of *E. acoroides* (L.f.) Royle were collected in March 2009 from the intertidal region of the Chinnapallam, Gulf of Mannar Biosphere Reserve, Tamilnadu, India (Latitude 8°35′-9°25′N; 78°08′-79°30′E) and immediately brought to the laboratory in plastic bags containing sea water in order to prevent evaporation. Then the plants were washed thoroughly with tap water to remove all sand particles and epiphytes. The samples were shade-dried at room temperature for five days until constant weight obtained and ground in an electric mixer. The powdered samples were then stored in refrigerator for future use.

2.3. Preparation of seagrass extracts

Dried finelly crushed leaves, root and rhizome (10 g) were extracted for 24 h in 200 mL of ethanol at room temperature under dark condition. Then the extraction was twice repeated and filtered through glass funnel and Whatmann No.1 filter paper. Each filtrate was concentrated to dryness under reduced pressure using a rotary flash evaporator.

2.4. Determination of total phenolic content

The total phenolic content of the extracts was measured by the modified Folin-Ciocalteu method[16]. An aliquot of the extract was mixed with 5 mL of Folin-Ciocalteu reagent (previously diluted with water 1:10 v/v) and 4 mL(75 g/L) of sodium carbonate. The tubes were vortexed for 15 sec and allowed to stand for 30 min at 40 ℃ for colour development. Absorbance was then measured at 765 nm using the PerkinElmer Lamda 25 UV-VIS Spectrophotometer. Total phenolic content was expressed as mg/g Tannic acid equivalent.

2.5. Determination of total proanthocyanidins

Proanthocyanidins determination was based on the procedure reported by Sun *et al*[17] in which a volume of 0.5 mL of 0.1 mg/mL of extract solution was mixed with 3 mL of 4% vanillin-methanol solution together with 1.5 mL of hydrochloric acid, and the mixture was allowed to stand for 15 min. The absorbance was measured at 500 nm. Total proanthocyanidin content was expressed as Tannic acid equivalents (mg/g).

2.6. Determination of antioxidant activity

2.6.1. Total antioxidant activity

Total antioxidant activities of crude extracts were determined according to the method of Prieto *et al*[18]. Briefly, 0.3 mL of sample solution (0.1 mg/mL) was mixed with 3.0 mL reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). Reaction mixture was incubated at 95 ℃ for 90 min in a water bath. Absorbance of all the sample mixture was measured at 695 nm. Total antioxidant activity is expressed as the number of equivalence of ascorbic acid. A calibration curve of ascorbic acid was prepared and the total antioxidant activity was standardized against ascorbic acid and was expressed as mg ascorbic acid equivalents per gram of sample on a dry weight (DW) basis.

2.6.2. DPPH radical-scavenging activity

The scavenging effects of samples for DPPH radical were monitored according to the method of Yen[19]. Briefly, a 2.0 mL of aliquot of test sample was added to 2.0 mL of 0.16 mM DPPH methanolic solution. The mixture was vortexed for 1 min and then left to stand at room temperature for 30 min in the dark, and its absorbance was read at 517 nm. The ability to scavenge the DPPH radical was calculated using the formulae given by Duan *et al*[20]. Synthetic antioxidants, Gallic acid and ascorbic acid were used as positive controls.

2.6.3. Ferric reducing antioxidant power (FRAP) assay

Reducing power of crude extract was determined by the method prescribed by Oyaizu[21]. Briefly, 1.0 mL of extract was mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL potassium ferricyanide (1%). Reaction mixture was incubated at 50 ℃ for 20 min. After incubation, 2.5 mL of trichloroacetic acid (10%) was added and centrifuged (650 g) for 10 min. From the upper layer, 2.5 mL solution was mixed with 2.5 mL distilled water and 0.5 mL $FeCl_3$ (0.1%). Absorbance of all the sample solutions was measured at 700 nm. Ascorbic acid is used as a positive control. FRAP value is expressed as the number of equivalence of gallic acid.

2.7. Statistical analysis

Three replicates of each sample were used for statistical analysis and the values were reported as mean±SD. Pearson's correlation and regression analysis were carried out using SPSS, version 16.0 software to study the relationship between antioxidant activities and total phenolic, proanthocyanidin content.

3. Results

The total phenolics and proanthocyanidins are presented

in Table 1. When compared to other parts of *E. acoroides*, leaves contain higher amount of phenol (0.323±0.028) followed by root. In this present study, the maximum proanthocyanidins was observed in leaves (0.570 0±0.000 3).

Table 1
Levels of total phenol and proanthocyanidin in *E. acoroides* (Mean[a] ± SD[b]) (mg TAE/g).

Species	Phenol	Proanthocyanidins
Leaf	0.323±0.028	0.570 0±0.000 3
Root	0.258±0.036	0.478 0±0.003 0
Rhizome	0.103±0.010	0.177 0±0.000 4

[a]Values are means of three replicate determinations; [b]SD, standard deviation.

Total antioxidant activities in the ethanolic extracts of *E. acoroides* are presented in Table 2. In this phosphomolybdenum method, molybdenum VI (Mo^{6+}) is reduced to form a green phosphate/Mo^{5+} complex. Higher activity of 11.77 mg ascorbic acid equivalent/g was observed in leaves and root followed by rhizome having the activity of 11.532 mg ascorbic acid equivalent/g. DPPH radical scavenging activities (%) of *E. acoroides* are presented in Table 2. Leaf showed significantly higher activity of 25.76% followed by root (20.26%) and rhizome (19.75%). The scavenging effect of standards on the DPPH radical decreased in the order: Ascorbic acid > Gallic acid, which was 57.03% and 55.67% respectively.

In this present study, leaves of *E. acoroides* had the highest ability for reducing Fe^{3+} than root and rhizome, which were similar to the results obtained in the total phenolic content. The positive control ascorbic acid showed significantly higher antioxidant activity than samples. However, the FRAP values of the samples were found to be in the following descending order: leaves (18.060±0.073) > root (6.520±0.018) > rhizome (4.770±0.084).

As far as we know, no seagrass have been investigated for the relationship between antioxidant activity and phenolic content. In this study, leaves, root and rhizome of *E. acoroides* were systematically evaluated for the first time, to establish the relationship between these two parameters. The correlation coefficient (R^2) between the antioxidant activities and total phenol, proanthocyanidin was determined and presented in Table 3. The correlation coefficient between the total phenolic content and the antioxidant capacities was found to be significant. There was a significant correlation between total phenolic content and total antioxidant activity (R^2=0.923), DPPH assay (R^2=0.509), FRAP assay (R^2=0.994). Similarly, significant correlation was found between the proanthocyanidin and total antioxidant activity (R^2=0.977), DPPH assay (R^2=0.464) and the FRAP assay (R^2=0.998). Thus, the phenolic compounds were major contributor to the antioxidant capacities of *E. acoroides*.

Table 2
Total antioxidant activity, DPPH assay and FRAP assay of *E. acoroides* (Mean[a] ± SD[b]).

Species	Total antioxidant activity (mg ascorbic acid)	DPPH assay (%)	FRAP assay(mg gallic acid/g)
Leaf	11.770±0.026	25.760±0.040	18.060±0.073
Root	11.770±0.026	20.250±0.020	6.520±0.018
Rhizome	11.532±0.003	19.750±0.035	4.770±0.084
Ascorbic acid	NA	57.030±0.030	122.880±1.535
Gallic acid	NA	55.670±0.020	NA

[a]Values are means of three replicate determinations; [b]SD, standard deviation; NA-Not analysed.

Table 3
Correlation between total phenolic content, proanthocyanidins and antioxidant assays.

Antioxidant assay	Total antioxidant activity		DPPH assay		FRAP assay	
	R^2	*P* value	R^2	*P* value	R^2	*P* value
Total phenolic content	0.923	0.125	0.509	0.493	0.994	0.047
Proanthocyanidins	0.977	0.096	0.464	0.523	0.998	0.019

4. Discussion

Phenolic compounds were commonly found in plants and have been reported to have several biological activities including potential antioxidants and free radical scavengers apart from primary defense role[22]. Seagrasses are particularly rich in proanthocyanidins (condensed tannins) [23]. The presence of phytoconstituents, such as phenols, flavonoids and tannin in seaweeds and seagrasses may be responsible for antioxidant activity in preventing a number of diseases through free-radical scavenging activity[24]. Earlier reports revealed that polyphenols of the seagrasses have the antioxidant activity[12-14]. It is also reported that the presence condensed tannins in seagrasses may act as deterrents against herbivore feeding as well as against fungal and bacterial invasion[25].

Published reports on the total antioxidant activity of seagrasses are not available. However, Kumaran[26] have reported total antioxidant activity in the range of 245 to 376 mg ascorbic acid equivalents/g in *Phyllanthus* species. Ganesan *et al*[27] noticed higher antioxidant activity (32.01 mg ascorbic acid equivalents/g) in ethyl acetate fraction of *Acanthophora spicifera (A. spicifera)*. Ye *et al*[28] noticed higher antioxidant activity (30.50 μ mol $FeSO_4$/mg) in ethanol extract of brown seaweed *Sargassum pallidum (S. pallidum)*. It has been reported that solvents used for extraction have dramatic effect on the chemical species[29].

The effect of antioxidants on DPPH radical scavenging is thought to be due to hydrogen donating ability. DPPH is a stable free radical and it accepts an electron or hydrogen radical to become a stable diamagnetic molecule. When a DPPH solution is mixed with a substrate acting as a hydrogen atom donor, a stable non-radical form of DPPH is obtained with simultaneous change of the violet color to pale

yellow[30]. Hence, DPPH (1,1-diphenyl- 2-picrylhydrazyl) has been used extensively as a free radical to evaluate reducing substances and is a useful reagent for investigating the free radical scavenging activities of compounds[20].

In the FRAP assay, antioxidants in the sample reduce ferric (III) to ferrous (II) in a redox-linked colourimetric reaction[31] that involves single electron transfer. The reducing power indicates that the antioxidant compounds are electron donars and reduce the oxidized intermediate of the lipid peroxidation process, so that they can act as primary and secondary antioxidants[19]. The positive control ascorbic acid showed significantly higher antioxidant activity than samples. Same trend has also been reported by Kumaran[26] in methanolic extracts of *Phyllanthus* species.

From the present findings it was concluded that the *E. acoroides* have strong antioxidant capacity influenced mostly by total phenolic content which was the major contributor to the antioxidant capacities of the seagrasses. Further studies are being carried out on other species of seagrasses of different habitats in order to provide more comprehensive data on the antioxidant activity. As well as the characterization of the principle antioxidant agents, which can be used to treat various oxidative stress-related diseases.

Conflict of interest statement

We declare that we have no conflict of interest.

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