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Research Article

Phytochemical analysis and *in vitro* antioxidant screening of sea grass-*Enhalus acoroides*

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ABSTRACT

The present study was conducted to determine the yield of extract, qualitative, quantitative analysis and antioxidant activity of the crude extracts of leaves of *Enhalus acoroides*. The extracts were prepared by using Hexane, Chloroform, Ethanol, Ethyl acetate and Water solvents. The yield of extract for all the five solvents was calculated and they were studied for qualitative analysis of phytochemical compounds. The crude extracts were evaluated for total phenolic and total flavonoids contents. The antioxidant activity of crude extract of *Enhalus acoroides* was assessed by FRAP assay and the scavenging activity towards DPPH radical, H_2O_2, NO and ABTS. The present study revealed the presence of major phytochemicals like alkaloids, phenols, flavonoids, steroids, tannins, saponins. Quantitative analysis of the total phenols were high in Aqueous which has 78.36 mgGAE/g and total flavonoids were high in Aqueous which has 57.52 mgQU/g. Free radical scavenging activities was found high in Aqueous extract with maximum percentage of inhibition.

Keywords: Antioxidant activity*; Enhalus acoroides;* Phytochemical Analysis; Quantitative analysis.

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 defence against oxidation that produce free radicals in food, chemicals and in living systems (Szabo MR *et al*., 2007). 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 (Sureda A *et al*., 2008). 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 (Kannan RR *et al*., 2010). Because of carcinogenicity of synthetic antioxidants, there is dearth for antioxidants from natural origin (Anandjiwala S *et al*., 2007). Natural antioxidants play a vital role in antioxidant defence 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, for the treatment of fever and skin disease, muscle pain, wounds and stomach problems, remedy against stings of different kind of rays, tranquillizer for babies (De la Torre-Castro M and Ronnback P, 2004). Seagrasses are known to produce secondary metabolites as defence mechanism under stress conditions and these compounds are found to be anti-oxidative in nature. However, research on the antioxidant activity of seagrasses has not been much carried out compared to the seaweeds, and initiated only recently (Liyana-Pathirana C and Shahidi F, 2006). Hence the present study aims to examine the qualitative and quantitative analysis and their antioxidant capacity of leaf extracts of *Enhalus acoroides* species.

Habitat and Ecology

Enhalus acoroides is found in the subtidal zone and is slow to produce new shoots but produces high biomass, being a very large seagrass. The siltier the water, the longer the leaves grow in order to capture more light. It is the only species that releases pollen to the surface of the water in sexual reproduction, which restricts its distribution to intertidal and shallow

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subtidal areas. It is a slow-growing, persistent species with a poor resistance to perturbation.

Enhalus acoroides is common in the major seagrass areas of Southeast Asia. It occurs in southern India, Sri Lanka, and the Lakshadweep Islands. It ranges from the Red Sea south to northern Mozambique, and in the Seychelles. In Thailand, it occurs in brackish water canals down to the lower intertidal and subtidal zones on mud, muddy sand and sandy coral substrates. In the Gulf of Thailand, it grows on coarse substrate ranging from medium and coarse sand to coral rubble at a depth of 0.5-1.0 m. In Indonesia, *E. acoroides* grows in a variety of different sediment types, from silt to coarse sand, in subtidal areas or localities with heavy bioturbation. In the Philippines, it colonizes turbid, quiet, protected areas such as bays and estuaries. In Peninsular Malaysia, it is common all around the coast on muddy shores and areas exposed at low tide.

Enhalus acoroides (L.f.) 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. 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 (Liyana-Pathirana C and Shahidi F, 2006). 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 (Hartog CD and Kuo J, 2006).

Montaño NME *et al*., 1999 made preliminary screening of antioxidant activities of *E. acoroides* collected from Gulf of Mannar Biosphere Reserve, India. de la Torre-Castro M and Rönnbäck P, 2004 listed the major sterol and fatty acid component of fresh leaves of *E. acoroides*. Ragupathi Raja Kannan R *et al*., 2010 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 (Gillan FT and Hogg RW, 1984)

MATERIALS AND METHODS

Collection and Authentication of Sea Grass

Sample has been collected from Devipattinam, Ramanadhapurm District on June, 2016 and authenticated by the expert of ICAR, Dr. N. Kaliaperumal M.Sc., Ph.D., Scientist- in-charge, CMFRI.

Preparation of Seagrass Extracts

The leaves of *Enhalus acoroides* were carefully separated, cleaned, shade dried, mechanically grinded and powdered. The powder was subjected to solvent extraction with Hexane, Chloroform, Ethanol, Ethyl acetate and Water. The extracts were concentrated by using the Rotary Evaporator and the yield of the extracts was noted with respect to the dried plant material.

Qualitative Phytochemical Analysis

The Bioactive compounds were analysed by the qualitative tests **(**Shu-Hua Qi *et al*., 2008**)** for five crude extracts – Hexane, Chloroform, Ethyl acetate, ethanol and Water.

Quantitative analysis

Determination of Total Phenolic Content

The important bioactive compounds were estimated using standard proven methods includes total phenol content (Gokhale SB *et al*., 1993), Total Flavonoid content (Singleton VL and Rossi JA, 1965) and Total Tannin content (Prieto P *et al*., 1999).

Antioxidant Assays

The most preferred antioxidant assays includes DPPH scavenging activity (Schanderl, S H, 1970), ABTS radical scavenging assay (Blois MS, 1958), $FeCl_3$ Scavenging Antioxidant Assay (Benzie IF, Strain JJ 1996), Superoxide Anion-Scavenging Activity (Liu *et al.* 1997) and Nitric Oxide-Scavenging Activity (Green LC *et al*., 1982) were performed by standard methods.

Statistical analysis

The results were statistically analyzed using a one-way analysis of variance (ANOVA). To evaluate the significance of the difference in means of different sections of the thallus, Duncan's multiple comparison tests was performed using SPSS statistical package (Version 17). The values are presented as the mean ± SD, and $p \leq 0.05$ was considered statistically significant

RESULTS AND DISCUSSION

Sea grasses produce enormous secondary metabolites to limit the invasion of microorganisms, epiphytes and predation (Marcocci L *et al*., 1994). Marine plants with bioactive phytochemicals performing important roles in survival and growth are now being seen as novel and dynamic drugs and agrochemicals (Wahl M. 2001.). *Enhalus acoroides* widely distributed in seabeds of Mandabam Coast, Tamil Nadu, India and has a significant role in the Ayurvedic medicine.

Phytochemical analysis is of paramount importance in identifying new source of therapeutically and industrially valuable compounds having medicinal

plants have been chemically investigated (Grode SH *et al*., 1983). In the present study, secondary metabolites were qualitatively and quantitatively analysed from *Enhalus acoroides*.

Yield of Extract

In the current investigation, the crude extract of *Enhalus acoroides* was prepared in the ascending order of Hexane>Chloroform>Ethyl acetate>Ethanol>Aqueous and were analysed. The yield of the different extract was found to be highest from Aqueous (2.76g) which is non polar followed by Ethanol (958mg), chloroform (543mg) and ethyl acetate (226mg) which hints the possibility of medium polar metabolites. Followed by hexane extract (162mg) which hints the highly polar metabolites (Table 1).

Qualitative Phytochemical Analysis

The qualitative analysis of bioactive compounds for the five extracts has been analyzed in this study and there was wide range of phytochemical compounds present in the five extracts as shown in table 2. The hexane being highly non-polar in nature was able to extract very less compound characterized like alkaloids, flavanoids, quinine, glycosides and terpenoids. Chloroform extract was found to have a wide range of bioactive compounds like phenols, flavonoids, quinone, tannins and steroids. The Ethanol extract was positive for flavanoids, quinone, sugars and terpenoids. The ethyl acetate extract was found to contain flavanoids, phenols, carbohydrates, quinone, sugars, terpenoids and saponins. The water extract was positive for phenol, quinine, tannins and terpenoids. The presence of bioactive constituents indicates that the *Enhalus acoroides* can be used in a multitude of ways for the beneficiary of population.

Quantitative determination

Total phenolic content

The estimation of total phenolic content was based on the absorbance of sample and Folin-Ciocalteau reagent mixture at 765 nm. Gallic acid was used as a standard compound and the total phenols were expressed as µg/mL gallic acid equivalent using the standard curve equation: y=0.0079x + 1.476, R2 = 0.9949, where y is the absorbance at 765 nm and x is the total phenolic content expressed in µg/gm. The Aqueous extract showed higher level of phenolic content was 78.36mgQU/g than the other extracts (Table 3 and Graph 1). The higher amount of phenol is important in regulation of plant growth, development and disease resistance. Phenols when mixed with the flavonoid compounds in plants are reported to show multiple activities like antioxidant, anti-carcinogenic, anti-inflammatory etc.

Flavonoid Quantitative Determination

The determination of flavonoids in the seagrasses was conducted using the Aluminium Chloride method with quercetin as standard. The Aqueous extract showed significantly higher level of flavonoids was 57.52mgQU/g than the other extracts (Table 4 and Graph 2). Earlier reports revealed that plant phenolic compounds including flavonoids are potent antioxidants with reported antimutageneic and anticarcinogenic effects (Gnanambal KME *et al*., 2013). Flavonoids have been used against the cancer causing tumors and it inhibits the promotion of growth and progression of tumors (Ambasta S.P eta l., 1986).

Total Tannin content

The presence of tannins in *Enhalus acoroides* was confirmed by the method of folin - denis method with tannic acid as standard. The chloroform extract showed the higher level of tannin content was 159.41 mg/g dw (Table 5 and Graph 3). Tannins are complex secondary metabolites having various medicinal properties but difficult to isolate in pure form. Tannins are polyphenols, have a large influence on the nutritive value of humans and animals foodstuff. Tannin contributes various medicinal properties such as antimicrobial, anti-inflammatory and astringent activity (Middleton E. and Kandaswami C, 1994). Tannins inhibit the pathogenic fungi and antimicrobial activity of extracts showed better activity by the presence of tannins (Stevens JF *et al*., 1992). Recent interest in phenolic compounds due to their protective role, through utilization of fruits and indigenous vegetables such as apple (Chung K.T *et al*., 1998), black caraway, carrot, cranberry, orange, tomato against oxidative damage diseases such as arteriosclerosis, cardiovascular, coronary heart disease, aging, stroke and cancer (Chang, C.H *et al*., 2006).

Antioxidant assays

DPPH Free Radical Scavenging Activity

Antioxidant scavenging property of the extract was studied by using DPPH activity. By increasing in the concentration of extract there is decrease the $DPPH^{\bullet}$ radicals by IC^{50}, which is a parameter widely used to estimate the antioxidant activity (Abdi, S. and A. Ali, 1999). $DPPH^{\bullet}$ is a stable free radical and that can accept an electron or hydrogen radical to become a stable diamagnetic molecule (Krishna TM *et al*., 2013). A freshly prepared $DPPH^{\bullet}$ solution is of deep purple colour with absorption maximum at 517 nm and in the presence of antioxidant, this colour disappears due to quenching of $DPPH^{\bullet}$ free radicals and converting them into a colourless product 2 ,2- dipenyl-1-picryl hydrazine (Bijaya LM and Bikash B, 2013). Hence $DPPH^{\bullet}$ is usually used as a substance to evaluate the antioxidant activity (Sumathy R *et al*., 2013). In the present study, the extracts had significant scavenging effects on the $DPPH^{\bullet}$ radical which was increasing with the increase in the concentration of the sample from 10-200 µg/m L. Similar trend of $DPPH^{\bullet}$ free radical scavenging activity was already documented well (Shah R *et al*., 2010). All the crude extracts of *E. acoroides*

Table 1: Physicochemical Evaluations

Solvent	Weight of the Powder (gm)	Weight of the Crude Extract (mg)	Crude Extract %	Colour of the Extract
Hexane	150	162	0.10	Dark Green
Chloroform	150	543	0.36	Dark Green
Ethanol	150	958	0.63	Dark Green
Ethyl acetate	150	226	0.15	Dark Green
Water	150	2.76	1.8	Dark Green

Table 2: Phytochemical Analysis of different crude extracts of *Enhalus acoroides*

Compounds	E1	E2	E3	E4	E5
Alkaloids	+	-	+	-	-
Flavonoid	+	++	+++	+	-
Phenol	-	+	++	-	++
Quinone	+	+	++	+	+
Tannins	-	+	+	-	+
Carbohydrates	-	-	++	+	-
Steroids	-	+	-	-	-
Glycosides	+	-	-	-	-
Terpenoids	+	-	++	+	+
Ninhydrin	-	-	-	-	-
Saponin	-	-	-	+	-

+ = Present, - = Absent E1 – Hexane extract, E2 – Chloroform extract, E3 – Ethyl Acetate extract, E4-Ethanol extract, E5 – Aqueous extract

Table 3: Total phenolic content of crude extract of *Enhalus acoroides*

Sample	Total phenolic content (mg) (gallic acid equivalents/g weight)
Hexane	43.11
Chloroform	74.68
Ethyl acetate	44.16
Ethanol	48.11
Water	78.36

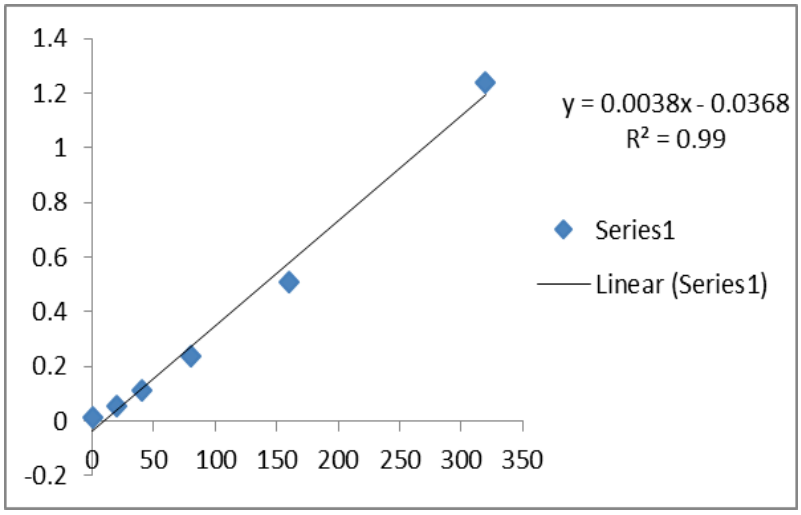

Figure 1: Linear regression of Total Phenolic content

were examined for antioxidant activity by DPPH method and different concentrations of the five extracts were analysed and Hexane extract was found to inhibit maximum percent of free radicals (26.88±0.43µg/ml) at 200µg/ml concentration than the other extracts where as standard compound ascorbic acid showed 99.76±0.11µg/ml free radical inhibition (Table 6). This might be due to the presence of higher flavonoids content, the most required bio compounds for scavenging activity in this extracts.

Total Antioxidant Activity By ABTS•+ Radical Cation Decolourization Assay

The reduction of the 2,2'azinobis (3-ethylbenzothiazoline sulphonate) radical cation

Table 4: Total flavonoid content of crude extract of Enhalus acoroides

Sample	Total flavonoid content (mg) (quercetin equivalents/g weight)
Hexane	8.08
Chloroform	37.99
Ethyl acetate	14.18
Ethanol	9.71
Water	57.52

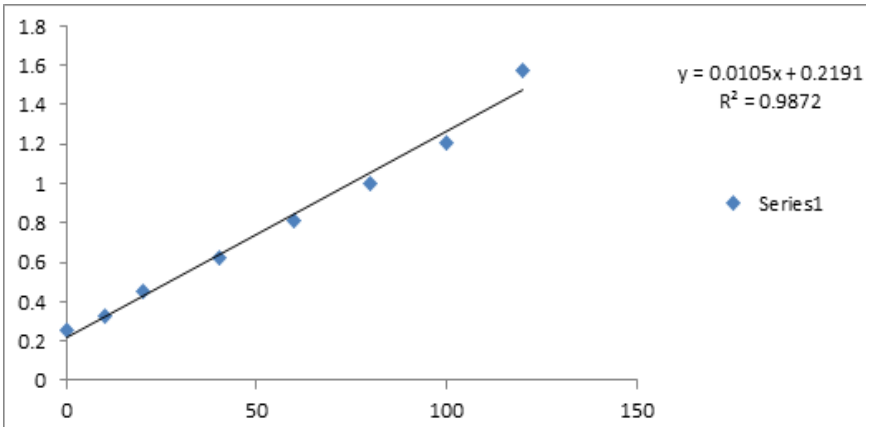

Figure 2: Linear regression of Total Flavonoid content

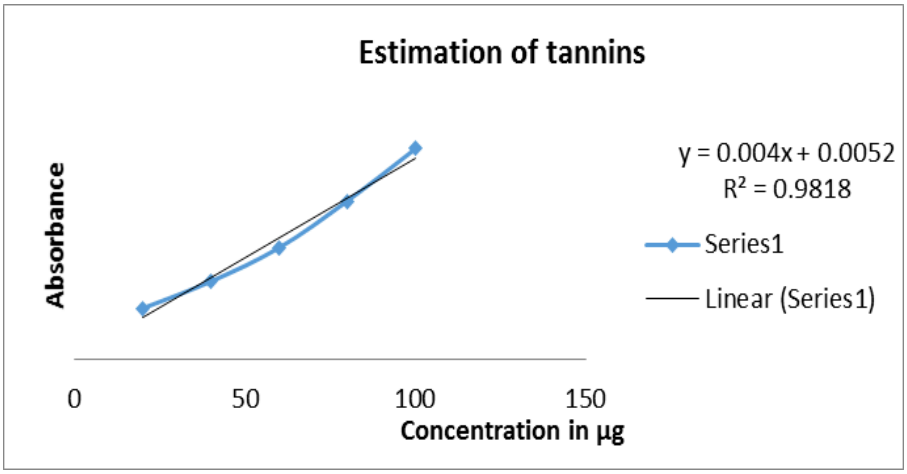

Figure 3: Linear regression of Total Tannin content

Table 5: Total tannin content of crude extract of *Enhalus acoroides*

Sample	Total tannin content (mg) (tannic acid equivalents/g weight)
Hexane	75.33
Chloroform	159.41
Ethyl acetate	52.4
Ethanol	57.4
Water	153.15

Table 6: Antioxidant activity of *Enhalus acoroides* determined by DPPH assay

Concentration µg	Standard (Ascorbic acid)	Hexane	Chloroform	Ethyl Acetate	Ethanol	Water
5	59.64 ±1.37	11.58 ±1.27	17.81±1.32	18.89± 0.70	15.06± 0.89	12.86±0.74
10	74.27 ±0.86	15.54± 0.72	20.97± 0.68	19.89±1.16	17.93±0.48	15.14±0.83
20	94.80± 0.24	17.85±1.20	26.00± 0.68	21.01± 0.95	20.09±0.91	17.81± 0.66
50	97.52± 0.18	19.37±0.96	28.96± 0.68	24.77± 0.49	23.45±0.66	21.01± 0.43
100	98.80± 0.11	24.29±0.45	31.28± 0.70	28.36± 0.72	26.08±0.54	26.28±0.56
200	99.76±0.11	26.88±0.43	32.92± 0.70	30.72±0.63	30.00±0.54	30.68±0.72

Table 7: Antioxidant activity of *Enhalus acoroides* determined by ABTS assay

Concentration µg	Standard (Ascorbic acid)	Hexane	Chloroform	Ethyl Acetate	Ethanol	Water
5	35.21±1.09	13.09±0.22	16.39±1.24	20.09± 1.27	22.30± 1.50	22.31±1.28
10	51.08±1.01	21.22± 1.20	21.29± 1.04	30.79±0.28	24.96±1.12	26.81±1.29
20	86.09± 0.08	27.10±1.42	25.60± 1.06	37.47± 1.38	28.24±0.99	31.86± 1.12
50	86.31± 0.02	33.17±2.08	35.05± 1.16	44.31± 1.57	29.34±0.62	45.66± 0.96
100	86.10± 0.33	46.72±2.10	45.51± 0.96	60.48± 1.10	32.86±0.95	81.27±1.28
200	86.30±0.05	61.43±1.23	60.52± 1.19	78.31±0.35	42.93±1.25	83.67±0.81

Table 8: Antioxidant activity of *Enhalus acoroides* determined by SO assay

Concentration µg	Standard (Ascorbic acid)	Hexane	Chloroform	Ethyl Acetate	Ethanol	Water
10	8.02±0.52	6.26±1.46	13.28±1.42	11.92± 0.97	3.99± 1.60	20.65±1.05
20	17.29±1.13	10.57±1.35	24.71± 1.75	15.08±1.65	9.22±1.12	25.71±0.98
50	27.26± 1.05	34.33±1.20	31.02± 0.77	24.31± 0.97	15.13±1.16	30.0±0.93
100	32.88±2.19	40.45±0.98	42.50± 1.14	34.43± 1.20	24.46±1.12	39.34±1.06
200	44.06± 1.33	42.70±1.05	52.18± 1.05	44.56± 1.85	36.94±0.91	44.91±1.36

Table 9: Antioxidant activity of *Enhalus acoroides* determined by NO assay

Concentration µg	Standard (Ascorbic acid)	Hexane	Chloroform	Ethyl Acetate	Ethanol	Water
500	21.76±1.84	5.18±0.27	4.61±1.59	3.21±1.05	7.16± 0.87	27.66±0.79
750	35.13±1.67	7.21±1.12	7.34±1.32	4.61±1.20	11.30±2.49	38.91±1.62
1000	41.95± 1.52	9.71±1.39	9.89±1.87	9.76± 1.65	21.63±2.31	48.28±0.60
1500	53.21±1.22	17.72±1.45	13.89±2.77	14.73± 1.10	32.10±2.99	50±0.82
2000	69.12±0.86	25.98±1.58	22.60±1.12	29.33±1.57	39.70±4.95	56.64±0.72

Table 10: Antioxidant activity of *Enhalus acoroides* determined by FRAP assay

Concentration µg	Standard (Ascorbic acid)	Hexane	Chloroform	Ethyl Acetate	Ethanol	Water
5	0.11±0.01	0.13±0.004	0.13±0.005	0.12±0.01	0.12±0.007	0.11±0.006
10	0.13±0.009	0.14±0.003	0.14±0.003	0.13±0.005	0.13±0.008	0.13±0.006
20	0.24±0.02	0.18±0.001	0.18±0.005	0.13±0.01	0.13±0.006	0.15±0.007
50	0.39±0.004	0.18±0.002	0.19±0.003	0.13±0.008	0.15±0.005	0.17±0.004
100	0.47±0.008	0.19±0.002	0.16±0.004	0.17±0.007	0.15±0.008	0.30±0.004
200	0.56±0.01	0.21±0.006	0.21±0.005	0.21±0.01	0.17±0.004	0.42±0.009

($ABTS^{\bullet+}$) has been widely used to measure the antioxidant capacity of natural extracts (Thambiraj J and Paulsamy S, 2012). $ABTS^{\bullet+}$, a stable free radical with the characteristic absorbance at 734 nm, was used to study the radicals scavenging effect of extracts of *Enhalus acoroides*. The presence of bioactive chemical compounds in the tested extracts that inhibit the potassium persulfate activity may reduce the production of $ABTS^{\bullet+}$. This study reveals that aqueous extracts of *Enhalus acoroides* exhibited higher $ABTS^{\bullet+}$ radical scavenging activity when compared to the standard Ascorbic acid (Table 7).

Superoxide Scavenging Activity

Superoxide anion is very harmful to cellular components (Korycka-Dahl M, 1978). Robak and Glyglewski, 1998 reported that flavonoids are effective antioxidants mainly because they scavenge superoxide anions. As shown in table 8, the superoxide radical scavenging activities of the plant extract and the reference compound are increased markedly with increasing concentrations. The results suggest that the chloroform extract is a more potent scavenger of superoxide radical which has 56.84±1.13µg/ml in 400µg/ml concentration than the standard quercetin.

Nitric Oxide Scavenging Activity Assay

Nitric oxide ($NO^{\bullet}$) is an important chemical mediator generated by endothelial cells, macrophages, neurons, etc. and is involved in the regulation of various physiological process. Nitric oxides formed during their reduction with oxygen or with superoxides such as NO^2, N^2O^4, N^3O^4 are very reactive. The excess concentration of $NO^{\bullet}$ will cause diseases in human beings which can alter the structural and functional behaviour of many cellular components. Nitrite ions react with Griess reagent and form a purple azo dye. The decrease in the formation of purple azo dye reflects the presence of scavengers in the test compounds. The Aqueous extracts of *Enhalus acoroides* which has maximum scavenging activity found to be an efficient scavenger of nitric oxide

radicals in sodium nitroprusside (Table 9). It clearly indicates that *E. acoroides* has a noticeable effect as scavenging nitric oxide radicals. Among the five extracts evaluated, the aqueous leaf extract of *E. acoroides* exhibited more activity, which may be due to the presence of water-soluble compounds like phenolics with potent free radical-scavenging effects.

Metal chelating activity assay

The FRAP assay was employed to estimate the antioxidant capacity of the samples *invitro*. According to Hodzic *et al*., 2009, FRAP assay had been used to determine antioxidant activity as it is simple and quick. Besides that, the reaction is reproducible and linearly related to molar concentration of the antioxidants. However, some disadvantage was found in this method as FRAP assay does not react fast with some antioxidants such gluthathione. Guo, C. *et al*., 2003 stated that FRAP assay still can be used for assessment of antioxidant activity in plants materials as humans only absorb limited amount of gluthathione. Higher FRAP values give higher antioxidant capacity because FRAP value is based on reducing ferric ion, where antioxidants are the reducing agent. Antioxidants are compounds capable of donating a single electron or hydrogen atom for reduction. Among the five extracts evaluated, the aqueous leaf extract of *E. acoroides* exhibited more antioxidant activity, which has 0.42±0.009µg/ml in 200µg/ml concentration when compared to the ascorbic acid used as the standard (Table 10).

CONCLUSION

It can be concluded that crude extract of *Enhalus acoroides* has effective in scavenging free radicals and has the potential source of natural antioxidants and this justified its uses in folkloric medicines. On the basis of response in terms of scavenging radicals and reducing power activity, it is concluded that the species, *E. acoroides* possessed potential antioxidant activity. It may be due to the presence of respective secondary metabolites such as phenolics, flavonoids, tannins etc. in the species. The strong correlation between the contents of total phenolics, flavonoids and tannins and radical scavenging activity indicates that these phytochemical constituents are major contributors to the antioxidant potential of this species. Therefore, this species can be attempted to derive the drugs of antioxidant properties. However, further studies by *invitro* and *invivo* models are still needed to confirm this property. It further reflects a hope for the development of many more novel anticancer agents or templates from sea grasses which in future may serve for the production of biologically improved therapeutic agents.

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