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PHYTOCHEMICALS AND *IN-VITRO* ANTIOXIDANT ACTIVITY OF *HALOPHILA BECCARII*

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Keywords:

Halophila beccarii, Antioxidant capacity, Phytochemical analysis, Scavenging activity, *In-vitro* models

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ABSTRACT: Free radicals induce numerous diseases by lipid peroxidation, oxidation of monosaccharides and DNA damage**.** In the present study, the marine plant seagrass *Halophila beccarii* was exploited to detect the phytochemical constituents and its antioxidant potential. The aqueous and organic extracts of *Halophila beccarii* portrayed the presence of various metabolites such as phenolic compounds, flavonoids, tannins, and saponins. The antioxidant properties of extracts of *Halophila beccarii* (Hydrocharitaceae) were studied in four *in-vitro* models *viz.* Total antioxidant activity, radical scavenging activity by DPPH reduction Assay, Scavenging of H_2O_2 and reducing power. The extract was found to contain large amounts of secondary metabolites such as phenolic compounds and flavonoids. Among all the extracts the methanolic extract showed significant antioxidant activity compared with standards. The experimental data provide ample evidence to utilize *Halophila beccarii* as a novel dietary agent for treating chronic metabolic diseases, such as diabetes and hyperlipidemia and could be used as a potential source for natural health products.

INTRODUCTION: Antioxidants boost the immunity of the body either through the prevention or delay in the oxidation of highly reactive oxygen species and controlling the oxidative stress. The important mechanism of antioxidants in the cell is to protect the cells from damage caused by the action of reactive oxygen species (ROS), such as superoxide radical ($O_2^{\cdot}$), hydroxy radical ($OH^{\cdot}$), peroxide radicals ($ROO^{\cdot}$) and nitric oxide radicals. The ROS generated during oxidative stress are detrimental to the body unless they are eliminated by the endogenous antioxidant system. Usually, the overexpression of free radicals overshadow the endogenous metabolizing system.

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During excessive metabolism and oxidative stress-free radicals are generated within our cells cause extensive damage to nearby cells, mitochondria, DNA that leads to age-related degenerative diseases, cancer, diabetes and other human diseases [1, 2]. Herbal products play an important role in balancing the pro-oxidant and antioxidant status and controlling oxidative stress.

There is upsurge interest in the utilization of plant-derived natural antioxidants in the drug development process due to nontoxic phytochemical compounds [3, 4]. Plant-derived products exhibit antioxidant mechanism due to the presence of phenolic compounds, flavonoids, saponins, tannins, vitamin E and carotenoids [4]. Literature is abounding with the antioxidant activity of terrestrial plants, but the research on marine-based plants is scanty and undermined [5]. In folk medicine, seagrass has been used for a variety of remedial purposes, *e.g.,* for the treatment of

fever and skin diseases, muscle pain, wounds and stomach problems [5, 6]. The present study focused on exploring the phytochemical compounds of sea-grass and their role in antioxidative mechanism [7].

MATERIALS AND METHODS:

Collection of Seagrass Sample: The seagrass plants were collected from the sea coast near Pulicat Lake at Nellore district, Andhra Pradesh, India. The seagrass plant was botanically identified as *Halophila beccarii* belongs to *Hydrocharitaceae* family. The collected plants were brought to the laboratory under aseptic conditions and washed with tap water and distilled water. The washed material was shade dried, and the dried samples were then ground to a fine powder using mixer grinder.

Preparation of Extracts: The extract was prepared by soaking 20 g of dried powder of *Halophila beccarii* in 200 ml of different solvents such as methanol, ethanol, and water for seven days at room temperature. The solution was then filtered using a Whatman No.1 filter paper, and the extract was evaporated to dryness with a Rota evaporator under reduced pressure at 40 ºC. The dried extracts were dissolved in 0.1% DMSO and stored at 4 ºC for further use. The aqueous, methanol and ethanolic extracts of *H. beccarii* was designated as *Halophila beccarii* aqueous extract (HBAE), *Halophila beccarii* methanolic extract (HBME) and *Halophila beccarii* ethanolic extract (HBEE) respectively.

Qualitative Screening for Phytochemicals: Both organic and aqueous extracts were used to detect the phytochemicals such as phenols and flavonoids, steroids and terpenoids, saponins, tannins, coumarins, carboxylic acids, and tannins. Phytochemical analysis of *H. beccarii* was carried out by adopting standard methods [8]. In brief, the alkaloids in the extract were analyzed by dissolving 0.5 g of seagrass extract in 5ml of methanol and 2N HCl and then filtered. The filtrate was treated with Meyer's and Wagner's reagents and the formation of precipitate denotes the presence of alkaloids. Flavonoids were confirmed by heating 0.5 g of HBAE, HBME and HBEE with 5 ml of ethyl acetate at 40-50 ºC on a water bath for 5 min. The filtrate was treated with 1 % aluminum chloride in methanol and the appearance of the yellow color is treated as a positive index for flavonoids.

The presence of Tannins was confirmed by boiling 0.5 g of all extracts in 20 ml of water, followed by the addition of a few drops of 5% $FeCl_3$ to the filtrate. Tannins were detected by the appearance of brownish-green or blue-black color. The saponins content of *H. beccarii* was shown by boiling the 0.5 g of extract in water and the filtrate was shacked vigorously and the formation of forth is the confining test for saponins. Cardiac glycosides were identified with the formation of the reddish-brown color ring at the junction of glacial acetic acid to $FeCl_3$ and conc. H_2SO_4 reaction mixture. Terpenoids were identified by the addition of 1 ml of chloroform, followed by conc. H_2S0_4 to 1 ml of extract. The reddish-brown color is a positive test for terpenoids. Similarly, phenols were identified with the blue or green color by the addition of a few drops of 10% $FeCl_3$ solution to the extracts.

Quantitative Analysis for Phytocompounds:

Total Phenols: The total phenolic content in the extracts of *H. beccarii* was determined with the Folin- Ciocalteu's reagent (FCR) by using gallic acid as a standard [9]. Different concentrations of both aqueous and organic extracts (0.5 ml) were mixed with 0.1 ml of FCR (diluted 1:2 S v/v) and incubated at room temperature for 15 min. After incubation 2.5 ml saturated sodium carbonate was added and again incubated for 30 min at room temperature. The absorbance was measured against the blank at 750 nm using a spectrophotometer (Shimadzu UV 160). The total phenol content was measured in terms of Gallic acid equivalent per gram sample.

Total Flavonoids: The flavonoid content was estimated by considering quercetin as a standard [10]. Different concentrations of HBAE, HBME, and HBEE (50, 100, 150, 200 and 250, 500 µg/ml) were added to 0.3 ml of 10% $AlCl_3$, and 2 ml of NaOH (1 Mol/l) and the final volume was made up to 10 ml with distilled water. The absorbance was read at 510 nm, and the total flavonoid content was expressed as quercetin equivalents (QE) per gram sample.

Total Saponins: To measure the saponin content about 250 µl of vanillin reagent (800 mg of vanillin in 10 ml of 99.5% ethanol) and 2.5 ml of 72% sulphuric acid was added to different concentrations (100 to 500 µg/ml) of HBAE,

HBME and HBEE [10]. The reaction mixture was heated at 60 ºC for 10 min, the absorbance was measured at 544 nm against Diosgenin.

Determination of Vitamin C & Vitamin E: The vitamin C content of *H. beccarii* extract was determined by the method of [11]. Briefly, 3.0 ml reagent containing 0.6 M sulfuric acid, 28 mmol sodium phosphate, and 4 mmol ammonium molybdate was added to 0.3 ml of the extracts and incubated at 95 °C for 90 min in a water bath. Absorbance was measured at 695 nm.

The amount of vitamin E present in extracts of *H. beccarii* was estimated by the method [12]. A drop of thiourea (10%) and 0.25 ml 2% di-nitro phenylhydrazine (in 9N H_2SO_4) was added to the HBAE, HBME, and HBEE and incubated at 37 °C for 3 h. After incubation 1.25 ml of 85%, H_2SO_4 was added under the ice-cold condition and kept at room temperature for 30 min. The absorbance was measured at 540 nm against a blank.

***In-vitro* Anti-Oxidant Activity:**

Total Antioxidant Activity: The total antioxidant activity of the various extracts of *H. beccarii* was analyzed as per the standard method [13]. In brief, 0.2 ml of extract at various concentrations ranging from 50-500 µg/ml was added to 3.0 ml reaction mixture containing 0.6 M sulfuric acid, 28 mmol sodium phosphate, and 4.0 mmol ammonium molybdate. The reaction mixture was incubated at 95 °C for 90 min on the water bath. After incubation, absorbance was measured at 695 nm. Ascorbic acid was used as standard [14].

DPPH Radical Scavenging Activity: The free radical scavenging activity of the extracts was measured by using 2, 2-diphenyl-1-picrylhydrazyl or 1, 1-diphenyl-2- picrylhydrazyl [15]. The reaction mixture consisted of 1.0 ml of the extract and 1.0 ml of DPPH dissolved in 0.3 mm methanol. After incubation for 10 min under dark conditions, the absorbance was measured at 517 nm. The percentage of scavenging activity was calculated.

Hydrogen Peroxide Radical Scavenging Assay: The Hydrogen peroxide scavenging ability of the HBAE, HBME, and HBEE were determined according to the method of [16]. The extracts (1.0 ml) with different concentrations (50 - 500 µg/ml) were added to 0.6 ml of hydrogen peroxide prepared in 0.1 M phosphate buffer saline (pH 7.4, 50 mm) and samples were incubated at 30 ºC. After 10 min of incubation, the absorbance was read at 230 nm. The percentage of inhibition was measured by the following formula

$$\text{Percentage inhibition} = \frac{\text{Ab of control} - \text{Ab of test}}{\text{Ab of control}} \times 100$$

Estimation of Reducing Power: Reducing power of the extract was determined by mixing the different concentrations of extracts (50-500 µg/ml) with 1.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide [13]. The reaction mixture was incubated at 50 °C for 20 min, and after completion of reaction time, 2.5 ml of trichloroacetic acid (10%) was added and centrifuged at 3000 rpm for 10 min. The supernatant (2.5 ml) was diluted with 2.5 ml distilled water and treated with 0.5 ml of 0.1% $FeCl_3$. The absorbance was measured at 700 nm, and the data was tabulated by comparing with standard Ascorbic acid.

Statistical Analysis: All the experiments were conducted in triplicate and results were tabulated as the mean of three independent experiments and standard deviation. Statistical analysis was carried out by multiple comparison tests by using graph pad prism software version 7.0 and p-value <0.05 were considered significant.

RESULTS:

Qualitative Screening for Phytochemicals: The seagrass collected from the marine sediment was identified as *H. beccarii* based on the morphological features of seagrass. The phytochemicals were extracted from *H. beccarii* aqueous and organic mode. The preliminary qualitative analysis indicated the presence of alkaloids, phenols, saponins, flavonoids, tannins, HBAE, HBME and HBEE of *Halophil beccarii* was shown in **Table 1**. However, the alkaloids and coumarins were detected only in HBME. Cardiac glycosides, flavonoids, glycosides, terpenoids were detected in all three extracts. Compared to other extracts, the methanolic extract was abundant with secondary metabolites with higher levels of flavonoids, glycosides, and saponins. Next, to methanol, the ethanol extract exhibited greater varieties of phytochemicals.

TABLE 1: QUALITATIVE ANALYSIS OF PHYTOCHEMICALS IN *H. BECCARII*

S. no.	Phytochemical constituents	HBAE	HBME	HBEE
1	Alkaloids	-	++	-
2	Cardiac glycosides	+	+	+
3	Coumarins	-	+	-
4	Flavonoids	+	++	+
5	Glycosides	+	++	+
6	Phenols	+	++	+
7	Saponins	-	++	+
8	Steroids	-	++	+
9	Tannins	-	++	+
10	Terpenoids	+	++	+

Measurement of Secondary Metabolites: Antioxidant compounds such as phenol, flavonoids, tannins, vitamin C and vitamin E contents were quantitatively analyzed and represented in **Table 2**. *H. beccarii* extracts contained a significantly higher amount of phenols, flavonoids, and saponins compared to tannins. The extract had a significantly higher amount of vitamin C and vitamin E.

H. beccarii was particularly rich in source of phenols and flavonoids. The number of phenolics varied significantly from 5.44 to18. 44 mg GAE/g in HBAE and HBME respectively. Among all extracts, methanolic extracts showed relatively abundant levels of phytochemicals compared to other solvents.

TABLE 2: BIOCHEMICAL COMPOSITION OF SEAGRASS *H. BECCARII*

S. no.	Parameter	Standard equivalent HBAE mg/g	Standard equivalent HBME mg/g	Standard equivalent HBEE mg/g
1	Phenols [1]	5.44 ± 0.035	18.44 ± 0.032	9.15 ± 0.025
2	Flavonoids [2]	3.25 ± 0.048	10.59 ± 0.035	7.05 ± 0.025
3	Saponins [3]	3.02 ± 0.025	11.02 ± 0.062	6.04 ± 0.082
4	Tannins [1]	0.26 ± 0.025	5.35 ± 0.045	2.15 ± 0.025
5	Vitamin C[4]	5.24 ± 0.047	11.96 ± 0.110	8.15 ± 0.055
6	Vitamin E[5]	4.45 ± 0.108	22.86 ± 1.202	17.50 ± 0.080

Values are expressed as mean ± SEM of three independent experiments, statistical significance 1% level ($p \leq 0.001$).
1. mg gallic acid/g; 2. mg quercetin/g; 3. mg Diosgenin/g; 4. mg ascorbic acid/g; 5. mg tocopherol/g

Antioxidant Activity of Seagrass Extracts: Four different methods have been adopted to measure the antioxidant capacity of *H. beccarii*. Total antioxidant activity was determined by phosphomolybdenum method, varied significantly with different extracts. Total antioxidant activity measured by phosphomolybdenum methods indicates both water-soluble and fat-soluble antioxidant capacity. Among the three extracts, HBME represented a greater amount of antioxidant compounds, equivalent to ascorbic acid, to effectively reduce stress **Fig. 1**. The data indicate dose-dependent total antioxidant activity.

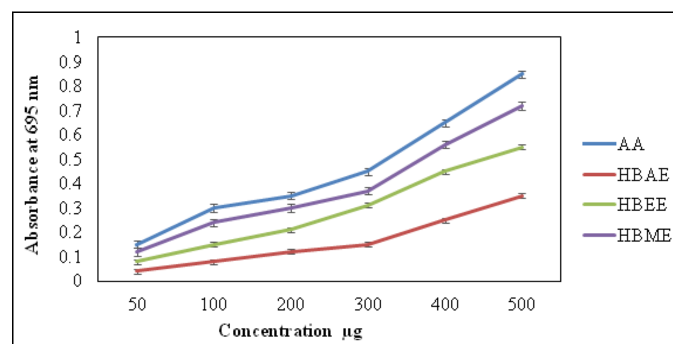

FIG. 1: ANTIOXIDANT ACTIVITY OF EXTRACTS FROM *H. BECCARII.* Values are expressed as mean ± SEM of three independent experiments, statistical significance 1% level ($p \leq 0.05$).

DPPH free radical scavenging has been widely used as a remarkable model to assess the antioxidant activity of plant extracts. The relative antioxidant activity to scavenge the free radical has been compared with standard ascorbic acid. In the DPPH assay, the extracts were able to reduce the stable radical DPPH to yellow colored diphenyl picrylhydrazine. The parameter IC_{50} has been used to compare the effective concentration of extract that causes 50% of inhibition of DPPH activity with the standard ascorbic acid. The IC_{50} value was found to be 200 µg/ml, 400 µg/ml and 500 µg/ml with HBME, HBEE and HBEE respectively **Table 3**. Both standard and extracts exhibited dose-dependent inhibition of DPPH activity.

The reducing power is a hallmark for electron donating activity of phenols and the antioxidants by donating electrons by reductions for the cleavage of free radicals and results in the reduction of ferric chloride Fe^{3+} to ferrous chloride Fe^{2+}. The experimental results showed that the reducing power of methanolic extract was higher than all other extracts **Fig. 2**. The reducing power of methanolic extracts was found to be nearly equivalent to the standard ascorbic acid.

The methanolic extract derived from *H. beccarii* proved to be a better source of antioxidant than other extracts. Therefore, the formation of Fe^{2+} was monitored by measuring the absorbance at 700 nm. All the extracts derived from *Halophila beccarii* exhibited a strong concentration-dependent inhibition of hydrogen peroxide. Among all the extracts, methanol extract showed prominent inhibition. The IC_{50} values for the aqueous, ethanol and methanol were found to be 450 µg/ml, 300 µg/ml, 150 µg/ml respectively **Fig. 3.**

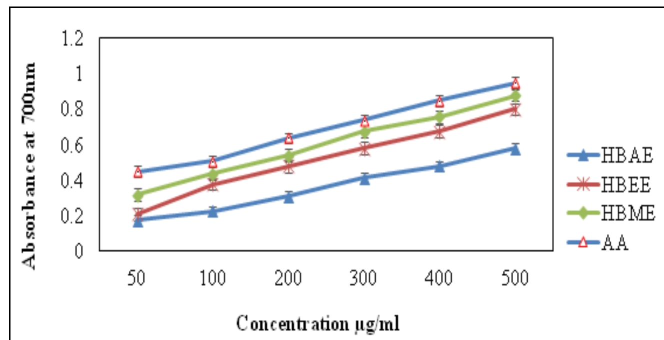

FIG. 2: REDUCING POWER OF EXTRACTS FROM *H. BECCARII*

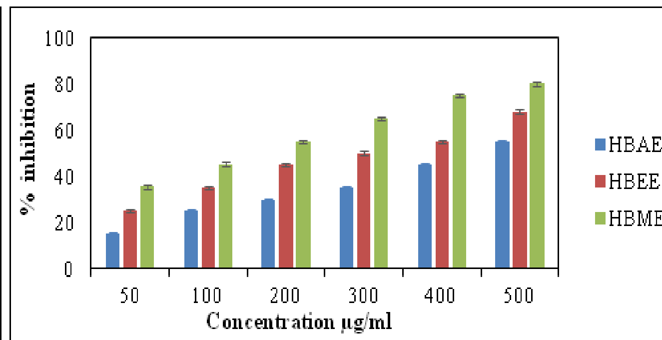

FIG. 3: H_2O_2 RADICAL SCAVENGING ACTIVITY OF EXTRACTS FROM *H. BECCARII*

Values are expressed as mean ± SEM of three independent experiments, statistical significance 1% level ($p \le 0.05$).

TABLE 3: SCAVENGING ACTIVITY (%) ON DPPH RADICALS OF *H. BECCARII*

Sample	Conc. µg/ml	DPPH scavenging Assay (%)	IC_{50} µg/ml
HBME	50	31.8 ± 0.03	
	100	40.4 ± 0.02	
	200	50.3 ± 0.02	200 µg/ml
	300	55.7 ± 0.07	
	400	62.8 ± 0.03	
	500	70.5 ± 0.03	
HBEE	50	20.04 ± 0.02	
	100	25.20 ± 0.04	
	200	35.05 ± 0.02	400 µg/ml
	300	45.25 ± 0.08	
	400	50.20 ± 0.05	
	500	55.20 ± 0.07	
HBAE	50	15.03 ± 0.02	
	100	20.02 ± 0.04	
	200	28.05 ± 0.02	500 µg/ml
	300	38.07 ± 0.08	
	400	45.01 ± 0.05	
	500	50.20 ± 0.05	
Ascorbic acid	50	50.03 ± 0.02	
	100	58.02 ± 0.04	
	200	65.05 ± 0.02	50 µg/ml
	300	72.07 ± 0.08	
	400	80.01 ± 0.05	
	500	87.05 ± 0.04	

Values are expressed as mean ± SEM of three independent experiments, statistical significance 1% level ($p \le 0.05$)

DISCUSSION: The present study analyzed the phytoconstituents and antioxidant activity of *H. beccarii*, which is used as folklore medicine [17]. Little information is available about the antioxidant activity of the experimental marine seagrass. The aqueous, methanol and ethanol extracts of *H. beccarii* yielded positive results for phenols flavonoids and saponins tannins, terpenoids vitamin C and vitamin E [18]. The phytochemical constituents are by results reported from terrestrial plants reported the presence of phenols, flavonoids, and tannins in seagrass [19]. The methanolic extract of *H. beccarii* showed higher amounts of phenols followed by saponins, flavonoids and very little amounts of tannins.

The analysis of phytochemical constituents in *H. beccarii* extract, given the main status, the phenolics are the main secondary metabolites that play a specific role in the maintenance of the human body [20]. Earlier reports indicated the presence of tannins at higher levels in *Halophila ovalis* than *H. beccarii.* The antioxidant potency of crude extract of seagrass has been reported a positive relationship between total phenolic compounds and free radical scavenging capacity according to reference [21].

The seagrasses are rich in source of tannins and used to treat diseases in traditional medicines, intake of tannins may prevent the onset of chronic diseases [22]. The antioxidative vitamins ascorbic acid and vitamin E are not synthesized by most mammals and human beings, but they are potent free radical scavengers; therefore they need to be supplemented in their diets. Vitamin E act as an antioxidant against peroxidation of cellular and subcellular fatty acids leading to the formation of phoenix free radicals.

When compared to vitamin E content vitamin C is lower because the formed free radicals may react with vitamin C to produce tocopherol. This might be the reason for lower content of vitamin E in seagrass than vitamin E. A similar identification has been made in the seaweed *Acanthophora spicifera* [19]. It was reported that the methanolic extract of seagrass has more potent phytochemicals than aqueous and ethanolic extract. Accumulation of phytochemicals and antioxidant activity is greatly influenced by environmental factors [23].

The antioxidant activity of various extracts of *H. beccarii* was determined using different methods. Reducing power is concentration dependent. The same method has also been adopted in the methanolic extract of higher plants [20]. To evaluate antioxidant activity, DPPH is often used as a substrate [24]. Total antioxidant activity was evolved by using ascorbic acid as a control.

In algae, terrestrial plants and seagrass the phytoconstituents like vitamins (C & E), phenolics (tannins, flavonoids, and phenol) have been acting as an antioxidant of scavenging free radicals like H_2O_2 [25]. The free radical scavenging and antioxidant activity were found in seagrass could be associated with their main phenolic compound [9].

CONCLUSION: The Present study shows that phenol content in the methanolic extracts of *H. beccarii* is high and these extracts exhibit strong antioxidant activities. The results would help to ascertain the potency of the crude extract from *H. beccarii* potential source of natural antioxidants. It can be used for minimizing or preventing lipid oxidation in pharmaceutical products and retarding the formation of toxic oxidation products.

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CONFLICT OF INTEREST: Nil

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