

ANTIOXIDANT ACTIVITY OF *HALOPHILA OVALIS* AND *HALOPHILA BECCARII* (HYDROCHARITACEAE): TWO IMPORTANT SEAGRASS SPECIES OF CHILIKA LAGOON, INDIA**SUBRAT KUMAR KAR¹, SUPRAVA SAHOO², BASUDEBA KAR², SOUMENDRA KUMAR NAIK³, PRATAP CHANDRA PANDA^{1*}**

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Received: 03 August 2018, Revised and Accepted: 24 November 2018

ABSTRACT

Objective: The present study was undertaken to evaluate the total phenolic and flavonoid content and the antioxidant property of two important seagrass species namely, *Halophila ovalis* and *Halophila beccarii* occurring in Chilika lagoon, Odisha, India.

Methods: Total Phenolic Content (TPC) of the extracts of *Halophila* species was determined by Folin-Ciocalteu method with little modifications and the total flavonoid content (TFC) was measured by aluminum chloride colorimetric assay. The antioxidant activity of different extracts was investigated by DPPH and ABTS radical scavenging activity. IC₅₀ values were calculated for the DPPH and ABTS methods.

Result: The study revealed that the methanol extract of *H. ovalis* has greater antioxidant activity than *H. beccarii*. Methanol extract of both the species (*H. ovalis* and *H. beccarii*) was found to possess high phenolic content at value of 70.25 mg GAE/g of extract and 48.53 mg GAE/g of extract respectively. Similarly flavonoid contents was found highest in methanol extract for both *H. ovalis* (76.82 mg quercetin equivalent/ g of extract) and *H. beccarii* (64.28 mg quercetin equivalent/ g of extract). The antioxidant activity of different extracts of these two species were evaluated using DPPH and ABTS radical assay. The methanol extract of both *H. ovalis* and *H. beccarii* showed high radical scavenging activity with IC₅₀ values of 37.77 µg/ml and 52.25 µg/ml for DPPH and 25.62 µg/ml and 45.45 µg/ml for ABTS respectively.

Conclusion: The study revealed the potential of the *Halophila* species as natural sources of antioxidants having considerable commercial importance.

Keywords: ABTS radical, 1, 1-diphenyl-2-picrylhydrazyl radical, Flavonoids, Phenolics.

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INTRODUCTION

Seagrasses, a group of marine flowering plants, inhabit the tidal and sub-tidal zones of shallow and sheltered localities of seas, gulfs, bays, backwaters, lagoons, and estuaries along temperate and tropical coastlines of the world [1,2]. With only about 72 species and 13 genera, seagrasses play key ecological roles in fisheries production, sediment accumulation, and stabilization [1,3,4] and have direct value to humanity as food, feed, green manure, and medicine [5-7]. Phytochemical analyses of seagrass species have shown that they are potential sources of antioxidants [8-11], antibacterial, antifungal and anti-inflammatory agents [8,12-14], and source of anticancer compounds [15].

Chilika, the largest tropical brackish water lagoon of Asia, is situated in the east coast of India in the state of Odisha. It is designated as a wetland of international importance under the Ramsar Convention and harbors rich flora and fauna characteristic of freshwater, brackish water, and marine habitats. As many as 5 seagrass species, namely *Halophila ovalis*, *H. ovata*, *Halophila beccarii*, *Halodule pinifolia*, and *H. uninervis* have been reported to occur in Chilika lagoon and the area under seagrass meadows are increasing after the opening of a new mouth connecting the lagoon to the Bay of Bengal [16]. As in other parts of the world, seagrasses of Chilika lagoon form dense meadows producing considerable biomass, provide excellent habitat for spawning fish and many juvenile marine invertebrates, perform multiple ecosystem services such as recycling of nutrients and stabilization of sediments. Although in a limited scale, the local fishermen communities use the whole plants of *Halophila* species as feed for buffaloes and goats

after repeated washing, as packing materials for crabs and as green manure in their fields after decomposition. The leaf powder mixed with turmeric paste is also applied externally by fishermen for treatment of skin diseases.

In the face, increasing demand for antioxidants of plant origin, the importance of seagrasses as the natural source of antioxidants has increased many-fold. The antioxidant activity of plants might be due to the presence of phenolic compounds, flavonoids, α -tocopherol, and carotenoids [17]. In spite of the biological potential of seagrasses, the antioxidant activity of several seagrass species has not yet been extensively studied [18] for different coastal habits of India. In the present study, the antioxidant potential of two seagrass species occurring in Chilika lagoon such as *H. ovalis* and *Halophila beccarii* has been assessed along with measurement of total phenolic and total flavonoid contents (TFC) in different solvent extracts. *H. beccarii* is a globally threatened species classified under "Vulnerable" category by IUCN, and the antioxidant activity of this species has so far not been evaluated. Although some seagrasses of the west coast and southern coastal regions of India have been investigated in respect of their antioxidant, antimicrobial and anti-inflammatory properties [5,6,8,10,12,14], none of the species occurring in east coast of India which also includes Chilika lagoon, have been assessed for their antioxidant potential.

The present paper reports the antioxidant activities of two important seagrasses (*H. ovalis* and *H. beccarii*) of Chilika lagoon, Odisha, India, along with an estimation of total phenolic and TFC in different solvent extracts.

MATERIALS AND METHODS

PLANT MATERIALS

Whole plants of *H. ovalis* (R.Br.) Hook.f. and *H. beccarii* Asch. belonging to the family Hydrocharitaceae were collected from sandy shores of Chilika lagoon close to Pathara village (19°37'10.1"N Lat., 85°08'45.9"E Long.; Alt. 11.6 m) of Ganjam district, Odisha by laying 1 m×1 m sample plots. The species were collected by our team and examined by Dr. P. C. panda and kept at RPRC herbarium having voucher no 10068 for *H. ovalis* and 10174 for *H. beccarii*. Plant samples were cleaned by repeated washing in lagoon water, removing clays, sands, and other epiphytic organisms growing on leaf blades. The plant samples were brought to the laboratory in zip lock polypack with the lagoon water to prevent dehydration. The samples then washed thoroughly with tap water to remove sands and debris, if any, leaves were removed manually and dried at room temperature.

Preparation of extract

The collected leaves were thoroughly washed, cut into pieces and dried for 15–20 days under shade. Then, the dried materials were grounded to a coarse powder. For extraction, different solvents such as methanol, n-hexane, and chloroform were added to 100 g of powdered leaves separately and placed in Soxhlet apparatus for 24 h. The extracts were filtered with Whatman 40 filter paper and then concentrated using a rotary evaporator to give rise to a semi-solid mass. Each solvent extraction method was repeated thrice for the purpose of accuracy. The residues obtained were stored in refrigerator for further analysis.

Determination of total phenolic content (TPC)

TPC of the extracts of two *Halophila* species was determined by the Folin–Ciocalteu method [19] using gallic acid as a standard phenolic compound. To record the readings within the standard curve range of 0.0–600 µg of gallic acid/ml, the extracts were diluted with distilled water to a known concentration. In a test tube, 250 µl of gallic acid solution or diluted extract was added to 1 ml of distilled water followed by the addition of 250 µl of Folin–Ciocalteu reagent. To allow complete reaction with the Folin–Ciocalteu reagent, the samples were shaken well for mixing and then allowed to stand at room temperature for 5 min. Then, 2.5 ml of aqueous solution of 7% Na₂CO₃ was added to the test tube, and the final volume was made up to 6 ml with distilled water. After incubating the samples for 90 min, the absorbance of the subsequent blue colored solution was measured using spectrophotometer at 760 nm. The result was expressed as mg of GAE/g of the extract using an equation that was obtained from the standard gallic acid graph. All the experiments were carried out in triplicates.

Determination of TFC

TFC of different extracts of two *Halophila* species was assessed by the protocol suggested by Ordon et al. [20] with little modification. This process is based on the establishment of a complex flavonoid-aluminum. 1 ml of ethanol solution of 2% AlCl₃ was mixed with 1 ml of extract solution and kept in the dark for 1 h at room temperature. After incubation, the absorbance of the solution was measured at 420 nm using ultraviolet–visible (UV-VIS) spectrophotometer. From the calibration curve of Quercetin, TFC was calculated and expressed in terms of mg quercetin equivalent/g of the extract. All the determinations were performed in triplicates.

1, 1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging activity

Different extracts of *Halophila* species were subjected to DPPH radical scavenging activity, using the method of Blois with little modification [21]. According to this method, 1 ml of extracts with different concentrations was added to 2 ml of 0.1mM of freshly prepared DPPH methanol solution. The vigorously shaken mixture was left to stand for 30 min in the dark at room temperature. Then, the colorimetric changes of the resulting solution, i.e. from deep violet color to light yellow color were measured by a UV/Visible spectrophotometer at 517 nm. In this experiment, ascorbic acid was used as positive control.

The absorbance of the DPPH radical without any samples (the control) was measured. All the observations were carried out in triplicate.

Radical scavenging activity was expressed as percentage inhibition of DPPH radical and was calculated using the following equation-

$$\% \text{Inhibition} = [(A_{\text{control}} - A_{\text{test}}) / A_{\text{control}}] \times 100$$

Where, A_{control} is the absorbance of the control and A_{test} is the absorbance of samples.

The antioxidant activity of each sample was expressed in terms of IC₅₀ value calculated from the graph after plotting inhibition percentage against sample concentration.

ABTS radical scavenging assay

ABTS radical scavenging activity of different extracts of *Halophila* species was determined by following the method of Re et al. [22]. The working solution was prepared by mixing the two stock solutions, i.e. 7 mM ABTS solution and 2.4 mM potassium persulfate solution in equal quantities. Then, it was allowed to react for 12 h at room temperature in the dark. To obtain an absorbance of 0.706±0.001 units at 734 nm using the spectrophotometer, the subsequent solution was then diluted by adding 1 ml of freshly prepared ABTS solution. ABTS solution was freshly prepared for each experiment. 1 ml of different extracts were mixed with 2.5 ml of the ABTS solution and it was allowed to react. Then after 7 min, the absorbance was measured at 734 nm. BHT was used as positive control. The ABTS scavenging capacity of the extract was compared with the positive control and percentage inhibition was calculated as:

$$\text{"ABTS radical scavenging activity (\%)} = [(A_{\text{control}} - A_{\text{test}}) / A_{\text{control}}] \times 100$$

Where A_{control} is the absorbance of ABTS radical+methanol; A_{test} is the absorbance of ABTS radical+sample."

RESULTS AND DISCUSSION

Total phenolic and TFC

In the present study, methanolic extract of *H. ovalis* was found to possess high phenolic (70.25 mg GAE/g of extract) and high flavonoid contents (76.82 mg quercetin equivalent/g of extract) as compared to *H. beccarii*. The phenolic content of *H. ovalis* of Chilika lagoon was significantly higher (70.25 mg GAE/g) than that of the same species collected from Mandapam coast, Tamil Nadu, India (0.424 mg GAE/g) as reported by Ragupathi et al. [10]. The TPC of methanol extract of *H. beccarii* was 48.53 mg GAE/g of extract, and TFC was 64.27 mg quercetin equivalent/g of extract whereas the extract of n-hexane showed the lowest TPC and TFC contents for both the species (Table 1).

TPC was calculated using the standard curve of gallic acid (Fig. 1) (standard curve equation: Y=0.004x+0.063, R²=0.998) and TFC was calculated using the standard curve of Quercetin (Fig. 2) (standard curve equation: Y= 0.013x+0.487, R²=0.886). It was reported that plant secondary metabolites such as phenolics and flavonoids are the potent free radical scavengers [23]. Plant antioxidants which include phenolic compounds showed significant free radicals scavenging activity. Thus, the antioxidant potential of a sample can be accredited mostly to its phenolic compounds [24].

Evaluation of antioxidant activity

Antioxidant activity of plant extracts of *H. ovalis* and *H. beccarii* was studied through DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) methods.

The antioxidant activity of different extracts obtained from *H. ovalis* and *H. beccarii* was evaluated using DPPH radical assay. Here, the extracts showed substantial DPPH radical inhibiting property at 200 µg/ml concentration (Table 2). The DPPH radical scavenging activity of both the *Halophila* species compared with that of standard ascorbic acid was calculated. It was evidenced from the result that

scavenging activity increased with the increase in the concentration of the samples.

The methanol extract of *H. ovalis* was able to reduce the stable violet DPPH radical to the yellow DPPH-H with low IC₅₀ value, i.e. 38.26µg/ml whereas the IC₅₀ value of methanol extract of *H. beccarii* is 94.26 µg/ml. Lower IC₅₀ value indicates a higher antioxidant activity. In both the species of *Halophila*, DPPH free radical scavenging capacity of extracts and positive controls was from strongest to weakest: ascorbic acid > methanol extract > chloroform extract > n hexane extract.

In the present study, the extracts of *Halophila* species were effectively scavenge the ABTS radical. The methanol, chloroform, and n-hexane extracts of both the species exhibited potent scavenging effects against ABTS (Table 3).

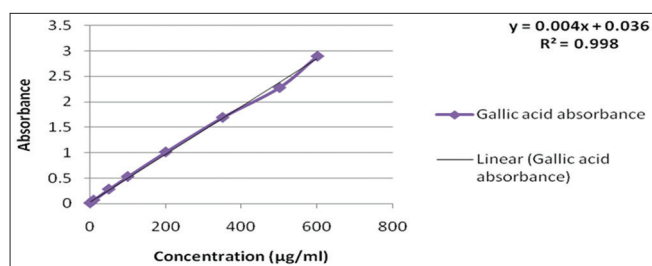


Fig. 1: Quercetin calibration curve for total flavonoid contents

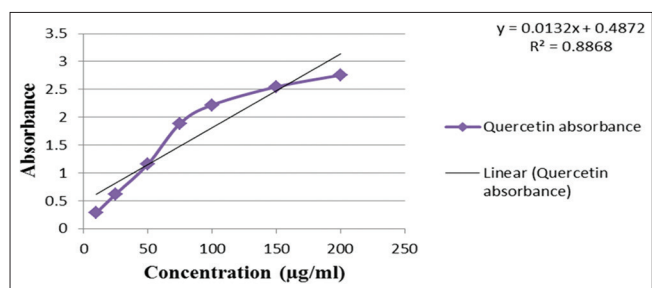


Fig.2 : Gallic acid calibration curve for total phenolic compounds

At 100 µg/ml concentration, the extracts exhibited higher inhibitory property against ABTS radicals. This assay is centered on the inhibition of the absorbance of radical cation ABTS⁺, which has a characteristic wavelength at 734 nm, by antioxidants. The principle involves the relationship between ABTS and potassium persulfate to produce the ABTS radical cation (ABTS⁺) which is a blue-green chromogen. The colored radical is changed back to colorless ABTS when it reacted with antioxidant reductant [25]. The IC₅₀ value of the methanol extract of *H. ovalis* and *H. beccarii* is 25.62 µg/ml and 45.45 µg/ml, respectively (Table 4).

The extracts were able to reduce stable free radicals and hence can be correlated with the higher phenolics and flavonoids contents that are known to be potential antioxidants [26,27]. It is well known that the antioxidant activity of plant extracts containing polyphenol components is due to the capacity to be donors of hydrogen atoms or electrons and to capture the free radicals. Hence, it can be concluded that the higher antioxidant activity of methanol extract of both the *Halophila* species observed in both DPPH and ABTS method might be due to higher phenolics and flavonoids content. The study showed that methanol extract of *H. ovalis* and *H. beccarii* collected from Chilika lagoon exhibited 50% of scavenging activity on DPPH radicals at 37.77µg/ml and 25.62 µg/ml and ABTS radicals at 52.25µg/ml and 45.45 µg/ml, respectively. Relatively less scavenging activity has been reported in *H. ovalis* from Mandapam coast, Tamil Nadu by Rengasamy *et al.* [10] and from Chunnambar estuary, Pondicherry by Yuvaraj *et al.* [14]. Hence, we consider that the higher antioxidant activity of the extracts might be due to its high content of phenolics and flavonoids in *H. ovalis* occurring in Chilika lagoon in eastern India. However, till date, there is no published report on the antioxidant activity of *H. beccarii* in India or elsewhere.

CONCLUSION

The antioxidant activity of extracts of *H. ovalis* and *H. beccarii* two seagrass species of Chilika lagoon was evaluated on the basis of DPPH and ABTS radical scavenging activities. The result of the present study revealed that the methanolic extract of *H. ovalis* of Chilika lagoon exhibited the highest antioxidant activity in comparison to the antioxidant potential of this species in other habitats reported so far. The methanolic extract of *H. ovalis* contained a high level of phenolics and flavonoids, which can be attributed to strong antioxidant activity observed against ABTS and DPPH radicals. This strong antioxidant property identifies the particular seagrass species as a potential source

Table 1: TPC and TFC of *H. ovalis* and *H. beccarii*

Solvent extracts	<i>H. ovalis</i>		<i>H. beccarii</i>	
	TPC (mg GAE/g)	TFC (mg quercetin equivalent/g)	TPC (mg GAE/g)	TFC (mg quercetin equivalent/g)
Methanol	70.25±0.34	76.82±0.11	48.53±0.32	64.27±0.84
N hexane	53.44±0.18	47.83±0.22	23.62±0.95	34.55±0.58
Chloroform	62.87±0.71	65.25±0.85	30.33±0.76	48.69±0.34

Data represented as mean±standard deviation (n=3), TPC: Total phenolic content, TFC: Total flavonoid contents, TPC: Total phenolic content, GAE: Gallic acid equivalents, *H. ovalis*: *Halophila ovalis*, *H. beccarii*: *Halophila beccarii*,

Table 2: Percentage of inhibition of *H. ovalis* and *H. beccarii* revealed from DPPH Assay

Concentration (µg/ml)	% Inhibition	Methanol		N hexane		Chloroform	
		Ascorbic acid (positive control)		Ascorbic acid (positive control)		Ascorbic acid (positive control)	
		<i>H. ovalis</i>	<i>H. beccarii</i>	<i>H. ovalis</i>	<i>H. beccarii</i>	<i>H. ovalis</i>	<i>H. beccarii</i>
1	4.32±0.48	9.96±0.63	8.43±0.69	3.87±0.35	3.75±0.67	5.22±0.8	6.48±0.43
10	74.81±0.33	21.63±0.48	17.75±0.53	9.18±0.7	7.75±0.22	10.58±0.49	10.57±0.38
20	80.15±0.74	33.87±0.22	32.65±0.38	25.46±0.83	18.54±0.56	26.61±0.15	19.66±0.86
50	88.72±0.43	66.93±0.7	40.81±0.91	39.55±0.96	33.95±0.64	38.97±0.7	29.28±0.32
100	91.45±0.18	99.55±0.46	53.06±0.13	48.34±0.77	40.21±0.76	53.19±0.55	40.34±0.61
200	99.52±0.4	99.98±0.23	89.38±0.78	60.52±0.56	60.78±0.37	83.76±0.62	68.73±0.18

Data represented as mean±standard deviation (n=3), *H. ovalis*: *Halophila ovalis*, *H. beccarii*: *Halophila beccarii*

Table 3: Percentage of inhibition of *H. ovalis* and *H. beccarii* revealed from ABTS assay

Concentration ($\mu\text{g/ml}$)	% Inhibition								
		Ascorbic acid (positive control)		Methanol		N hexane		Chloroform	
				<i>H. ovalis</i>	<i>H. beccarii</i>	<i>H. ovalis</i>	<i>H. beccarii</i>	<i>H. ovalis</i>	<i>H. beccarii</i>
1	5.32 \pm 0.54			12.96 \pm 0.31	10.35 \pm 0.42	2.87 \pm 0.37	4.56 \pm 0.74	5.22 \pm 0.9	8.83 \pm 0.58
10	67.61 \pm 0.29			29.96 \pm 0.23	20.75 \pm 0.38	10.85 \pm 0.43	11.67 \pm 0.44	19.58 \pm 0.24	14.54 \pm 0.82
20	80.52 \pm 0.9			43.67 \pm 0.19	36.23 \pm 0.15	27.68 \pm 0.71	21.48 \pm 0.31	33.91 \pm 0.45	22.86 \pm 0.67
50	86.28 \pm 0.47			70.34 \pm 0.63	54.71 \pm 0.34	42.35 \pm 0.7	43.95 \pm 0.55	53.22 \pm 0.74	37.78 \pm 0.55
100	95.33 \pm 0.34			99.55 \pm 0.51	69.64 \pm 0.63	58.34 \pm 0.3	60.15 \pm 0.12	64.79 \pm 0.4	46.34 \pm 0.63
200	99.42 \pm 0.42			99.98 \pm 0.66	86.83 \pm 0.93	69.52 \pm 0.23	68.77 \pm 0.47	80.76 \pm 0.8	65.73 \pm 0.92

Data represented as mean \pm standard deviation (n=3), *H. ovalis*: *Halophila ovalis*, *H. beccarii*: *Halophila beccarii*

Table 4: IC₅₀ values of *H. ovalis* and *H. beccarii*

Solvent extracts	DPPH ($\mu\text{g/ml}$)		ABTS ($\mu\text{g/ml}$)	
	<i>H. ovalis</i>	<i>H. beccarii</i>	<i>H. ovalis</i>	<i>H. beccarii</i>
Methanol	37.77	52.25	25.62	45.45
N hexane	100.20	102.02	52.45	51.96
Chloroform	53.14	102.74	30.34	100.71
Positive control (ascorbic acid)	5.55		6.23	

H. ovalis: *Halophila ovalis*, *H. beccarii*: *Halophila beccarii*

of natural antioxidant for protection against several oxidative stress-related diseases. Besides, the antioxidant activity of *H. beccarii* reported here for the first time will open up similar research in other parts of the world and help in bioprospecting and conservation of this globally threatened plant species.

AUTHORS' CONTRIBUTIONS

PCP conceived the project and taxonomically identified the species. PCP and SKN supervised and guided the research work and preparation of the manuscript. SKK collected and processed the samples and performed analysis of extracts with different solvents. SS and BK studied the scavenging activity and analyzed the data. BK, SKK, and SS interpreted the data and prepared the manuscript. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors report no conflicts of interest regarding this manuscript.

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