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Research Journal of Agricultural Sciences
An International Journal

P- ISSN: 0976-1675
E- ISSN: 2249-4538

Volume: 13
Issue: 01

Res. Jr. of Agril. Sci. (2022) 13: 260–265

CARAS

Res. Jr. of Agril. Sci. (Jan-Feb 2022)
13(1): 260–265

ISSN: 0976-1675 (P)
ISSN: 2249-4538 (E)

www.rjas.org

Full Length Research Article

Studies on Phytochemical, Proximate and Antioxidant Potential of *Halodule pinifolia*

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Received: 20 Dec 2021 | Revised accepted: 28 Jan 2022 | Published online: 21 Feb 2022

A B S T R A C T

Seagrasses have a long history of being used for a variety of remedial purposes, such as treatment of fever, skin diseases, muscle pains, wounds and stomach problems. Hence it is essential to study their bioactive metabolites and medicinal properties when considering their food applications. The present study was designed to evaluate the antioxidant potential of different extract of *Halodule pinifolia*. The free radical scavenging activities of the seagrass were evaluated by various *in vitro* methods. Phytochemical screening revealed the presence of carbohydrates, reducing sugars, alkaloids, saponins, phenolic compounds and flavonoids in aqueous seagrass extract. The antioxidant activity determination revealed that at 100 µg/ml, that seagrass extract had the highest antioxidant activity on DPPH free radicals. This study suggests that *H. pinifolia* extracts exhibit great potential for antioxidant activity and may be useful for their nutritional and medicinal functions.

***Key words*:** *Halodule pinifolia*, Seagrass extract, Phytochemical, Proximate, Antioxidant activity

Natural products have been an important resource for the maintenance of life for ages. Several life-saving drugs have been developed from the plants. The plant kingdom has provided an endless source of medicinal plants first used in their crude forms as herbal teas, syrups, infusions, ointments, liniments and powders. Herbal remedies and alternative medicines are used throughout the world and in the past herbs often represented the original sources of most drugs. Marine species are known to produce a large number of structurally diverse secondary metabolites [1]. 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·), hydroxy radical (OH·), peroxide radicals (ROO·) 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 over expression of free radicals overshadows the endogenous metabolizing system.

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 [2-3]. Herbal products play an important role in balancing the pro-oxidant and antioxidant status and controlling oxidative stress. 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 [4-5]. With only about 72 species and 13 genera, seagrasses play key ecological roles in fisheries production, sediment accumulation, and stabilization [6] and have direct value to humanity as food, feed, green manure, and medicine [7-8]. Phytochemical analyses of seagrass species have shown that they are potential sources of antioxidants [9-10], antibacterial, antifungal and anti-inflammatory agents [11-12], and source of anticancer compounds [13]. The present study the antioxidant activities of important seagrasses (*Cymodocea serrulata*) of Thanjavur, Tamil Nadu, India, along with an estimation of phytochemical analysis.

MATERIALS AND METHODS

Algal samples will be collected from Thanjavur district, East costal region, Tamil Nadu. The w*et al*gal species were identified by standard according to their morphologies [14-15]. Wet algal species will be first washed with sea water to remove

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the debris like sand, sea shells, pieces of wood and tiny stones. It will be shade dried for 24 hours and then finally dried in a tray drier at 60°C to remove the water content. Dry algae obtained will be finely chopped into pieces and then ground into fine powder using mortar and pestle. Microwave drying makes the drying process faster without any degradation of cell components.

Preparation of extract

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.

Phytochemical screening

Qualitative phytochemical screenings were performed using standard procedures [16-17]. The occurrence of phytochemicals in the crude extracts of *Cymodocea serrulata* was determined.

Quantitative analysis

The extract was subjected to quantitative analysis for the determination of secondary phytochemical constituents like determination of total alkaloids [18], total phenolic content [19], total flavonoids content [20], estimation of total tannins [21] and estimation of total saponins [22].

Proximate analysis

Proximate analysis such as moisture content, Ash content [23], crude fibre, crude lipid [24], Protein [25] and carbohydrate [26] was determined.

In vitro antioxidant activity

Antioxidant activity of the extract was determined by various methods such as DPPH radical scavenging activity [27], Superoxide radical scavenging activity [28], Hydrogen Peroxide Scavenging Activity [29] and Total antioxidant capacity [30].

RESULTS AND DISCUSSION

Preliminary phytochemical screening

The phytochemicals were analyzed qualitatively by using standard protocols in different solvent extract of *Halodule pinifolia*. The protein, reducing sugar, phenol, tannins, amino acid and steroids were found in all the extracts. The flavonoids, anthraquinones and terpenoids were present in ethanol and acetone extracts. Tanins, alkaloids, amino acids, steroids and phenol were present in the hexane extract of *H. pinifolia*. The saponins, resins and glycosides were present only in the ethanol extracts of sea grass *H. pinifolia*. This is consistent with the findings of Ragupathi *et al.* [31] who had reported the qualitative analysis of the above phytoconstituents in the methanolic extracts of five seagrasses like *Enhalus acoroides*, *Thalassia hemprichii*, *Halodule pinifolia*, *Cymodocea serrulata* and *Cymodocea rotundata* from Chinnapallam coast of Tamil Nadu. Athiperumalsami *et al*. [32] screened four seagrasses such as *Halophila ovalis*, *S. isoetifolium*, *C. serrulata* and *H. pinifolia* and reported 15 phytochemicals from benzene and petroleum ether extract of *S. isoetifolium* collected from Gulf of Mannar. The results of the present study are also in line with the results of Girija *et al*. [33] who reported the presence of ten phytoconstituents in the methanol extracts of *H. pinifolia* collected from the study site.

Table 1 Qualitative phytochemical analysis for the extracts of *H. pinifolia*

Phytochemicals	Solvents		
	Ethanol	Acetone	Hexane
Proteins	+	+	+
Resins	+	-	-
Tannins	+	+	+
Saponins	+	+	+
Flavonoids	+	+	+
Alkaloids	+	+	+
Amino acids	+	+	+
Steroids	+	+	+
Reducing sugar	+	+	+
Glycosides	+	+	+
Anthraquinones	+	-	+
Terpenoids	+	+	+
Phenol	+	+	+

+ Present - Absent

Quantitative phytochemical

The quantitative analysis of the phytoconstituents was also done in the sea grass. The phytochemicals such as flavonoids, phenols, alkaloids, tannin and saponins were quantitatively estimated in different solvent extracts of sea grass (Table 2). The highest alkaloids content (49.5 mg/100g) was found in the ethanolic extracts and followed by acetone (40.1 mg/100g) and hexane (38.3 mg/100g) extracts of *H. pinifolia.* The flavonoids content was showed the highest value in ethanolic extract (69.5 mg/100g) followed by acetone (56.8 mg/100g) and hexane extracts (48.7 mg/100g). The highest phenol and sapanins content were present the ethanolic extract (38.2 mg/100g and 32.9 mg/100g) when compared with hexane and acetone extracts respectively. The highest content of tannin was found (35.9 mg/100g) in ethanolic extracts and moderate level was present in the acetone (29.2 mg/100g) and hexane (26.4 mg/100g) extracts respectively. From this present study the ethanol extracts showed the higher values of flavonoids (69.5 mg/100g), alkaloids (49.5 mg/100g), saponins (32.9 mg/100g), tannins (35.9 mg/100g) and phenol (38.2 mg/100g) when compared to other solvent extracts.

Data depicted in (Table 2) indicated the quantitative phytochemical composition of seagrass and the results revealed that the presence of alkaloid, phenolic, flavonoids, tannins and saponins. The pharmacological action of alkaloids are widespread antimicrobial and anti-diarrheal effects. Phenolic

compounds in plants provide an array of natural sources of antioxidants for use in foods and nutraceuticals [34]. Total Flavonoid content of all spices are nearly related which indicated that flavonoids exert their antioxidative activity by effectively scavenging various free radicals. Saponins and tannins have a broad spectrum of biological activity such as cytotoxic activity, antioxidant, antimicrobial and *in vivo* antitumor activities [35].

Table 2 Quantitative phytochemical composition of *Halodule pinifolia*

Parameters	Ethanol (mg/100g)	Acetone (mg/100g)	Hexane (mg/100g)
Alkaloids	49.5 ± 0.45	40.1 ± 0.26	38.3 ± 0.38
Flavonoids	69.5 ± 0.21	56.8 ± 0.48	48.7 ± 0.19
Phenols	38.2 ± 0.24	35.9 ± 0.12	33.4 ± 0.34
Tannin	35.9 ± 0.12	29.2 ± 0.26	26.4 ± 0.18
Saponin	32.9 ± 0.27	30.2 ± 0.15	27.7 ± 0.32

Each value is the Mean ± SD of three replicates

Proximate compositions

The proximate compositions of the seagrass are summarized in table 3. From this present study the ethanol extracts showed the higher values of carbohydrate (45.12%) and moisture content (22.8%) and moderate concentration of protein (9.42%) and ash (7.8%) and low concentration of fat (5.3%) and crude fibre (8.9%) when compared to other solvent extracts. *H. pinifolia* have relatively average moisture content but that can also show some possible microbial activities during storage [36]. The ash content 8.9% indicates that the seagrass are rich in mineral elements. Crude Lipids are the principal sources of energy and *H. pinifolia* lipid should provide some amount of energy. The crude protein content of 9.42% obtained in present study is higher compared to 6.30% in water Spinach and then it indicates that the leaves are a poor source of daily proteins. The crude fibre content of 8.9% is low compared to other seagrass [37], but the value is within the range of 0.70 – 12.0% for most leafy vegetables. Dietary fibre helps to reduce serum cholesterol level, risk of coronary heart disease, colon, breast cancer and hypertension [38]. The carbohydrate content of the leaves is considerably this indicates that the plant can contribute meaningfully to the daily energy requirement [39].

Table 3 Proximate composition of *Halodule pinifolia*

Parameters	Ethanol (%)	Acetone (%)	Hexane (%)
Moisture content	22.8 ± 0.16	22.1 ± 0.21	21.7 ± 0.12
Total Ash	7.8 ± 0.35	7.6 ± 0.28	7.3 ± 0.15
Crude Fibre	8.9 ± 0.15	8.6 ± 0.31	8.1 ± 0.26
Crude lipid	5.3 ± 0.26	4.9 ± 0.28	4.5 ± 0.13
Protein	9.42 ± 0.33	8.96 ± 0.25	8.22 ± 0.16
Carbohydrates	45.12 ± 0.26	42.28 ± 0.12	41.02 ± 0.31

Each value is the Mean ± SD of three replicates

Antioxidant activity

Antioxidant capacity of *Halodule pinifolia* was checked by different assays because of the lack of a validated assay that can reliably measure the antioxidant capacity of foods, herbs and biological samples. The antioxidant activity of seagrass extract of *Halodule pinifolia* as measured by the ability to scavenge DPPH free radicals was compared with the standard ascorbic acid. The results of DPPH assay revealed that leaf extract exhibited significant antioxidant activity (Table 4-8). The DPPH scavenging effect was found to increase with increasing concentrations. At 100µg/ml, the highest percentage inhibition (22.9%) was shown by different extract compared to the reference antioxidant ascorbic acid (24.8% inhibition).

Table 4 DPPH radical scavenging activity of different concentrations of *Halodule pinifolia*

Concentrations (µg)	DPPH radical scavenging activity (%)			
	Ascorbic acid	Hexane	Acetone	Ethanol
200	50.95 ± 1.99	38.63 ± 1.64	35.61 ± 1.74	58.63 ± 2.87
400	83.75 ± 3.25	48.49 ± 1.99	44.65 ± 2.16	59.452 ± 2.99
600	86.62 ± 3.46	52.87 ± 2.29	47.94 ± 2.21	60.00 ± 3.06
800	88.53 ± 4.06	55.89 ± 2.41	55.06 ± 2.49	61.095 ± 3.35
1000	90.28 ± 4.67	56.43 ± 2.63	58.35 ± 2.68	61.917 ± 3.49

Each value is the Mean ± SD of three replicates

Table 5 Hydroxyl radical scavenging activity of different concentrations of *Halodule pinifolia*

Concentrations (µg)	Hydroxyl radical scavenging activity (%)			
	Gallic acid	Hexane	Acetone	Ethanol
200	31.15 ± 1.58	20.00 ± 1.18	16.07 ± 0.78	47.14 ± 1.76
400	49.27 ± 1.75	32.85 ± 1.43	27.85 ± 0.90	47.85 ± 1.81
600	71.25 ± 2.58	38.57 ± 1.61	32.14 ± 1.02	50.00 ± 2.02
800	75.36 ± 2.89	42.50 ± 1.66	41.42 ± 1.48	51.42 ± 2.17
1000	80.67 ± 3.21	43.21 ± 1.75	45.71 ± 1.73	53.57 ± 2.25

Each value is the Mean ± SD of three replicates

Table 6 Nitric oxide radical scavenging activity of different concentrations of *H. pinifolia*

Concentrations (µg)	Nitric oxide radical scavenging activity (%)			
	Gallic acid	Hexane	Acetone	Ethanol
200	57.44 ± 2.34	23.536 ± 1.43	18.681 ± 0.94	17.994 ± 0.72
400	64.53 ± 2.65	26.016 ± 1.54	24.908 ± 1.09	20.791 ± 0.86
600	68.08 ± 3.09	29.340 ± 1.35	27.810 ± 1.15	24.907 ± 0.98
800	71.63 ± 3.31	29.763 ± 1.22	30.818 ± 1.43	27.810 ± 1.19
1000	79.07 ± 3.85	33.826 ± 1.55	34.301 ± 1.55	34.353 ± 1.62

Each value is the Mean ± SD of three replicates

Table 7 Superoxide radical scavenging activity of different concentrations of *H. pinifolia*

Concentrations (µg)	Superoxide radical scavenging activity (%)			
	Ascorbic acid	Hexane	Acetone	Ethanol
200	33.33 ± 2.43	59.53 ± 3.1	53.846 ± 2.64	57.230 ± 2.37
400	41.66 ± 2.63	64.77 ± 3.39	56.384 ± 3.06	65.692 ± 3.06
600	66.66 ± 3.14	67.76 ± 3.89	58.615 ± 3.18	66.923 ± 3.11
800	75.00 ± 3.56	71.07 ± 4.25	62.307 ± 3.39	73.615 ± 2.81
1000	91.66 ± 5.09	73.84 ± 4.41	70.461 ± 3.51	77.230 ± 2.94

Each value is the Mean ± SD of three replicates

Table 8 Reducing power of different concentrations of *H. pinifolia*

Concentrations (µg)	Superoxide radical scavenging activity (%)			
	Ascorbic acid	Hexane	Acetone	Ethanol
200	0.262 ± 0.009	0.229 ± 0.012	0.283 ± 0.011	0.251 ± 0.01
400	0.525 ± 0.02	0.232 ± 0.015	0.286 ± 0.013	0.258 ± 0.009
600	0.588 ± 0.025	0.244 ± 0.016	0.291 ± 0.015	0.269 ± 0.007
800	0.660 ± 0.026	0.269 ± 0.02	0.310 ± 0.009	0.333 ± 0.012
1000	0.893 ± 0.036	0.335 ± 0.023	0.351 ± 0.007	0.438 ± 0.011

Each value is the Mean ± SD of three replicates

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 [40]. Seagrasses are particularly rich in proanthocyanidins (condensed tannins) [32]. 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 [41]. Earlier reports revealed that polyphenols of the seagrasses have the antioxidant activity [42]. 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 [43]. Published reports on the total antioxidant activity of seagrasses are not available. However, Kumaran and Karunakaran [44] have reported total antioxidant activity in the range of 245 to 376 mg ascorbic acid equivalents/g in Phyllanthus species. Ganesan *et al*. [45] noticed higher antioxidant activity (32.01 mg ascorbic acid equivalents/g) in ethyl acetate fraction of *Acanthophora spicifera*. Ye *et al*. [46] noticed higher antioxidant activity (30.50 mmol FeSO4/mg) in ethanol extract of brown seaweed *Sargassum pallidum*. It has been reported that solvents used for extraction have dramatic effect on the chemical species [47].

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 [48]. 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.

In the FRAP assay, antioxidants in the sample reduce ferric (III) to ferrous (II) in a redox-linked colourimetric reaction [49] 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. The positive control ascorbic acid showed significantly higher antioxidant activity than samples. Same trend has also been reported by Kumaran and Karunakaran [44] in methanolic extracts of Phyllanthus species.

CONCLUSION

On the basis of the results obtained in the present study, it is concluded that ethanol extract of *H. pinifolia* has potent anti-oxidant activities. Thus the *H. pinifolia* extract may be attributed to the presence of phenolic compounds and flavonoids etc., therefore, further investigation is 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.

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