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Extraction, purification and study on antioxidant properties of fucoxanthin from brown seaweeds

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ABSTRACT

Five brown seaweeds such as Sargassum wightii, Sargassum ilicifolium, Sargassum longifolium, Padina sp. and Turbinaria sp. were selected for fucoxanthin extraction and purification. Among the various solvents used 90% acetone was found to be good for extraction of carotenoids. The crude pigments extracted were initially screened through TLC (Thin-layer chromatography) and then fucoxanthin was separated and purified using Silica column chromatography (230-400 mesh, Merck). The fucoxanthin eluted using n-hexane and acetone in the ratio of 7:3 was sensitive to light and temperature. The purified sample of fucoxanthin was later checked with free radicals for its antioxidant property.

Keywords: Brown seaweeds, fucoxanthin, silica column chromatography, antioxidant property

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INTRODUCTION

Seaweeds, a heterogeneous group of plants with long life history, constitute a commercially important renewable resource. Species of *Sargassum, Padina, Dictyota* and *Gracilaria* are used as fertilizers, food additives and animal feed. Since past two decades extensive research has been done on bioactive compounds present in seaweeds which include sulfated polysaccharides, phlorotannins and diterpenes [1]. The other types of bioactive compounds present in seaweeds are the photosynthetic pigments including carotenoids and xanthophylls. Among these pigments, fucoxanthin forms an important constituent of brown seaweeds. Fucoxanthin is the main non-provitamin A carotenoid present in brown seaweeds which belongs to the group of xanthophylls and exhibits potent antioxidant activity. Apart from pigments, brown seaweeds also have polyphenols [2-5]. In India, Gulf of Mannar region is found to be a rich source of brown seaweeds throughout the season.

Although seaweeds possess wide applications in food and pharmaceutical industries, the antioxidant activities of many types of seaweeds in the South Indian coastal area are still unexplored. Reactive oxygen species (ROS) and oxidative stress have proved to be toxic to human health as they oxidize biomolecules which leads to cell damage and cell death. In order to overcome these situations antioxidants are important in food items as supplements to prevent cell death. Fucoxanthin is a natural pigment that can replace the synthetic antioxidants [10]. Though numerous seaweeds have been studied for their antioxidant activities [6-9] comparatively less data is available on the antioxidant activities of fucoxanthin extracted from brown seaweeds.

The aim of the present work was to estimate the pigments present in five different brown seaweeds such as *Sargassum wightii, Sargassum ilicifolium, Sargassum longifolium, Padina gymnospora* and *Turbinaria ornata* and to study the antioxidant activity of crude pigments and fucoxanthin using DPPH as free radicals.

EXPERIMENTAL SECTION

Collection of algae

Brown seaweeds such as *Sargassum ilicifolium, Sargassum longifolium, Sargassum wightii, Padina gymnospora* and *Turbinaria ornata* were collected from Mandapam, Gulf of Mannar coast, Rameswaram, India. The collected samples were cleaned well with sea water to remove the extraneous matter such as epiphytes, sand particles, pebbles and shells and brought to the laboratory in sterile bags. The samples were then washed thoroughly with tap water and distilled water and spread in dark at room temperature for drying.

Sample preparation

Dried seaweeds were ground using mixer without producing heat and converted to powder. The powdered sample was stored in dark condition for further use.

Extraction of pigments

One gram each of powdered seaweed sample was weighed and dissolved in 10ml of three solvents such as 90% acetone, 100% acetone and 90% ethanol. The samples were incubated for overnight at 4℃ in a dark place and then centrifuged at 8000rpm for 15 minutes. Extraction was repeated three times till the sample became colorless. The procedure was carried out in triplicates. The sample was stored in amber color bottles to avoid degradation by light.

Analysis

The pigments extracted using different solvents were quantified using UV-Visible spectrophotometer (Varian 300) by reading the absorbance at their respective wavelengths and using the formulae given below.

Formulae

(Arnon, 1949) [11]

Chl a (mg g^{-1}) = [12.7 (A_{663}) −2.69 (A_{645}) V] / (1000×W)

Total Chl (mg g^{-1}) = [20.2 (A_{645}) + 8.02 (A_{663}) V] / (1000×W)

(Jeffrey et al, 1961) [20, 23]

Chlorophyll C1+C2 (mg g^{-1}) = [24.36×A_{630}−3.73×A_{664}]

(Jensen and Jensen, 1959 & Duxbury and Yentsh, 1956) [12]

Carotenoids (mg g^{-1}) = [7.6 (A_{480}) − 1.49 (A_{510}) V] / (1000×W)

(Seely et al, 1972) [13]

Fucoxanthin (mg g^{-1}) = A_{470} −1.239 (A_{631}+A_{581}−0.3 ×A_{664}) − 0.0275×A_{664} /141

Where, A = Absorbance at particular wavelength

V = Total volume of the pigment extract

W = Weight of the sample used for extraction

The values were noted and scanned for peak labeling. The baseline correction was made through control sample.

Screening and purification

Thin Layer Chromatography

Thin layer chromatography was used to calculate the Rf value of the photosynthetic pigments chlorophyll, carotenoids and fucoxanthin present in the extracted samples. In TLC pigments in the mixture are separated on the basis of their differences in solubilities and partition co-efficient in a binary solvent system. Silica gel coated plates (Merck -10×6cm) and the developing solvent (n-hexane: acetone) in a ratio of 7:3 were used for TLC [24].

Initially the chromatography sheets were pre-saturated with the solvent. 5µl of the sample was then carefully applied on the plates and the samples were allowed to dry. The loaded plates were then placed in a pre-saturated tank with caution such that the applied sample does not dip in the solvent system. The set up was left undisturbed and the solvent was allowed to move up till it reached 9cm. The plates were then removed from the tank and the colour spots were marked immediately. Four major pigment spots such as carotenes, chlorophyll *a*, fucoxanthin and

chlorophyll *c* were observed on the plate. The Rf values were noted in the TLC plates and calculated by the standard formula given below

[Rf = Distance moved by the pigment / Distance moved by the solvent]

Column chromatography

Column chromatography is a method of separating the pigments according to their density. A glass column cleaned with acetone and initially packed with glass wool or cotton at the bottom end was used for separation and purification. Silica gel (230-400 mesh) mixed with the solvent n-hexane was poured immediately in to the column after continuous stirring without any breakage or bubbles. The column was left undisturbed for one day for proper binding of silica and later 5ml of the crude pigment sample was loaded in the column. After the binding of pigments in the silica column, the eluting solvent n-hexane and acetone in the ratio 7:3 [25] was added frequently for separation and purification of fucoxanthin from crude extracts [see Fig 1]. The fractions obtained were collected and stored in brown bottles under 4-10℃ for further use.

Antioxidant activity

DPPH (1,1-diphenyl-2-picrylhydrazyl) stock (1M) was prepared using 0.394 grams of DPPH in 1 ml each of 90% ethanol, 90% acetone and 100% acetone separately and DPPH working solutions (0.15 mM) were prepared from 15µl stock mixed with 100 ml of respective solvents. 2ml of 0.15mM/l DPPH radical was taken in a test tube, 1 ml of sample extract was added and vortexed for 30 seconds. Reaction mixture was kept in dark at room temperature for 20 minutes. Control was prepared without adding the sample extract. Absorption was read at 517nm and the anti-oxidant activity was calculated using the standard formula of [26-28]

Total Anti-oxidant activity=1-(Absorbance of sample/Absorbance of control) ×100

Statistical Analysis

All the experiments were carried out in triplicates and the average values were subjected to One-way Anova by the Tukey-HSD method.

RESULTS AND DISCUSSION

Pigment extraction and quantification

Pigments such as chlorophylls and carotenoids were quantified from five different brown seaweeds such as *Sargassum wightii, Sargassum ilicifolium, Sargassum longifolium, Padina gymnospora* and *Turbinaria ornate*, and the fucoxanthin present in *Sargassum ilicifolium* was purified using silica column chromatography. The solvents used for extraction were 90% acetone, 100% acetone and 90% ethanol. Among the three solvents, 90% acetone was found to have good extraction efficiency. Chlorophyll *a*, chlorophyll *b*, and total chlorophyll contents were found to be high in *Padina gymnospora* compared to other seaweeds. Pigments extracted using 100% acetone showed values lower than that of 90% acetone and higher than that of 90% ethanol extracted samples.

Pigments extracted from *Sargassum ilicifolium* with 100% acetone showed higher quantity than 90% ethanol, but overall extraction process showed that 90% acetone is the most efficient solvent to extract pigments than 90% ethanol. 100% acetone showed lesser extraction efficiency probably due to difficulty in binding to the powdered seaweed as compared to 90% acetone. Moreover, the evaporation time for 100% solvent is very less compared to 90% solvent. Fucoxanthin content was found high in 90% acetone extract of *Padina gymnospora* and *Turbinaria ornata* compared to other seaweeds, but comparatively lesser in 100% acetone extract (Table 1).

Purification of fucoxanthin

Extracted pigments were purified using silica column chromatography. The solvent system n-hexane and acetone (7:3) was used. Chlorophyll *a* pigment (blue green) was eluted first followed by carotenoids and fucoxanthin (yellow orange), and finally chlorophyll *c* (fluorescent green) as shown in Fig 1. The fucoxanthin eluted was stored in dark under 4℃ for further use. The fraction of yellow orange colour was subjected to spectral analysis and based on the absorbance maximum at 446nm it was confirmed to be fucoxanthin (Fig 2).

Thin layer chromatography

The extracted crude pigments and purified fucoxanthin pigment were checked through Thin-layer chromatography using the solvent n-hexane and acetone in the ratio 7:3. Rf values of separated pigments were calculated and compared to standard Rf values (Table 2) [11].

Antioxidant activity

Antioxidant activity was tested for the crude and purified extracts by using 0.15mM DPPH free radical. Percentage of DPPH scavenging activity was found to be high for *Sargassum longifolium* extracted in 90% acetone, rest of the samples showing lesser activity. In 100% acetone extract the activity was similar for *Sargassum wightii, Sargassum ilicifolium* and *Sargassum longifolium*, less for *Padina gymnospora* and high for *Turbinaria ornata*. *Sargassum ilicifolium* and *Sargassum longifolium* exhibited equal extent of activity in 90% ethanol whereas *Sargassum wightii* and *Turbinaria ornata* showed higher activities and *Padina gymnospora* comparatively very less (Table 3). The fucoxanthin separated from *Padina gymnospora* showed higher scavenging activity than that of *Sargassum ilicifolium* (Table 4).

Table 1. Quantification of photosynthetic pigments (mg g^{-1} DW) in different solvents

S.No	Seaweeds	Chl a	Chl C_1+C_2	T Chl	Carotenoids	Fucoxanthin
	90% Acetone					
1.	*Sargassum wightii*	0.75±0.074^{b}	0.02±0.016^{b}	0.77±0.08^{b}	0.09±0.015^{a}	0.12±0.010^{a}
2.	*Sargassum ilicifolium*	0.96±0.087^{c}	0.09±0.012^{c}	1.05±0.09^{c}	0.19±0.015^{b}	0.23±0.023^{b}
3.	*Sargassum longifolium*	0.54±0.032^{a}	0.034±0.006^{b}	0.57±0.03^{ab}	0.07±0.006^{a}	0.09±0.006^{a}
4.	*Padina* sp.	1.13±0.092^{c}	0.133±0.015^{d}	1.26±0.10^{c}	0.41±0.035^{c}	0.38±0.046^{c}
5.	*Turbinaria* sp.	0.50±0.044^{a}	-0.01±0.002^{a}	0.50±0.04^{a}	0.41±0.035^{c}	0.38±0.046^{c}
	90% Ethanol					
1.	*Sargassum wightii*	0.36±0.045^{b}	0.03±0.006^{a}	0.39±0.045^{b}	0.06±0.006^{b}	0.08±0.006^{b}
2.	*Sargassum ilicifolium*	0.45±0.040^{b}	0.7±0.015^{b}	0.52±0.026^{c}	0.13±0.017^{c}	0.15±0.012^{c}
3.	*Sargassum longifolium*	0.19±0.012^{a}	0.02±0^{a}	0.21±0.012^{a}	0.03±0.006^{a}	0.03±0.006^{a}
4.	*Padina* sp.	0.65±0.021^{c}	0.14±0.010^{c}	0.79±0.029^{d}	0.26±0.006^{d}	0.25±0.006^{d}
5.	*Turbinaria* sp.	0.20±0.038^{a}	0.01±0.001^{a}	0.21±0.032^{a}	0.03±0.006^{a}	0.04±0.006^{a}
	100% Acetone					
1.	*Sargassum wightii*	0.47±0.03^{b}	0.0±0.012^{b}	0.47±0.032^{b}	0.04±0.006^{b}	0.08±0.010^{b}
2.	*Sargassum ilicifolium*	0.63±0.058^{c}	-0.02±0.002^{a}	0.63±0.058^{c}	0.09±0.006^{d}	0.16±0.012^{d}
3.	*Sargassum longifolium*	0.21±0.026^{a}	-0.002±0.001^{b}	0.21±0.026^{a}	0.02±0.006^{a}	0.03±0.006^{a}
4.	*Padina* sp.	0.29±0.01^{a}	0.003±0.001^{b}	0.29±0.011^{a}	0.11±0.006^{d}	0.12±0.006^{c}
5.	*Turbinaria* sp.	0.46±0.026^{b}	-0.01±0.001^{ab}	0.46±0.026^{b}	0.07±0.006^{c}	0.11±0.006^{c}

All the values are mean ±SD of triplicates. Significant differences ($p<0.05$) with different seaweeds for different solvents are indicated by different superscript lower letters.

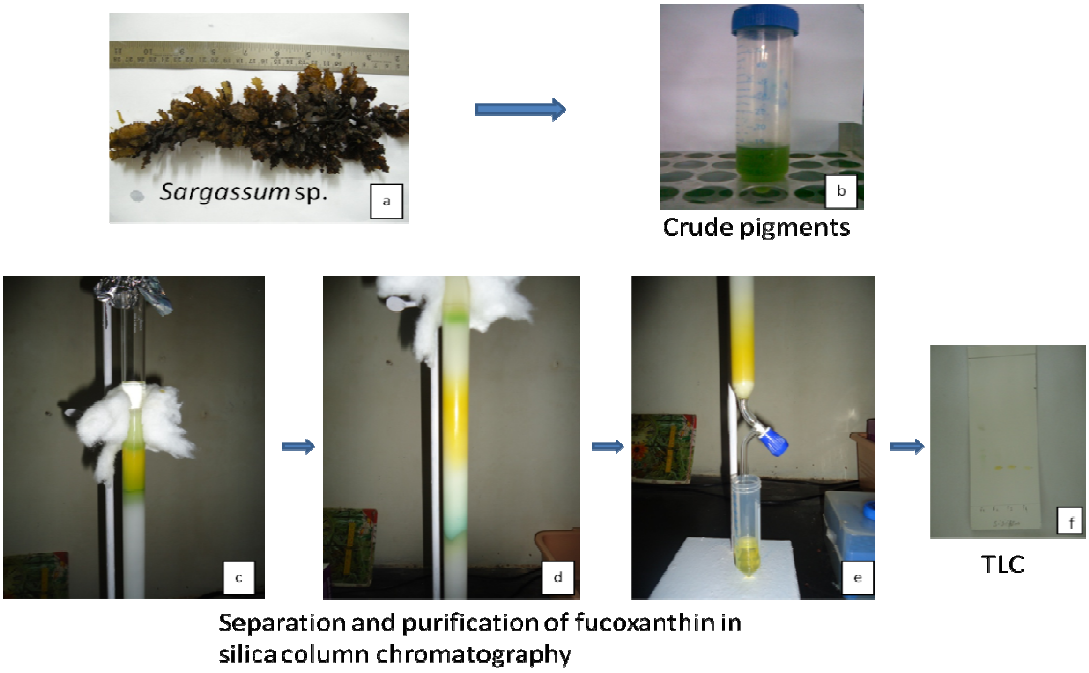
Table 2. Rf values of extracted pigments in TLC plate

Seaweeds	90%acetone	90%ethanol	100%acetone	Fucoxanthin
Sargassum ilicifolium	0.08	0.1	0.21	0.21
	0.23	0.26	0.35	
	0.35	0.4		
		0.6		
Sargassum longifolium	0.01	0.083		
	0.35	0.26		
		0.41		
		0.58		
Sagassum wightii	0.06		0.36	
	0.13			
	0.26			
	0.35			
Padina gymnospora	0.08	0.26	0.21	
	0.23	0.28	0.35	
	0.35	0.4		
Turbinaria ornata	0.1	0.28	0.21	
	0.26		0.35	

Antioxidants such as ascorbic acid are molecules capable of slowing down and preventing the oxidation of other molecules [15]. Fucoxanthin is a major carotenoid in brown algae and it has high anti-oxidation property [16-17]. The results of the studies conducted on this alga clearly indicate that fucoxanthin isolated from *Sargassum* sp. possesses prominent antioxidant activity against H_2O_2- mediated cell damage and which might probably be a

potential therapeutic agent for treating or preventing several diseases implicated with oxidative stress [18]. Cho et al. [19] suggested that the strong antioxidant activity of the extracts from *Enteromorpha prolifera* is because of a chlorophyll compound, pheophorbide 'a', rather than phenolic compounds.

Fig 1. Overview of fucoxanthin separation and purification from brown seaweed



a- *Sargassum wightii, b- Crude extract of pigments, c-e- Separation and purification of Fucoxanthin, f- Checking Fucoxanthin purity by TLC.*

Table 3. Anti-oxidant activity of different seaweed extracts

S.No	Seaweeds	% DPPH anti-oxidant activity
	90% acetone	
1.	*Sargassum wightii*	41.33±3.78^{b}
2.	*Sargassum ilicifolium*	29±1.0^{a}
3.	*Sargassum longifolium*	57.67±6.65^{c}
4.	*Padina sp.*	24.67±1.52^{a}
5.	*Turbinaria sp.*	43±3.46^{b}
	90% ethanol	
1.	*Sargassum wightii*	47±1.0^{c}
2.	*Sargassum ilicifolium*	43.33±2.08^{bc}
3.	*Sargassum longifolium*	43±2.0^{b}
4.	*Padina sp.*	28±1.0^{a}
5.	*Turbinaria sp.*	58.33±0.57^{d}
	100% acetone	
1.	*Sargassum wightii*	34±1.73^{b}
2.	*Sargassum ilicifolium*	34±2.64^{b}
3.	*Sargassum longifolium*	34.67±3.21^{b}
4.	*Padina sp.*	23±2.64^{a}
5.	*Turbinaria sp.*	38.33±1.52^{b}

All the values are mean±SD of triplicates. Significant differences ($p<0.05$) with different solvent extract in different seaweeds are indicated by different superscript lower letters

Fig 2. Scan result of yellow fractions from *S.ilicifolium*

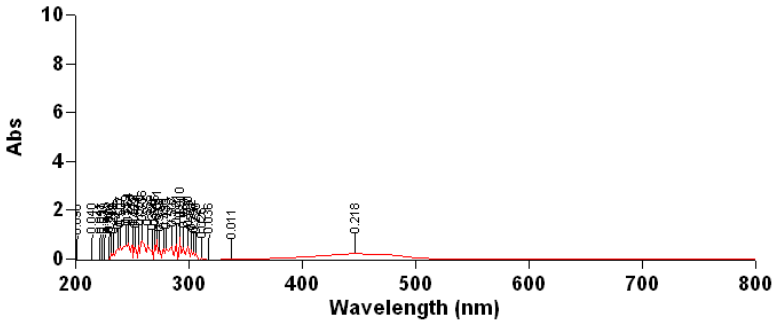
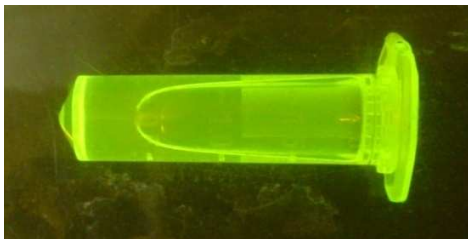


Table 4. Anti-oxidant activity of purified fucoxanthin

S.No	Seaweeds	% DPPH anti-oxidant activity
	90% acetone	
1.	*Sargassum wightii*	ND
2.	*Sargassum ilicifolium*	33.33±1.15
3.	*Sargassum longifolium*	ND
4.	*Padina sp.*	37±1.00
5.	*Turbinaria sp.*	ND

ND- Not detected

Fig 3. Fluorescent property of fucoxanthin under UV light



CONCLUSION

Research on bio-active compounds of seaweeds including pigments has increased in recent years. Antioxidant activity is one of the most studied, due to the interest of pigment compounds both as preservatives and protectors against oxidation in food and cosmetics and also due to their health implications, mainly in relation to their potential as functional ingredients. Brown seaweeds have higher antioxidant potential in comparison with red and green seaweeds and contain compounds not found in terrestrial sources. In-vitro antioxidant chemical methods, used as a first approach to evaluate potential agents that prevent lipid oxidation in foods, confirm that the crude extracts, fractions and pure components of brown seaweeds are comparatively similar or superior to synthetic antioxidants. The carotenoid, fucoxanthin present in brown seaweeds with its strong antioxidant activity has wide applications in various nutraceutical and pharmaceutical arenas as a commercially important bio-active compound [29-31]. Fucoxanthin also has its own fluorescent property (green colour) and can be used as a fluorescent marker in various diagnostic purposes in future. The spent biomass which is generated after the pigment extraction can be utilized in a better way in phycocolloid extraction and as a compost material in crop improvement.

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