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Phytochemical analysis and antioxidant activity of seaweed extracts

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Abstract
In the present study, phytochemical composition and *in vitro* antioxidant properties of ethanolic extracts of four seaweeds, *Gracilaria tenuistipitata*, *Padina gymnospora*, *Padina tetrastromatica* and *Stoechospermum marginatum* were investigated. The total phenol, flavonoid, carbohydrate, protein, sulphate and uronic acid contents of the ethanolic extracts of seaweeds were determined by standard methods. The antioxidant properties of the extracts were evaluated by DPPH and FRAP assays. The total phenol, flavonoid and carbohydrate contents were found to be significantly higher in *S. marginatum* extract. The sulfate content was found to be higher in the *G. tenuispitata* while the protein content was found to be highest in *P. tetrastromatica*. All the seaweed extracts tested were found to have antioxidant activities and it was found to be highest in *S. marginatum* extract. The results indicated that *S. marginatum* extract contained higher polyphenolic compounds with high antioxidant property and has a potential for developing natural antioxidants and could be lead for development of bioactive molecule.

Keywords: seaweed, antioxidant, free radicals, macroalgae, phytochemical, DPPH

Introduction

Reactive oxygen species (ROS) which includes free radicals such as superoxide anion radical, hydroxyl radical, hydrogen peroxide, singlet oxygen, nitric oxide radical and lipid peroxides are produced during normal cellular physiological or biochemical processes (Singh *et al*, 2015 and Jayanthi and Lalitha, 2011) [18, 7]. They induce oxidative stress causing cellular damage and play a central role in the pathogenesis of various diseases conditions including aging, inflammation, carcinogenesis, atherosclerosis, cardiovascular diseases, rheumatoid arthritis and neurodegenerative diseases (Sasikumar and Kalaisezhiyan, 2014) [17]. Antioxidants protects the cells from the damage caused by oxidative stress either by preventing formation of free radicals or by scavenging free radicals and thus progression of oxidative stress induced diseases can be prevented by supplementing with natural antioxidants.

Marine floras including microflora, microalgae, macroalgae, and flowering plants have been used for medicinal purposes in India, China, the Near East and Europe since ancient times (Boopathi and Kathiresan, 2010) [2]. Marine alagae, commonly called as seaweeds have gained attention in the recent years and research is focused in search of phytochemicals from marine algae owing to their potent antioxidant, antibacterial, antiviral, antifungal, anti-inflammatory and anticancer properties. The phytochemicals having strong antioxidant activities have been identified from marine macroalgae (Ponnan *et al,* 2017) [14] and therefore seaweeds represents one of the important sources of natural antioxidants. Hence, the present study was aimed at evaluation of phytochemical constituents and antioxidant activities of four seaweeds collected from the coastal areas of Tamil Nadu, India.

Materials and Methods

Collection of seaweeds and preparation of extract

Three seaweeds namely, *Stoechospermum marginatum*, *Padina gymnospora* and *Padina tetrastromatica* were collected from the Gulf of Mannar Region of Madapam Coast and *Gracilaria tenuistipitata* was collected from Muttukadu Lagoon, Tamilnadu, South-East Coast of India. The seaweeds were authenticated by the botanist of Botanical Survey of India, India.

The sea weeds were washed thoroughly, air dried and powdered. The ethanolic extracts of seaweeds were prepared by continuous hot percolation at 55 °C in soxhlet apparatus. The extracts were then vacuum concentrated, air dried and stored at 4 °C. The yield of the ethanolic extracts of *G. tenuispitata*, *S. marginatum*, *P. gymnospora* and *P. tetrastromatica* were 7.24%, 8.76%, 2.13% and 2.78%, respectively.

Qualitative phytochemical screening
The extracts were screened qualitatively for the presence of phytochemicals as per the methods (Khan *et al,* 2011 and Deyab *et al*, 2016) [9, 4].

Estimation of Total Phenolic content
The total phenolic content of the extracts was determined by the Folin-Ciocalteau method (Maurya and Singh, 2010) [12]. To 0.5 ml of seaweed extract (1 mg/ml), 2.0 ml of 7.5% sodium carbonate and 2.5 ml Folin-Ciocalteau reagent were added and the absorbance was measured after 30 minutes at 760 nm. The concentration of total phenolics was calculated using gallic acid standard curve and expressed as mg of Gallic acid equivalents (GAE) / gram of extract.

Estimation of total flavonoid content
The total flavonoid content of the extracts were determined by aluminium chloride method (Kamtekar *et al*, 2014) [8]. To 0.5 ml of seaweed extract, 2.0 ml of distilled water and 0.15 ml of 5% sodium nitrite were added and incubated for 5 minutes. To this, 0.15 ml of 10% aluminium chloride was added. After 6 minutes, 1 ml of 1 M NaOH was added. The volume was made up to 5 ml using distilled water, vortexed and incubated for 15 minutes. The development of orange yellowish colour was measured at 510 nm. The concentration of total flavonoid content was calculated using catechin standard curve and expressed as mg of Catechin / 100 gram of extract.

Estimation of carbohydrate content
The total soluble carbohydrate content of the extracts was determined by anthrone method (Jawsir *et al*, 2014) [6]. To 1 ml of seaweed extract, 5 ml of 2.5 N HCl was added and kept in boiling water bath for 3 hours. After cooling, the extracts were neutralized with sodium carbonate, the volume was made up to 10 ml with distilled water and centrifuged at 5000 rpm for 15 min. To 1 ml of supernatant, 4 ml of anthrone reagent was added and kept in boiling water bath for 8 minutes. The absorbance was measured at 490 nm. The carbohydrate content was calculated from calibration curve obtained using D-glucose as a standard. The results were expressed as g /100 g extract or%.

Estimation of total protein content
The protein content of the extract was determined by Lowry method (Lowry *et al*, 1957) [11]. To 1.0 ml of extract, 5.0 ml of alkaline copper reagent was added and incubated at room temperature for 30 minutes. To this, 0.5 ml of Folin-Ciocaltaeu reagent was added and the absorbance was measured at 660 nm after 10 minutes of incubation. The protein content was calculated from the calibration curve obtained using Bovine Serum albumin as a standard. The results were expressed as g /100 g extract or%.

Estimation of Sulphate Content
The total sulfate content of the extract was determined by Barium Chloride gelatin method (Jaswir *et al*, 2014 and Dodgson and Lloyd, 1961) [5, 6]. To 0.5 ml of extract (1 mg/ml), 0.75 ml of distilled water was added and hydrolyzed with 5 ml of 1 N HCl at 105 °C for 5 h. The solution was allowed to cool and 200 µl of which is mixed with 3.8 ml of 3% trichloroacetic acid and 1 ml of barium chloride gelatin solution. After 15 minutes, the absorbance was measured at 360 nm. The total sulphate content of the extract was determined using using K_2SO_4 as a standard and expressed as g /100 g extract or%.

Estimation of Uronic acid content
The uronic acid content of the extract was estimated by carbazole-sulfuric acid method (Navya and Khora, 2017) [13] using D-glucuronic acid as a standard. To 0.5 ml of extract and standard, 3.0 ml of sodium tetraborate reagent in concentration sulphuric acid was added and heated at 100 °C for 10 min. After cooling, 100 µl of carbazole reagent in absolute ethanol was added and mixed well. The solutions were reheated at 100 °C for 5 min and then cooled rapidly. The absorbance was read at 525 nm using UV visible spectrophotometer. The total uronic acid content of the extract was expressed as g /100 g extract or%.

Evaluation of Antioxidant Activity
DPPH radical scavenging assay
The free radical scavenging activity of seaweed extracts were measured using DPPH (2,2-diphenyl-1-picryl hydrazyl) (Singh *et al*, 2015) [18]. One mL of various concentrations of extract was added to one mL of 0.1 mM solution DPPH in methanol. The solutions were mixed and incubated in dark for 30 minutes at room temperature. After 30 min, absorbance was measured at 517 nm using UV-vis spectrophotometer. The free radical scavenging activity of the extracts was expressed as the effective concentration required for 50% of the DPPH radical reduction (IC_{50}) obtained from the plot of graph of scavenging activity against the concentration of the extract.

Ferric Reducing antioxidant power (FRAP) Assay
The total antioxidant activity of the sample was determined using the ferric reducing antioxidant power (FRAP) assay (Singh *et al*, 2015) [18]. The FRAP reagent was prepared fresh by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6-tripyridyl triazine) and 20 mM FeCl3 in the ratio of 10:1:1. To 0.5 ml of seaweed extract, 0.5 ml of water and 2.0 ml of FRAP reagent were added, vortexed and incubated at 40 °C for 30 minutes. The absorbance was read to 593 nm. The antioxidant capacity was expressed in FRAP units, mmol Fe^{2+} per gram of extract and it was calculated by linear regression curve of $FeSO_4$ standard.

Statistical analysis
The results were expressed as Mean ± S.D. and the data were analyzed by One Way analysis of variance followed Duncan's post hoc analysis using IBM SPSS version 2.0 for windows.

Results
Phytochemical analysis
The results of the phytochemical screening of seaweed extracts were given in table 1. The phytochemicals present in all the seaweed extracts were phenol, flavonoid, carbohydrate and reducing sugars, protein and aminoacids, saponin and steroid. Tannins, terpenoids, saponin and glycosides showed varied distribution in different seaweed extracts.

Table 1: Qualitative phytochemical screening of seaweed extracts

Parameter	*G. tenuistipitata*	*P. gymnospora*	*P. tetrastromatica*	*S. marginatum*
Alkaloids	-	-	-	+
Tannins	+	+	-	+
Phenols	+	+	+	+
Terpenoids	-	+	-	+
Flavonoids	+	+	+	+
Saponins	+	+	-	-
Glycosides	+	-	-	+
Carbohydrates and Reducing sugars	+	+	+	+
Proteins and Amino acids	+	+	+	+
Steroids	+	+	+	+
Quinones	-	-	-	-
Anthocyanin and betacyanin	-	-	-	-
Coumarins	-	-	-	-

+ - Present; - Absent

Total Phenol and flavonoid content

Total phenol and flavonoid content of the seaweed extracts were given in table 2. The total phenolic content of seaweed extracts showed statistically significant difference (*P*<0.01). The total phenolic content was signicantly higher in the ethanolic extract of *S. marginatum* (89.18 mg GAE/g extract) followed by *G. tenuispitata* (57.20 mg GAE/g extract), *P. tetrastromatica* (46.87 mg GAE/g extract) and *P. gymnospora* (31.00 mg GAE/g extract).

Flavonoids was found to be significantly higher in *S. marginatum* extract (11.92 mg catechin equivalent/100 g extract) followed by the extracts of *P. tetrastromatica* (10.00 mg catechin equivalent/100 g extract), *G. tenuispitata* (9.28 mg catechin equivalent/100 g extract) and P. *gymnospora.* (6.03 mg catechin equivalent/100 g extract).

Table 2: Quantitative analysis of phytochemicals in seaweed extracts

Name of the Seaweed	Total Phenolic Content (mg GAE/g extract)	Total Flavonoid Content (mg Catechin Equivalent/100 g extract)
G. tenuistipitata	$57.20 \pm 0.59^{c**}$	$9.28 \pm 0.27^{b**}$
P. gymnospora	$31.00 \pm 0.26^{a**}$	$6.03 \pm 0.35^{a**}$
P. tetrastromatica	$46.87 \pm 0.21^{b**}$	$10.00 \pm 0.23^{c**}$
S. marginatum	$89.18 \pm 0.17^{d**}$	$11.92 \pm 0.90^{d**}$

Values are expressed as mean ± S.D. (n=6). ** Significant at *P*<0.01. Means bearing similar superscript do not differ significantly

Biochemical composition

The carbohydrate, protein, uronic acid and sulphate content of the seaweed extracts were given in table 3**.** The carbohydrate content was higher in the *S. marginatum* extract (65.64%) and it differs significantly (*P*<0.01) from other seaweed extracts. The protein content of seaweed extracts ranged from 8.08 to 11.08%. It was found to be higher in the *P. tetrastromatica* extract and it differs significantly from *P. gymnospora* and *S. marginatum.* The protein content of *G. tenuistipitata* and *P. tetrastromatica* did not differ significantly. The *P. gymonospora* extract had low concentration of protein (8.08%) and it differs significantly from the other seaweeds.

The sulfate content of seaweed extracts ranged from 4.21% to 7.66%. Statistical difference (*P*<0.01) was observed between the seaweed extracts. The sulfate content was found to be higher in the *G. tenuispitata* extract (7.66%) followed by the extracts of *S. marginatum* (5.87%), *P. tetrastromatica* (5.55%) and *P. gymnospora* (4.21%).

The uronic acid content was found to be highest in *S. marginatum* extract (8.16%) and it differs significantly from the other seaweed extracts. The uronic acid content of *G. tenuispitata* (5.55%) differs significantly from the other seaweed extracts. The uronic acid content of *P. tetrastromatica* (5.52%) did not differ significantly from *P. gymonospora* (5.50%) and *G. tenuispitata* (5.55%).

Table 3: Biochemical composition of seaweed extracts

Name of the Seaweed	Total carbohydrate (g/100g or%)	Total Protein (g/100g or%)	Sulfate (g/100g or%)	Uronic acid (g/100g or%)
G. tenuistipitata	$44.74 \pm 0.31^{b**}$	$10.92 \pm 0.54^{c**}$	$7.66 \pm 0.14^{d**}$	$5.55 \pm 0.02^{b**}$
P. gymnospora	$56.50 \pm 0.68^{c**}$	$8.08 \pm 0.39^{a**}$	$4.21 \pm 0.27^{a**}$	$5.50 \pm 0.01^{a**}$
P. tetrastromatica	$33.74 \pm 0.80^{a**}$	$11.08 \pm 0.19^{c**}$	$5.55 \pm 0.13^{b**}$	$5.52 \pm 0.02^{ab**}$
S. marginatum	$65.64 \pm 0.45^{d**}$	$10.44 \pm 0.04^{b**}$	$5.87 \pm 0.11^{c**}$	$8.16 \pm 0.05^{c**}$

Values are expressed as mean ± S.D. (n=6). ** Significant at *P*<0.01. Means bearing similar superscript do not differ significantly

***In vitro* antioxidant activity**

In this study the antioxidant activity of the extract were analyzed by two methods, DPPH method and FRAP assay and the results were presented in table 4 and fig 1.

In DPPH method, S. *marginatum* demonstrated significantly higher antioxidant activity with an IC_{50} of 0.402 ± 0.033 mg/mL compared to other seaweed extracts. This was followed by the *G. tenuistipitata* (IC_{50} 0.510 ± 0.019 mg/mL), *P. tetrastromatica* (IC_{50} 0.773 ± 0.013 mg/mL) and *P. gymnospora* (IC_{50} 0.747 ± 0.013 mg/mL). No significant difference was observed between the IC_{50} values of *P. tetrastromatica* and *P. gymnospora*.

With the FRAP method, *S. marginatum* showed highest antioxidant activity (112.66 ± 0.70 mmol Fe^{2+}/g) and it was statistically significant (*P*<0.01) from the other seaweed extracts. This was followed by *G. tenuistipitata* (74.50 ± 0.63

mmol Fe^{2+}/g) *P. gymnospora* (46.17 mmol Fe^{2+}/g) and *P. tetrastromatica* (43.33 mmol Fe^{2+}/g). Statistically significant difference was observed between the FRAP values of seaweed extracts at (P<0.01).

Table 4: *In vitro* antioxidant activity of the seaweed extracts

Name of the Seaweed	DPPH (IC_{50}, mg/ml) (n=3)	FRAP value (mmol Fe^{2+}/g) (n=6)
G. tenuispitata	$0.510 \pm 0.019^{b**}$	$74.50 \pm 0.63^{c**}$
P. gymnospora	$0.773 \pm 0.013^{c**}$	$46.17 \pm 0.88^{b**}$
P. tetrastromatica	$0.747 \pm 0.013^{c**}$	$43.33 \pm 0.41^{a**}$
S. marginatum	$0.402 \pm 0.033^{a**}$	$112.66 \pm 0.70^{d**}$

Values are expressed as mean ± S.D. ** Significant at P<0.01. Means bearing similar superscript do not differ significantly

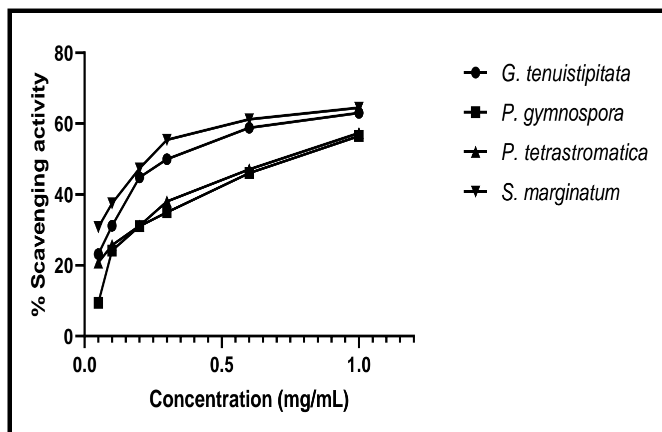

Fig 1: Scavenging activity of the extracts in DPPH assay

Discussion

The phenolic compounds are a large group of phytochemicals which have received considerable attention because of their potent antioxidative properties and protective against cancer and heart diseases (Sasikumar and Kalaisezhiyan, 2014) [17].

In the present investigation, *S. marginatum* extract showed higher concentration of total phenol and total flavonoid content than the other seaweed extracts. Higher phenolic and flavonoid content were reported (Kokilam *et al*, 2013; Tseng *et al*, 2014) [10, 19] in the methanolic extract of *P. tetrastromatica* and in the aqueous extract of *G. tenuistipitata* compared to the results of the present investigation and these variations could be due to the method of extraction and the solvent used. In the present investigation, higher carbohydrate content was recorded in *S. marginatum* (65.64%) followed by *P. gymnospora* (56.50%) and least in *P. tetrastromatica* (33.74%). The carbohydrate content of *Padina* species obtained in the present study was higher than the earlier reports (Ravi and Subramanian, 2017) [15]. The protein content in brown seaweeds is generally lower ranging from 5 to 15% of dry weight of seaweed (Kokilam *et al*, 2013) [10] and it was in agreement with the results of the present study. Various factors including geographical distribution, habitats, maturity, seasons and the environmental conditions, such as water, temperature, salinity, light, and nutrients also affects the composition composition of the seaweeds (Arumugama *et al,* 2017) [1].

Seaweed polysaccharides were reported to have various bioactivities including antitumor, antiviral, antibacterial, antioxidant, antimutagenic activities. The antioxidant activity of seaweed polysaccharides are closely related to their physicochemical properties, such as molecular weight, sulphate content, uronic acid content and polyphenol content (Wang *et al*, 2020) [20].

In the present study, sulphate content was found to be highest in *G. tenuistipitata.* The anticancer activity of fucoidan, a polysaccharide commonly found in seaweeds was significantly influenced by its sulphate content and earlier reports (Chen *et al*, 2004) [3] suggested that increased negative charges caused by high sulphate content in the polysaccharide influences the fucoidan-protein complexes involved in the cell proliferation and suppresses the cell growth. The sulphate content of seaweeds also influence the antioxidant and anticancer activities of the extracts.

Most of the polysaccharides isolated from seaweed are acid complex carbohydrates, composed of uronic acid and the uronic acid affects the physicochemical properties of the polysaccharides including solubility and thereby influences the biological activity of the polysaccharides (Chen *et al*, 2004; Wang *et al* 2016) [3, 20]. Several studies have reported that antioxidant activity of seaweed polysaccharides are highly correlated with the uronic acid content (Chen *et al*, 2004; Zhou *et al*, 2008) [3, 21]. In the present study, uronic acid content was found to be highest in the *S. marginatum* and it contributes to the antioxidant activity activity of the extract.

In the present study, the antioxidant activity of the seaweed extracts was analyzed by two methods, viz., DPPH and FRAPS method. In both assays, the antioxidant activity of ethanolic extract of *S. marginatum* was greater followed by G. *tenuistipita*. The antioxidant activity of the extracts increases with increasing concentration of the extracts and exhibited maximum activity at 1 mg/mL. The crude extracts contain many bioactive compounds and hence the antioxidant capacity of seaweed extracts could be due to its phenol, flavonoid, polysaccharides content present in the extracts. Several studies reported strong positive correlation between antioxidant activity and total phenol content, flavonoid and polysaccharide contents of the extracts (Chen *et al*, 2004; Rebaya *et al*, 2014; Wang *et al* 2016) [3, 16, 20].

Conclusion

In the recent years, search for the natural antioxidants have been increased due to the fact that they can be used in the treatment of chronic disease conditions and as a dietary supplement. The results obtained demonstrated that seaweed extacts have potent antioxidant activity. Among the four seaweeds, ethanolic extact of *S. marginatum* exhibited higher antioxidant activity. The antioxidant activity of marine algae obtained in the present study could be due polyphenols, flavonoids and polysaccharides. The present data suggests that *S. marginatum* and *G. tenuistipitata* can be used as a good source of natural antioxidants and it requires further studies for isolation of bioactive compounds.

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Conflicts

The authors declare that they have no conflicts of interest

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