Research Article

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# EXTRACTION AND ISOLATION OF PHLOROTANNINS FROM BROWN SEAWEED *TURBINARIA ORNATA* (TURNER) *J. AGARDH* AND ITS ANTIOXIDANT ACTIVITY

**Girija K, Hemalatha A, Saranya C, Parthiban C and P Anantharaman***
CAS in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai– 608502, India

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**Abstract:** The crude extract and fractions of *Turbinaria ornata* collected from Mandapam coast were evaluated for their antioxidant potentials. The antioxidant potential was determined using total antioxidant capacity, total phenolic content and DPPH radical scavenging activity. The ethyl acetate fraction obtained from the crude methanolic extract by different solvent extractions exhibited prominent antioxidant activity. The ethyl acetate fraction was further fractionated with column chromatography to yield 36 fractions. All the column purified fractions were subjected to thin layer chromatography analysis and the results produce 8 fractions. The antioxidant activity of crude methanolic extract and sub fractions were evaluated. The F5 fraction exhibited higher total phenolic content and antioxidant properties. On the basis of TLC, UV-Vis and FT-IR spectral analysis F5 fraction was found to contain phenolic compounds especially phlorotannins. To the best of our knowledge, the present study is the first report on the extraction and isolation of phlorotannins and this was responsible for antioxidant activity from brown seaweed *Turbinaria ornata* from India.

**Keywords:** *Turbinaria ornata;* Phlorotannins; UV-Vis Spectrophotometry; FT-IR; TLC.

## INTRODUCTION

The marine life offering enormous resources for novel compounds[1], and it has been classified as the largest remaining reservoir of natural molecules to be evaluated for their bioactivity[2]. In recent years, the seaweeds serve as an important source of bioactive natural substances[3,4]. Phlorotannins, a group of phenolic compounds which are restricted to polymers of phloroglucinol, have been identified from several brown algal families such as Alariaceae, Fucaceae and Sargassaceae[5]. Many studies have shown that phlorotannins are the only phenolic group detected in brown algae[6, 7]. The multifunctional antioxidant activity of polyphenols is highly related to phenol rings which act as electron traps to scavenge peroxy, superoxide-anions and hydroxyl radicals. Phlorotannins from brown algae have up to eight interconnected rings and they are therefore more potent free radical scavenger than other polyphenols derived from terrestrial plants, including green tea catechins, which only have three to four rings[8].

More than 5000 polyphenolics, including over 2000 flavonoids have been identified, and the number is still growing [9] and that are synthesized from carbohydrates via shikimate pathway. Thus, phenolic compounds are ubiquitous in the plant kingdom being found in all fruits and vegetables in virtually all parts of the plant. Recently, phenolics have been considered powerful antioxidants *in vitro* and proved to be more potent antioxidants than Vitamin C and E and carotenoids[10, 11].

A series of polyphenolic compounds such as catechins (e.g. gallocatechin, epicatechin and catechin gallate), flavonols and flavonol glycosides have been identified from methanol extracts of red and brown algae[12-14]. Earlier, reported that phlorotannins in *Sargassum kjellamanianum*[15], *Sargassum siliquastrum* [16] and *Ecklonia stolonifera* [17] have antioxidative activity.

The present work was undertaken to extraction and purification of phlorotannins from brown seaweed *Turbinaria ornata* collected from the Mandapam coast of Tamil Nadu, India and its antioxidant properties. This study is part of a programme on screening of seaweeds for a variety of biological activities, with interesting and potentially useful therapeutic activities.

## MATERIALS AND METHODS

**Sample collection and preparation:**

Live and healthy samples of edible brown seaweed *Turbinaria ornata* were collected from the intertidal region of Mandapam, Gulf of Mannar, Tamilnadu, India (latitude 9°18'N and longitude79°6'E). Collected samples were immediately brought to the laboratory in new plastic bags containing natural sea water to prevent evaporation. Plants were washed thoroughly with tap water to remove extraneous materials and shade dried. Dry plant material was ground in an electric mixer and stored at 4°C until future use.

**Extraction and purification of phenolic compounds:**

Fine powder of dry seaweed (25g) was extracted

***Corresponding Author:**
**Dr. P. Anantharaman Ph.D.,**
Associate Professor,
CAS in Marine Biology,
Faculty of Marine Sciences, Annamalai University,
Parangipettai - 608 502, Tamilnadu, India.

with 450 ml of methanol at room temperature for 24 h. The extraction procedure was repeated thrice and the extract was filtered through Whatmann No. 1 filter paper. The filtrate was concentrated to dryness under reduced pressure using a rotary evaporator (crude extract). This extract was washed with hexane and dichloromethane (three times each), and then extracted with ethyl acetate (three times each). The ethyl acetate phase (crude) was concentrated and stored at 4°C until future use. The ethyl acetate fraction was further fractionated with coloumn chromatography because it showed high antioxidant activity. The column was prepared using 100g of silica (60–200µm mesh size) and the mixture was stirred until it became less viscous. Then the fractions were eluted by starting with 100% hexane, chloroform/methanol in the following ratios: 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, ethyl acetate 0:10, ethyl acetate/ methanol 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9 and 100%

**Thin-Layer Chromatography:**

TLC was performed on a silica gel plate (5 × 20 cm, Kieselgel 60F, 0.25 mm, Merck). An aliquot of each fraction was spotted on the silica gel plate with a solvent system of chloroform/ethanol/acetic acid/water (98:10:2:2 v/v). Then the chromatogram was developed and dried for few minutes. It was visualized under ultraviolet (UV) light wavelength 365nm spots were marked. Then $R_f$ values for each bands were measured.

**Determination of total phenolic content:**

Total phenolic content was estimated as gallic acid equivalents (GAE) according to the Folin-Ciocalteu reagent[18]. The seaweed fraction (0.1 mL) was diluted with deionized water (7.9 mL). Folin-Ciocalteu phenol reagent (0.5 mL) was added, and the contents were mixed thoroughly. After 1 min, 1.5 mL of 20% sodium carbonate solution was added, and the mixture was mixed thoroughly. The mixture was allowed to stand for 1 h. After 1 h of incubation at 37°C, the absorbance of the blue color produced was measured at 750nm, using the PerkinElmer Lamda 25 UV-VIS Spectrophotometer. Phenolic content was expressed in milligrams per gram of dry weight (seaweed fraction) based on a standard curve of gallic acid (GA), which was expressed as milligrams per gram of gallic acid equivalent (GAE).

**Total antioxidant activity:**

Total antioxidant activities of the all the fractions were determined according to the method of Prieto *et al.*,[19]. Briefly, 0.3ml of sample was mixed with 3.0ml reagent solution (0.6 M sulfuric acid, 28mM sodium phosphate and 4 mM ammonium molybdate). Reaction mixture was incubated at 95°C for 90 min under water bath. Absorbance of all the sample mixture was measured at 695nm. Total antioxidant activity is expressed as the number of equivalence of ascorbic acid. A calibration curve of ascorbic acid was prepared and the total antioxidant activity was standardized against ascorbic acid and was expressed as mg ascorbic acid equivalents per gram of sample on a dry weight (DW) basis.

**DPPH radical-scavenging activity:**

The scavenging effects of samples for DPPH radical were monitored according to the method of Yen and Chen[20]. Briefly, a 2.0 ml of aliquot of test sample was added to 2.0 ml of 0.16 mM DPPH methanolic solution. The mixture was vortexed for 1 min and then left to stand at room temperature for 30 min in the dark, and its absorbance was read at 517 nm. The ability to scavenge the DPPH radical was calculated using the following equation.

$$\text{Scavenging effect (\%)} = [(A_{sample} - A_{sample\ blank}) / A_{control}]X\ 100$$

**Ultraviolet and Visible (UV-Vis) Spectrophotometry:**

UV-Visible spectra for active fractions were recorded on a PerkinElmer Lambda 25 UV VIS spectrophotometer equipped with 1.0 cm quartz cells. The width of excitation slits were set to 1.0 nm. The spectra collected with subsequent scanning spectra from 450 to 200nm at 1.0 nm increments.

**Fourier Transform- Infrared (FT-IR) Analysis:**

Spectra of F5 fraction was determined using an FT-IR spectrophotometer (Shimadzu, Japan.) with KBr pellets in the range 4000–400 $cm^{-1}$.

**Statistical analysis:**

Data were analyzed using the SPSS package for Windows (Version 10). Values were expressed as mean ± standard Deviation (SD).

## RESULTS

**Extraction and Fractionation:**

About 5.452g of yield was obtained from methanolic extract of *Turbinaria ornata*. The combined methanolic extract was further fractionated and retained 0.397 g of ethyl acetate fraction, 0.216g of dichloromethane fraction and 0.124g of hexane fraction (Table 1). The higher antioxidant ethyl acetate fraction was further fractionated in a silica gel column chromatography and 36 fractions were collected. Thin layer chromatography analysis of these fractions and the analytical results produce eight sub fractions namely F1, F2, F3, F4, F5, F6, F7 and F8. Table 1 shows the Rf values of spots from the TLC chromatograms of the eight sub fractions.

**Total phenolic content:**

In the present study, the total phenolic content of the various fractions was determined using Folin-

Ciocalteu method and the results are presented in Table.1. The Total phenolic content of all the crude extract and all the subfractions were expressed as mg Gallic acid equivalent (GAE) per gram of the extract or fraction. The F5 subfraction having the highest amount of phenolic contents (1.13mg/g) ranging from 0.02±0.015 to 1.13±0.02. They varied significantly and decreased in the following order: F5 > ethyl acetate > F2 > F3 > methanol > F1 > F7 > F8 > dichloromethane > F6 > F4. The presence of antioxidant phlorotannins in subfraction F5 of *T. ornata* could cause it to have higher phenolic content compared to other fractions.

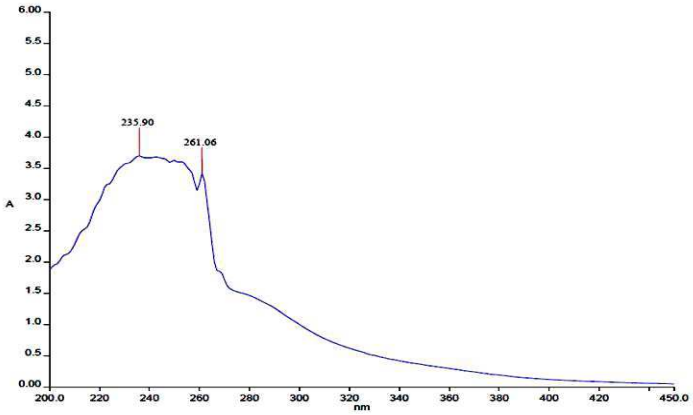


**Fig.1:** UV- Visible spectral identification of column purified fraction F5.

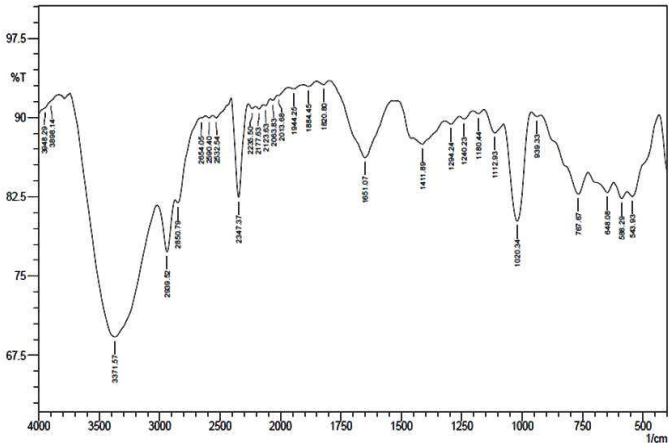


**Fig. 2:** FT-IR spectral identification of column purified fraction F5.

**Antioxidant properties:**

In the present study the antioxidant activity of methanol extract-1.24±0.02, fractions of DCM-1.18±0.03 and ethyl acetate-1.41±0.02 and sub fractions (F1, F2, F3, F4, F5, F6, F7 and F8) were analyzed and results are presented in Table.1. Higher activity of (1.44±0.01) mg ascorbic acid/g extract was observed in F5 fraction. The total antioxidant activities were significantly different among the fractions. DPPH radical scavenging activities (%) of crude methanolic extract and sub fractions are presented in Table.1 In the present study, higher DPPH radical scavenging activities was recorded in F1 (74.66%) followed by ethyl acetate (71.66%) and F2 (63.33%). The scavenging effect of standards on the DPPH radical decreased in the order: BHT >ascorbic acid > gallic acid, which was 75.66%, 69.27% and 57.28% respectively.

**Table 1:** Extraction yield of crude extract and fractions (% dry weight), Rf values of TLC spots and antioxidant activities.

| Fractions | Extraction Yield (g or mg) | Rf values | Total Phenolic content(mg) | Total Antioxidant Activity(mg) | DPPH Radical Scavenging Assay (%) |
|---|---|---|---|---|---|
| Methanol | 5.452 g | 0.89 | 1.24±0.01 | 1.24±0.02 | 56.66% |
| DCM | 216mg | 0.74 | 0.58±0.02 | 1.18±0.03 | 47.66% |
| Ethyl acetate | 397 mg | 0.88 | 1.05± 0.01 | 1.41±0.02 | 71.66% |
| F1 | 23.1 mg | 0.74 | 0.66±0.02 | 0.37±0.02 | 74.66% |
| F2 | 25.4 mg | 0.90 | 0.85±0.03 | 1.35±0.03 | 63.33% |
| F3 | 13.1 mg | 0.88 | 0.77±0.01 | 1.23±0.02 | 55% |
| F4 | 1.3 mg | 0.82 | 0.02±0.01 | 1.36±0.01 | 62% |
| F5 | 3.3 mg | 0.87 | 1.13±0.02 | 1.44±0.01 | 61% |
| F6 | 9.2 mg | 0.76 | 0.03±0.005 | 1.17±0.05 | 58.33% |
| F7 | 10.2 mg | 0.91 | 0.58±0.02 | 1.42±0.02 | 47% |
| F8 | 5.3 mg | 0.38 | 0.46±0.01 | 0.24±0.02 | 43% |

**Identification of phlorotannins:**

The UV-Visible spectrum for active column purified fraction F5 was recorded in 235.90 and 261.06 it's indicates the presence of phenolic compounds especially phlorotannins. The UV spectral properties are given in Fig. 1. The FT-IR spectral analysis of F5 fraction of *T. ornata* revealed that the spectral range of obtained functional group ranged between 400 and 4000 $cm^{-1}$ shown in Fig. 2. From the results, it was observed that the peaks 543.93, 588.29 and 648.08 $cm^{-1}$ may be due to C-Br bond. Followed by peak signals recorded in 939.33 $cm^{-1}$ may be due to nitrogen grouping (N-O). The peaks 1112.93, 1180.44, 1240.23 $cm^{-1}$ and sharp peak observed at 1020.34 $cm^{-1}$ may be due to the presence of carbon, oxygen (C-O) bond. The peak 1294.24 to 2230.50 $cm^{-1}$ may possible presence of carbon (C=C) double bond. Sharp peak observed in 2347.37 represents the possible presence of nitrogen, hydrogen atoms (N-H) bond. The vibration stretch recorded at 2850.79 and 2939.52 $cm^{-1}$ represents presence of (C-H) bond. Finally a broad band at 3371.57 $cm^{-1}$ may be phenol hydroxyl group ($OH^{-}$).

## DISCUSSION

In this study, the antioxidant activity of crude methanolic extract and sub fractions were evaluated. Phenolics are secondary metabolites that play a role in the maintenance of the human body[21]. Earlier reports revealed that seaweed extracts, especially polyphenols, have antioxidant activity[16, 22, 23]. The polyphenols of seaweeds such phlorotannins[24], which are bi-polar in nature, and mostly found in brown seaweeds[25], such as in *T. ornata* function as anti-oxidative components due to the presence of multiple

phenolic groups. In the phosphomolybdenum method, molybdenum VI ($Mo^{6+}$) is reduced to form a green phosphate/$Mo^{5+}$ complex. Ye *et al.,*[26] reported higher antioxidant activity (30.50µmol $FeSo_4$/mg) in the ethanol extract of the brown seaweed *Sargassum pallidum*. 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 the simultaneous change of the violet color to pale yellow[27]. The various phlorotannins can be observed that able to overcome the sensitivity problem inherent in the detection of endogenous radicals in biological systems. Furthermore, several phlorotannins that purified from *Ecklonia cava* are responsible for antioxidant activities and shown protective effects against hydrogen peroxide-induced cell damage [28]. In addition, eckol, phlorofucofuroeckol A, dieckol, and 8, 80-bieckol have shown a potent inhibition of phospholipid peroxidation at 1µM in a liposome system[29] and these phlorotannins have significant radical scavenging activities against superoxide and DPPH radicals effectively compare with ascorbic acid and α-tocopherol.

In this study, the antioxidant activity of crude methanolic extract and sub fractions were evaluated. The F5 fraction was exhibited higher total phenol content and antioxidant properties. The present findings suggest that F5 fraction contain phlorotannins, based on the UV-Vis and FT-IR spectral analysis. The present findings appear phlorotannin (Phenol) compound responsible for the high antioxidant activities in this seaweed (*T. ornata*). Phenolic compounds (phlorotannin) could elevate the value of seaweeds as functional ingredients in pharmaceuticals or functional foods.

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