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ASSESSMENT OF PHYTOCHEMICAL CONSTITUENTS AND INVITRO ANTIOXIDANT PROPERTIES OF FLAVONOID RICH FRACTIONS OF PADINA TETRASTROMATICA: A MARINE BROWN SEA WEED.

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Abstract

Brown seaweed *Padina tetrastromatica* is chief constituents of nutraceutical food and are regularly consumed by the Asian countries due to their large health benefits. Since earlier studies had been proved that *P. tetrastromatica* have pharmacological activities like antibacterial, antimicrobial, antifungal, antidiabetics, and cytotoxic activity. The present study was aimed to screen antioxidant activity of flavonoid rich fraction of *P. tetrastromatica* (FRPD). Extraction was done by maceration for 48 hours using methanol by modern sophisticated instruments. These extract was made into flavonoid rich fraction by partitioning with petroleum ether (60-80^{0}C) and n- hexane. The flavonoid rich extract was confirmed by phytochemical screening and the flavonoid contents are quantified by total phenolic and flavonoid content estimation. The total flavonoid and total phenolic content of the obtained fraction was found to be 174.71mg of quercetin equivalent, 246.28 mg GAE/g respectively. Antioxidant efficacies are also screened by DPPH and H_2O_2 radical scavenging assay and the total antioxidant capacity was also performed. The IC_{50} value of the antioxidant activity was found to be 121.92 µg/ml, 254.73 µg/ml, 190.99 µg/ml respectively. This preliminary investigation might be useful to screen the plant in molecular levels.

Keywords: *Padina tetrastromatica*, Total phenol and flavonoid content, Antioxidant assay, DPPH, H_2O_2 radical scavenging assay, Flavonoid rich fraction.

1. Introduction

Marine species are increasingly viewed as a major source of food and health ingredients. Seaweeds, also known as marine macroalgae, are fast-growing, highly efficient photosynthetic species that have a simple life cycle. Seaweeds are classified into three groups, based on their color: Rhodophyceae (red seaweed), Chlorophyceae (green seaweed), and Phaeophyceae (brown seaweed). The color of seaweeds is attributed to pigments such as phycobilins for red, chlorophyll for green, and fucoxanthin for brown algae [1]. *P. tetrastromatica* are olive green or dark green in colour, dichotomously or irregularly branched; margins smooth or slightly undulate, apex enrolled, surface smooth; sporangia present in double sporangial lines, sporangial lines and hair lines alternate to each other; attached with the help of a small, compact, holdfast, 0.5 – 1.5 cm broad and 0.7 – 2.0 cm long; Thallus modified into many lobes, upto three fourth of the thallus, many clefts present on the thallus; thalli 7-15 cm long, 7-12 cm broad at the apex, 10 – 12 cm broad at the middle and 7.5 – 10.0 cm broad at the base Hence seaweeds are utilized for its biological activities for food materials and biomedical applications. Antioxidant, dietary fiber, essential fatty acid, vitamins and minerals are the rich source of bioactive compounds obtained from seaweeds [2].

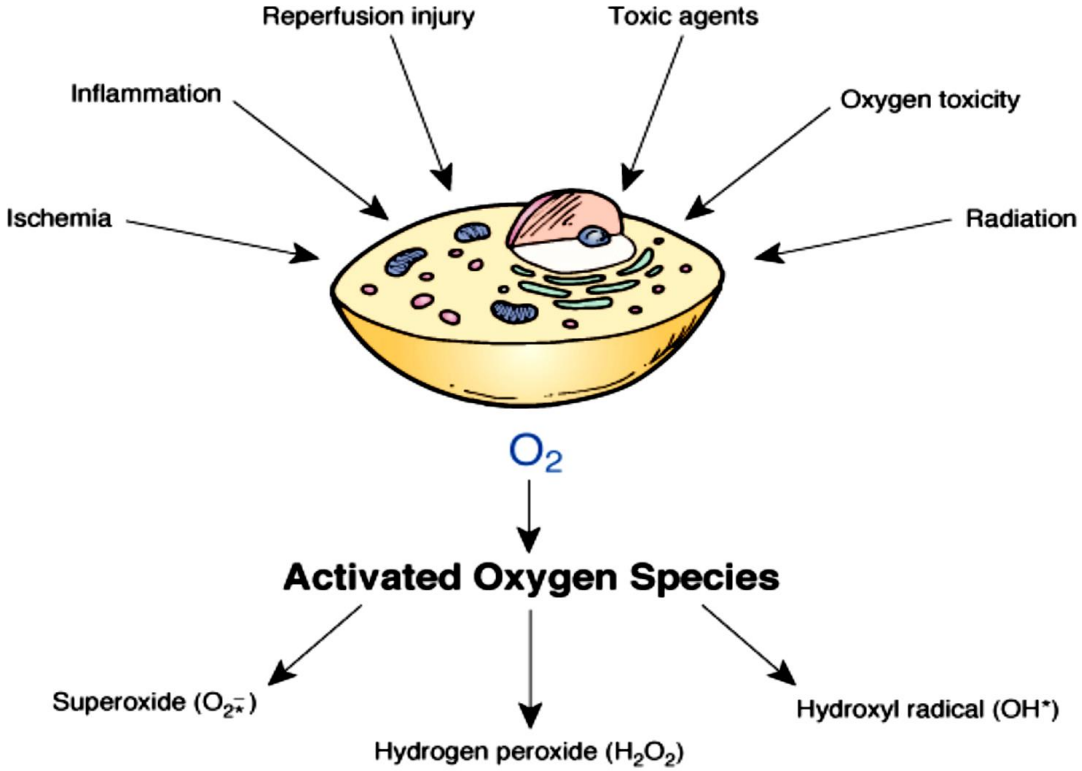


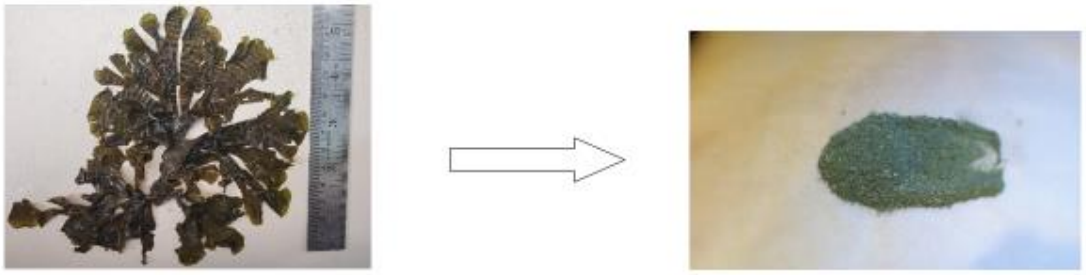
Figure.1 Free radicals liberation during tissue pathogenesis

Oxidative stress is an imbalance between the systemic manifestation of reactive oxygen species (ROS) and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Reactive oxygen species are produced by living organisms as a result of normal cellular metabolism [3]. At low to moderate concentrations they function in physiological cell processes, but at high concentrations, they produce adverse modifications to cell components such as lipids, proteins and DNA [4, 5, 6]. Oxidative stress from oxidative metabolism cause base damage, as well as strand breaks in DNA. Base damage is mostly indirect and caused by reactive oxygen species generated, examples O_2^- (superoxide radical), OH (hydroxyl radical) and H_2O_2 (hydrogen peroxide) (figure.1). Some reactive oxidative species act as cellular messengers in redox signaling. Thus, oxidative stress can cause disruptions in normal mechanisms of cellular signaling. In humans, oxidative stress is thought to be involved in the development of cancer and other chronic diseases. Flavonoids are major polyphenols present abundantly found in brown seaweed when compared to other red seaweed and green seaweed [7]. In Padina tetrastromatica, the earlier study have shown that seaweed has antibacterial, antimicrobial, antifungal, antidiabetics, and cytotoxic activity, but no one has revealed the potency of the flavonoid present in it. Thus present study was aimed to quantify flavonoid content and revealing its antioxidant activity by DPPH assay, H2O2 radical scavenging assay.

2. Materials and methods

2.1. Sample collection and preparation

P. tetrastromatica was collected from the coastal region of Gulf of Mannar region, Tamilnadu, India at low tide areas. The collected seaweed has been washed with tap water in order to remove dirt and other adhering matter in the seaweed and they were shadow dried and grounded in an electric blender (figure.2) until the required fine quality is obtained. These powdered samples are stored in an airtight container for future use.



Fresh sample of Padina tetrastromatica Powder form

Figure.2 Fresh sample and powder of *Padina tetrastromatica*

2.2. Reagents and chemicals

Methanol, Petroleum ether, n- hexane was obtained from Merck, India. Lead acetate, NaOH, Gallic acid, Aluminium chloride, and Potassium acetate was obtained from HiMedia Laboratories, Mumbai, India. Ascorbic acid, Quercetin, Mayes's reagent, Folin ciocalteu reagent, Ferric chloride, DPPH and H2O2 were obtained from Sigma Aldrich, USA.

2.3. Extraction procedure

The fine powder of *P. tetrastromatica* (5g) was macerated with 150ml of methanol (1:3) [8, 9]. The above mixture is stirred at 220rpm for 2hr, and then they are incubated in an orbital shaking incubator (Remi orbital shaking incubator, cis 60 plus, Mumbai) at room temperature at 100rpm for 48hrs. Again the mixture is extracted with ultrasonic extractor (Lark ultrasonic extractor, Lark innovative fine teknowledge, India for 15min [10], and then the solution is centrifuged at 1000rpm (Remi centrifuge 24 BL, India for 10min [11]. Filter the above mixture; filtrate was stored in amber colored bottle. The extraction process was repeated thrice until no more color discharged from the marc. The flavonoid isolation was carried out by the following method method[12] with slight modification initially powdered sample was extracted with methanol by maceration, then they are subjected to orbital shaking incubator, after which they are filtered through whatmann no.1 filterpaper. After extraction filtrate was partitioned by Petroleum ether, followed by n-hexane and they are allowed stand for 30min, from which aqueous layer obtained is a rich fraction of flavonoid. The fraction was considered as flavonoid rich fraction only after confirmation of flavonoid by phytochemical screening and quantified by phytochemical estimation.

2.4. Qualitative phytochemical screening of *Padina tetrastromatica*

Phytochemicals are naturally present in the seaweed and are biologically significant for play an essential role in defensive mechanism. Preliminary phytochemical screening is a part of chemical evaluation where the presence of the secondary metabolite such as alkaloid, flavonoid, steroid, tannin, saponins were screened as per the standard protocol [12, 13, 14, 15, 16]. Especially in the phytochemical screening, flavonoid are focused after the crude extract have been made into flavonoid rich fraction.

2.5. Quantitative estimation of phytochemicals in *Padina tetrastromatica*

a) **Estimation of Total Phenolic Content (TPC)**

The total phenol content was measured using the Folin–Ciocalteu method [17]. Extract of *P. tetrastromatica* (100µl) was mixed with 2 ml of 2% Na_2CO_3 and allowed to stand for 2 min at room temperature. Then, 100µl of 50% Folin–Ciocalteu phenol reagent (add company name) was added. After incubation for 30 min at room temperature (26^0C) in darkness, the absorbance was read at 720 nm in Elisa spectramax plus 384 Microplate reader (Molecular device LLC, USA). Total Phenolic Content values were expressed as mg Gallic acid equivalent. The experiments were performed in triplicate with control and standard to plot regression analysis.

b) **Estimation of Total Flavonoid Content (TFC)**

Aluminum chloride colorimetric method was used in the determination of Total Flavonoid Content. Quercetin was used as the standard [18,19]. 60 µL of methanol was mixed with 20 µL of extract or standard, 4 µL of 10 % aluminum chloride, 4 µL of 1.0 M potassium acetate and 122 µL of MilliQ water. The mixtures were incubated at room temperature (26^0C) for 30 min before reading the absorbance at 415 nm in Elisa spectramax plus 384 Microplate

reader (Molecular device LLC, USA). Total Flavonoid Content was expressed as mg of quercetin equivalent/g. The experiments were performed in triplicate with control and standard to plot regression analysis.

2.6. Antioxidant assays

a) Total antioxidant capacity

Phosphomolybdate assay [20] was used to measure the total antioxidant capacity of the flavonoid rich fraction of *Padina tetrastromatica*. To a reagent solution; sulphuric acid (0.6 M), sodium phosphate (28 mM) and ammonium molybdate (4 mM); 100 µl of each sample was added and incubated at 95°C in a water bath for 90 min. After cooling to room temperature, the absorbance was recorded at 765 nm in Elisa spectramax plus 384 Microplate reader (Molecular device LLC, USA) against reagent blank. Total antioxidant capacity of the ascorbic acid was also estimated for reference. The total antioxidant capacity was determined by using following formula

$$\text{Total antioxidant capacity (\%)} = \frac{\text{Abs control} - \text{Abs sample/standard}}{\text{Abs control}} \times 100$$

Where Abs means Absorbance.

b) DPPH free radical scavenging assay

1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) was used to measure the radical scavenging ability of the flavonoid rich fraction of *Padina tetrastromatica*. The reaction mixture containing 3ml of DPPH solution were mixed with 100µg of different concentrations (100-500 µg/ml) of the extract and shaken vigorously and incubated at dark condition for 30min. Ascorbic acid was used as the standard and the absorbance of the resulting solution was measured at 517nm in Elisa spectramax plus 384 Microplate reader (Molecular device LLC, USA)[17]. The percentage of inhibition has been calculated by using formula

$$\text{Percentage of inhibition (\%)} = \frac{\text{Abs control} - \text{Abs sample/standard}}{\text{Abs control}} \times 100$$

Where Abs means Absorbance.

c) Hydrogen peroxide scavenging assay

The free radical scavenging activity of flavonoid rich fraction of *Padina tetrastromatica* by hydrogen peroxide radical scavenging assay is followed as follows. In which 10 mM hydrogen peroxide solution was prepared in phosphate buffer (0.1 M, pH 7.4). 1ml of the extract was rapidly mixed with 2 ml of H_2O_2. The absorbance was measured at 230 nm after 10 min of incubation at 37^0C against a blank without H_2O_2 [19] in Elisa spectramax plus 384 Microplate reader (Molecular device LLC, USA). The percentage of scavenging of H_2O_2 was calculated using the formula

$$\text{Percentage of inhibition (\%)} = \frac{\text{Abs control} - \text{Abs sample/standard}}{\text{Abs control}} \times 100$$

Where Abs means Absorbance.

3. Results and discussion

The selected seaweed *P. tetrastromatica* was extracted with methanol by maceration technique, after which extract was made into flavonoid rich fraction by partitioning with petroleum ether, and n- hexane. The flavonoid rich fraction was confirmed by the standard phytochemical screening and their content also quantified by total flavonoid and phenolic estimation. The current extraction procedure had shown better yield for flavonoid and phytochemical screening have reveal the presence of active constituents like alkaloid, flavonoid, carbohydrates, saponins. The results of the phytochemical screening was displayed in Table.1

3.1. Phytochemical screening of *Padina tetrastromatica*

Table.1 Phytochemical screening of *Padina tetrastromatica*

S.No	Name of the Compound screened	Test performed	Result obtained
1.	Alkaloid	Mayer's Test	+
2.	Steroid	Liebermann's Test	-
3.	Carbohydrate	Molisch's Test	+
4.	Protein	Biuret Test	-
5.	Saponin	Foam Test	+
6.	Phenolic compound	Ferric chloride Test	+
7.	**Flavonoid**	a)NaOH Test b)Lead Acetate Test	++ ++

[- Absent, +Present , ++ Strongly present]

3.2. Estimation of total phenolic content

The methanolic extract of *P. tetrastromatica* was made into flavonoid rich fraction by partitioning method and the Total Phenolic Content of the extract was measured. In general methanol is consider as most suitable solvent for the extraction of polyphenolic compounds due to its ability to inhibit the action of polyphenol oxidase that leads to the oxidation of polyphenols [21]. Total phenolic content of the different concentration of the methnolic extract was done and standard gallic acid is compared with that of sample (Table.2 & Figure 3). The highest Total Phenolic Content value of the extract was represented as 246.28 mg GAE/g.

Table. 2 Total Phenolic Content of FRPD of *Padina tetrastromatica*

S.no	CONCENTRATION in µl	STANDARD (Gallic acid)	SAMPLE (FRPD)
1.	10	1.07 ± 0.005	0.12 ± 0.002
2.	15	1.88 ± 0.005	0.22 ± 0.003
3.	20	2.46 ± 0.004	0.40 ±0.001

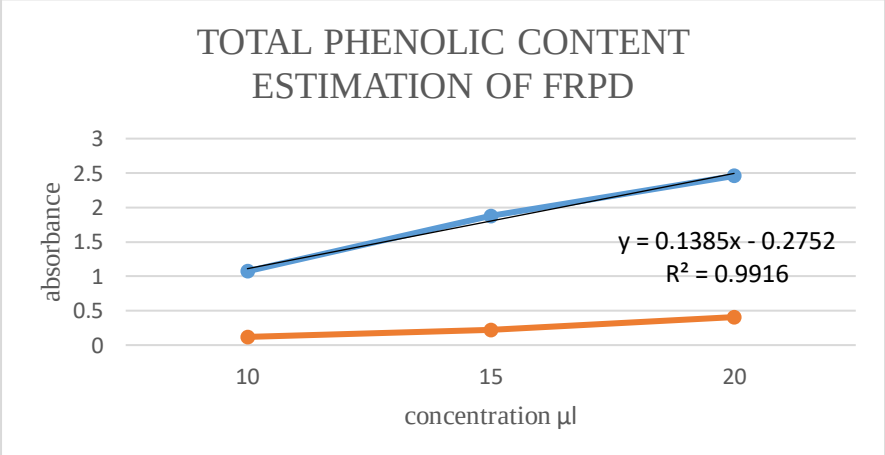


Figure 3. Histogram showing the total phenol content of different concentrations of *P. tetrastromatica*.

3.3. Estimation of total flavonoid content

Flavonoid is the major subclass of polyphenol and are active as natural antioxidant in plants. Generally flavonoid occurs as glycoside containing several polyphenolic hydroxyl group on their ring structure. The total flavonoid Content of the methanolic extract of *P. tetrastromatica* was done and results are represented in Table 3 & Figure 3 & 4. The total flavonoid content of the methnolic extract was found to be 174.71mg of quercetin equivalent.

Table.3 Total Flavonoid Content of FRPD of *Padina tetrastromatica*

S.No	CONCENTRATION (in µl)	STANDARD (Quercetin)	SAMPLE (FRPD)
1.	10	1.5 ± 0.005	0.13 ± 0.003
2.	15	2.7 ± 0.016	0.16 ± 0.001
3.	20	3.5 ± 0.008	0.24 ± 0.002

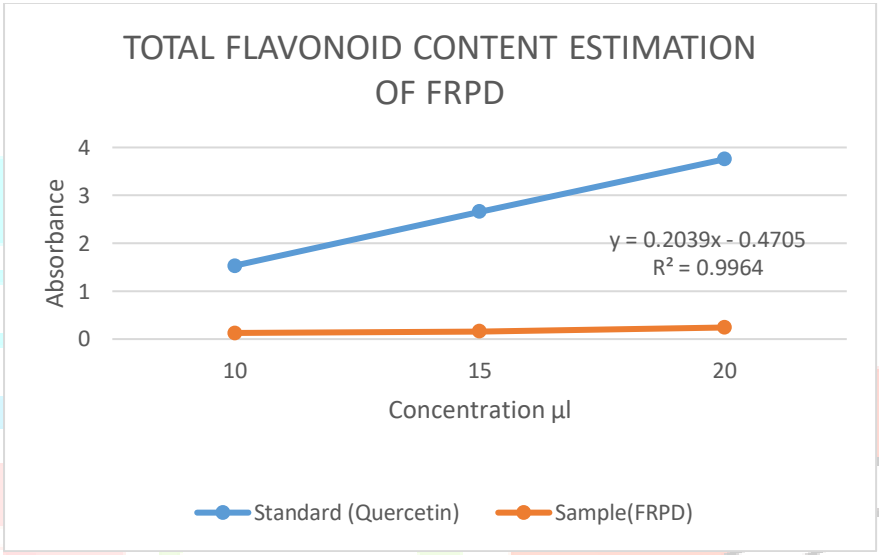


Figure 4. Histogram showing the total flavonoid content of different concentration of *P. tetrastromatica*.

3.4. Total antioxidant capacity

Flavonoids otherwise called as polyphenolic compound act as natural antioxidants in plant. The flavonoid rich fraction of the *P. tetrastromatica* was demonstrated with total antioxidant assay (Figure. 5). The percentage of inhibition was measured. All the data were expressed as Mean± SEM (Table.4). The highest percentage of inhibition was found in Total antioxidant assay as 71.71± 0.20. IC_{50} value was found to be 190.99 µg/ml. All the groups were compared by non-parametric t-test. Probability value was found to be significant and they are mentioned as *(significant), ** (more significant), *** (highly significant), **** (extremely significant)

Table.4 Total antioxidant capacity of FRPD

CONCENTRATION µg/ml	% of Inhibition	
	STANDARD (Ascorbic acid)	SAMPLE (FRPD)
100	43.18 ± 0.53	39.19 ± 0.53
200	55.63 ± 0.35	54.1 ± 0.72
300	64.43 ± 0.35	60.67 ± 0.53
400	78.04 ± 0.88	77.11 ± 0.35
500	84.58 ± 0.70	82.27 ± 0.20

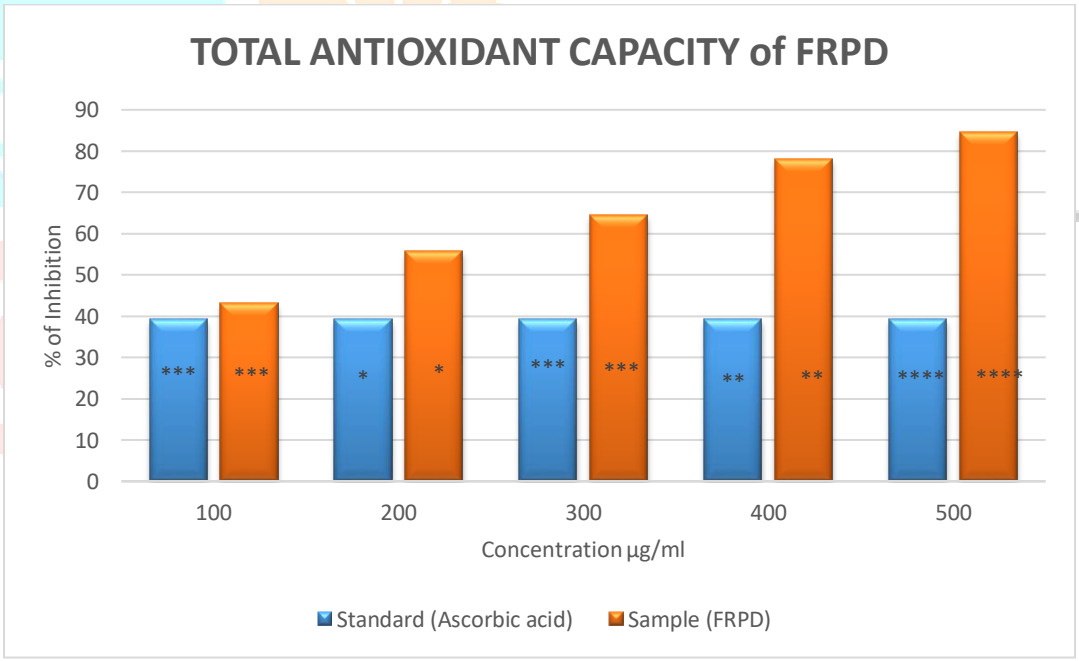


Figure. 5 Total antioxidant capacity of FRPD

3.5. DPPH free radical scavenging assay

The free radical scavenging assay using the DPPH radical is a preliminary test for the analysis of the antioxidant potential of the extracts. Antioxidant activity is related to the presence of the bioactive compound such as flavonoid (Figure.6). The flavonoid rich extracts percentage of inhibition was expressed as Mean± SEM (Table.5) and IC_{50}value was found to be 121.92 µg/ml and the highest value of inhibition was observed as 73.80±0.74. All the groups were compared with non-parametric t-test. Probability value was found to be significant and they are mentioned as *(significant), ** (more significant), *** (highly significant), **** (extremely significant).

Table.5 DPPH free radical scavenging Assay of FRPD

CONCENTRATION µg/ml	% of Inhibition	
	STANDARD (Ascorbic acid)	SAMPLE (FRPD)
100	67.13 ± 0.71	47.85 ± 0.71
200	77.37 ± 0.54	55.25 ± 0.22
300	80.71 ± 0.94	62.97 ± 0.54
400	84.99 ± 0.71	73.05 ± 0.89
500	88.21 ± 0.71	80.94 ± 0.74

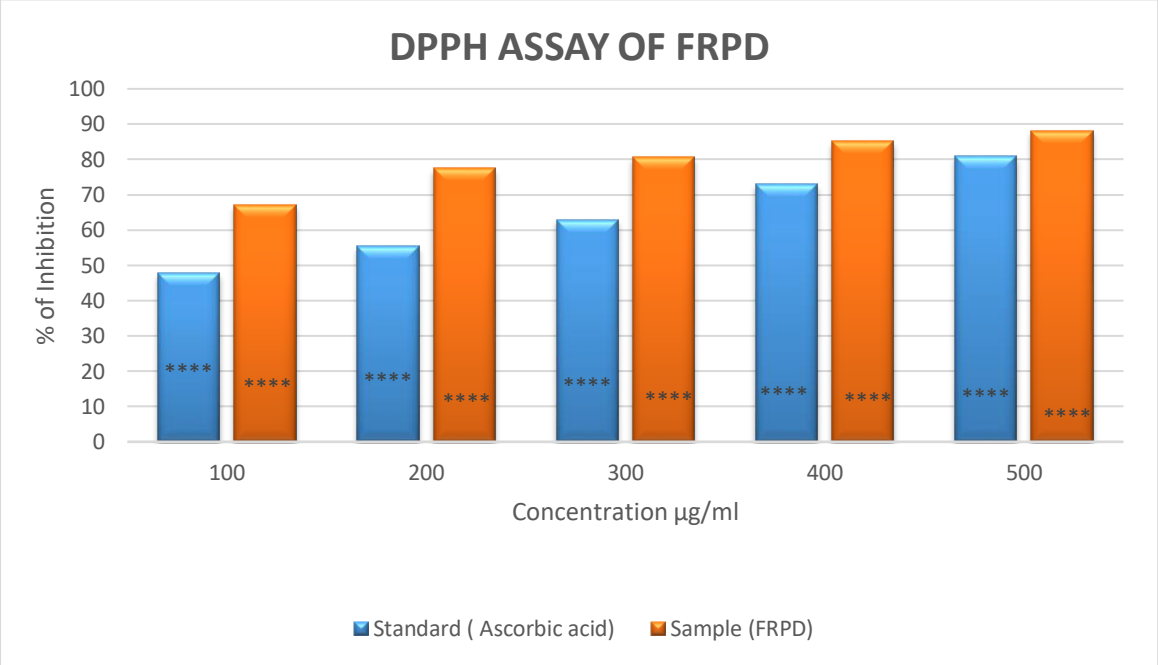


Figure. 6 DPPH assay of FRPD

3.6. Hydrogen peroxide scavenging assay

The antioxidant activity of flavonoid rich fraction of the *P. tetrastromatica* was demonstrated with Hydrogen peroxide scavenging assay (Figure.7). The percentage of inhibition of the following fraction was measured. All the data were expressed as Mean± SEM (Table.6). The highest value of percentage of inhibition in Total antioxidant assay was recorded as 75.52± 0.35. IC_{50} value was found to be 254.73 µg/ml. All the groups were compared by non-parametric t-test. Probability value was found to be significant and they are mentioned as *(significant), ** (more significant), *** (highly significant), **** (extremely significant)

Table.6 H_2O_2 Radical Scavenging assay

CONCENTRATION µg/ml	% of Inhibition	
	STANDARD (Ascorbic acid)	SAMPLE (FRPD)
100	44.51 ± 0.53	36.12 ± 0.53
200	55.82 ± 0.53	41.95 ± 0.70
300	63.28 ± 0.70	53 ± 0.53
400	72.95 ± 0.72	68.88 ± 0.70
500	81.81 ± 0.70	79.02 ± 0.35

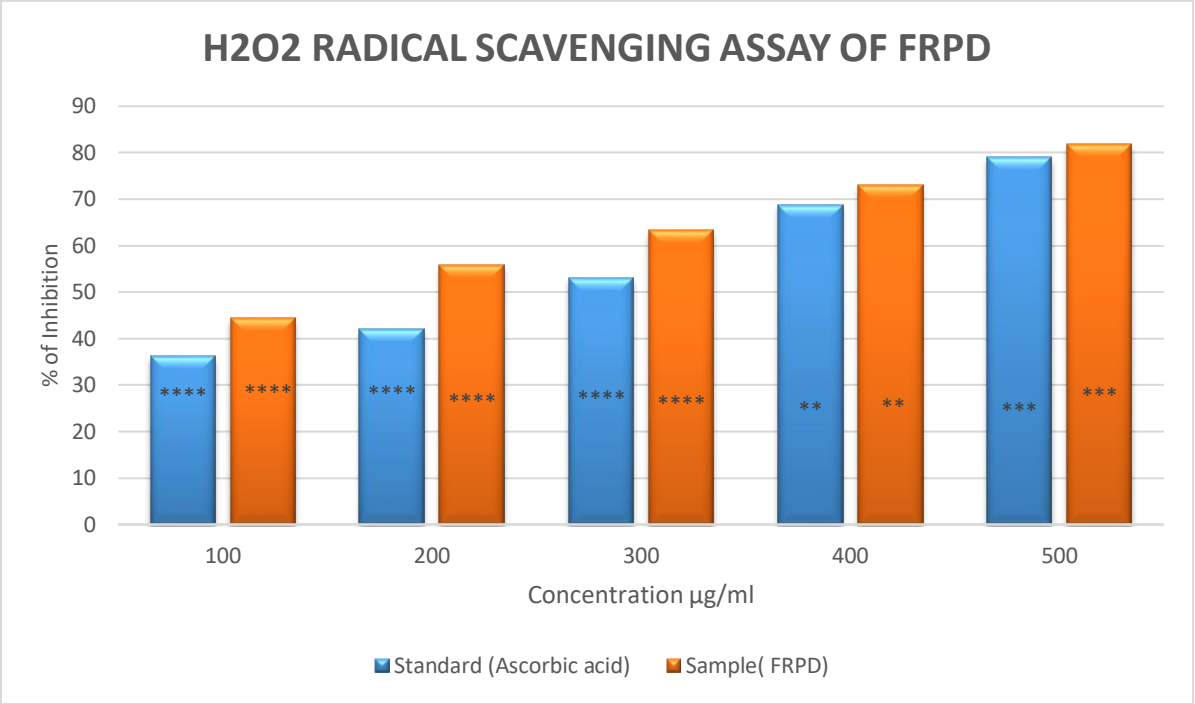


Figure. 7 H_2O_2 scavenging assay of FRPD

3.7. IC_{50} value

IC_{50} value is the concentration of the antioxidant required to leads 50% inhibition in the undertaken antioxidant assay. The performed antioxidant assay showed considerable inhibitory value in Total antioxidant capacity, DPPH Assay, H_2O_2 radical scavenging assay (table.7).

Table.7 IC_{50}value of antioxidant assay of FRPD

IC_{50} VALUE	TAC	DPPH	H_2O_2
	190.99 µg/ml	121.92 µg/ml	254.73 µg/ml

4. Conclusion

The present study shown that flavonoid rich fraction of the *P. tetrastromatica* had substantial amount of flavonoid whereas total flavonoid content and phenolic content revealed that the amount of flavonoid and phenolic content in the extract as 246.28 mg GAE/g, 174.71mg of quercetin equivalent respectively for 1g of the standard. Antioxidant activity of the following extract was carried by the standard methods by DPPH assay method, total antioxidant assay, hydrogen peroxide scavenging assay method and the results were highly significant when compared with standard. The IC_{50} value of those methods were calculated and compared which shows that fraction contain significant antioxidant activity as that in the standard. At the outset, the revealed flavonoid antioxidant activity shows the richness of flavonoid content present in the brown seaweed.

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