In Vitro Correlation Studies On Anticancer, Antioxidant Activity And Phenolic Content Of Green Marine Seaweeds From Gulf Of Mannar

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Abstract: Large number of existing studies have emphasized that thousands of natural polyphenols are represented from plant domain, occupying almost 70% of the globe, ocean incorporates enormous number of novel products with unique biological activities that may be useful in cancer drug development. Therefore four marine seaweeds were isolated, identified authenticated and they were analyzed for various antioxidant properties and the percentage of reaction in cell viability was also calculated to determine the potentiality of the secondary metabolites. The free radical scavenging activity was determined using DPPH and FRAP, and anticancer activity by MTT against HepG2 cancer cell line. It is evident from the results, among all four marine algal seaweeds, C.racemosa (46.32±0.55%) showed highest scavenging activity in DPPH and C.verticillata (61.83±0.012µM) in FRAP assay. In addition, a significant anticancer activity was observed in ethanolic crude extracts of C.scalpelliformis (78.52%) and C.verticillata (85.69%) of cell viability at 25µg/ml. Hence, the present study confirms that the green marine macro algae possess antioxidant and anticancer properties. Further analysis has to be carried out to determine the various bioactive compounds responsible for these biological functions.

Index Terms: Green marine macro algae, antioxidant activity, anticancer activity.

1. INTRODUCTION

Ocean occupies almost more than 70% of the world's earth surface. Natural compounds synthesized by a broad variety of marine organisms subsidize rich source of bioactive compounds and share approximate one half of the total world's bio-divergence (Yende et al., 2014). Approximately 9600 species of seaweeds are reported so far and there is a considerable debate over their classification. Seaweeds are usually classified based on their pigments into three broad groups: red (Rhodophyta), green (Chlorophyta), and brown (Phaeophyta) algae. Green algae are grouped within the phylum Chlorophyta, with more than 6000 species reported as macro and micro algae, found in freshwater to marine habitats. Of this, very few green macro algae have been reported from the oceans (Yiwen, 2015). Various ailments and bioactive compounds have been isolated from plant sources are known secondary metabolites which are involved in plant chemical defense systems, that are formed throughout the millions of years during which plants have co-existed with their attackers (Wink et al., 1999). Even though, the polyphenols are synthesized from terrestrial plants, the marine seaweeds are comparable in some aspects but contrasting in their chemical structures.

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These compounds possess a unique structure that is not observed in terrestrial plants. Based on the basic understanding of the natural oxidants from the plants, various marine bioactive compounds are derived from the seaweeds, are considered to be an excellent source of novel chemical constituents that are not found in terrestrial sources. Very less literature records have been observed on the beneficial properties of the marine seaweeds derived polyphenols and only few have reported the role of seaweed polyphenols in protection of cancer associated with Oxidative stress (Yoon et al., 2011). Hence our present experimental study addressed the role of different green marine seaweeds against oxidative stress and cytotoxic properties.

2. PROCEDURE

2.1 Collection and identification of Green marine macro algae
Green macro algae were collected from the deep ocean of Mandapam, Rameshwaram in Tamil Nadu. The green macro algae were then cleaned in running tap water to remove all the extraneous materials like epiphytes, sand, pebbles and shells. After drying, the washed seaweeds were blotted on a blotting paper and spread out at room temperature (37°C). The shade dried macro algae were then ground to fine powder and stored at 4°C for further analysis.

2.2 Extraction of sample for phytochemical analysis
Shade dried macro algae were then subjected to various solvents to extract the phytochemical constituents present in it. The samples were then subjected to maceration with 3 solvent systems (Methanol, Ethanol and Aqueous). The extracts were then filtered through Whatman filter paper & the filtrates were collected and poured on to a Petri dish. The solvents were allowed to evaporate and the phytocompounds were collected and stored in a sterile vial for further analysis.

2.3 Qualitative analysis of phytoconstituents
Various qualitative chemical tests have been performed to find out the phytochemical profile of the algal extracts. The green

macro algal extracts of different solvents has been analyzed for the presence of phenols, flavanoids, alkaloids, tannin, steroids, glycosides and saponins following standard protocol. Extracts were dissolved individually in hydrochloric acid.

2.3.1 Qualitative analysis

Test for Phenols (Ferric chloride test)
To 0.5ml of filtrate, few drops of 10% ferric chloride were added. Bluish black color confirms the presence of Phenols.

Test for Flavanoids (Alkaline reagent)
To 0.5ml of filtrate, few drops of 10% Sodium hydroxide was added. Intense yellow color forms and disappears confirms the presence of flavanoids.

Test for alkaloids (Mayer's Test)
To 0.5ml of filtrate, few drops of dil.hydrochloric acid and Mayer's reagent were added. White/yellow or creamy precipitate confirms the presence of alkaloids.

Test for Saponins (Froth test)
To 0.5ml of filtrate, 2ml of distilled water was added and 2-3 drops of Olive oil was added and vigorously shaken. Formation of emulsion confirms the presence of saponins.

Test for tannin (Ferric chloride test)
The extract was dissolved with 5ml of distilled water, to this few drops of neutral 5% ferric chloride solution was added and dark green color indicates the presence of tannin.

Test for Steroids (Salkowski test)
To 0.5ml of extracts, 1ml of chloroform was added along with few drops of concentrated sulphuric acid was added on the sides of the test tube. Formation of reddish brown ring confirms the presence of steroids.

Test for Glycosides (Liberman's test)
To 0.5ml of extract, 1ml of chloroform and few drops of acetic acid were added. Formation of violet to blue to green color confirms the presence of glycosides.

2.4 Determination of total phenol content (Folin-ciocaltaeu method)
The determination of total phenol content was performed using standard folin-ciocaltaeu reagent assay (Massoumeh et al 2014). To 100µl of extract, 100µl of FC reagent was added & incubated for 5mins in dark. After the incubation, 80µl of 7.5% sodium carbonate was added and again incubated for 2hrs in dark. Then 100µl of distilled water was added to it. The absorbance of the samples was read at 765nm. Gallic acid was used as a standard and the total phenolics were expressed as mg/g gallic acid equivalents (GAE).

2.5 IN VITRO ANTIOXIDANT ASSAY

2.5.1 DPPH free radical scavenging activity- (2, 2 – Diphenyl – 1- picrylhydrazl)
Antioxidant activity of the algal extract was studied using DPPH method (Blios et al., 1958). DPPH is a stable free radical which works on the principle of reducing capability of the antioxidants. When the antioxidants react with the DPPH, reduction of DPPH to DPPH-H takes place due to the decolorization of the mixture with respect to the number of electrons reduced. Increased decolorization represents maximum reduction by antioxidants. Different concentrations of the algal extracts were prepared ranging from 200-1000 µg/ml with the respective solvent. 1ml of each different concentration of algal extracts was mixed with 2ml of 0.1mM DPPH dissolved in methanol. The mixture was incubated at dark for 30 mins at room temperature. After incubation the absorbance was measured at 517nm using UV-Vis spectrophotometer. 1 ml of methanol and 2ml of DPPH was taken as positive control. Ascorbic acid was taken as standard. The percentage of free radical scavenging activity was calculated using the formula.

$$\text{Scavenging activity (\%)} = \frac{\text{Abs C-Abs T}}{\text{Abs C}} \times 100$$

2.5.2 FRAP assay (Ferric ion reducing antioxidant power)
The ability to reduce ferric ions was measured using the method followed by Benzie and Strain (1996). FRAP assay works on the principle of reducing property of the antioxidants from ferric 2, 4 6-tripyridyl-s-triazine complex (Fe^{3+}-TPTZ) to its ferrous colored form (Fe^{2+} -TPTZ). FRAP reagent was prepared by mixing 2.5ml of 10mM TPTZ (2, 4 6-tripyridyl-s-triazine) solution in 40mM hydrochloric acid and 2.5ml of 20mM of ferric chloride and 300mM of sodium acetate buffer (pH 3.6) at a ratio of 1:1:10 in volume. Different concentrations of algal extracts were prepared and 500µl of the extract was incubated with 1000µl of FRAP reagent. The reaction mixture was incubated at 37°C for 30mins and the absorbance was measured at 593nm. Freshly prepared ascorbic acid was used as the standard. The ferric reducing antioxidant capacity of the algal extracts was calculated from the linear calibration curve and the values are expressed as µM/g of sample.

2.6 Antiproliferative assay- (MTT-4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide)
HepG2 Liver cancer cell lines (1×10^6) was seeded onto the 96 well tissue culture plate and incubated at 37°C with 5% CO_2. After incubation, algal extracts of various concentrations were added to the culture plate and incubated for 72hrs at 37°C. After preincubation of cells with algal extracts, the cells were removed and washed with phosphate buffer saline (pH 7.4). 100µl/well (5mg/ml) of MTT was added and incubated for 4-5hrs. After a period of incubation, 100µl of DMSO was added to all the wells and the absorbance was measured at 570nm in ELISA reader using DMSO as the blank. The percentage of cell viability was calculated using the following formula (Rabia Alghazeer et al., 2018).

$$\text{\% of cell viability} = \frac{\text{Abs T}}{\text{Abs C}} \times 100$$

Abs C = Absorbance of Control
Abs T = Absorbance of Test sample

3. RESULTS AND DISCUSSION

3.1 Morphological Identification of marine Seaweeds
The marine seaweeds collected from Gulf of Mannar was identified and authenticated by Botanical Survey of India (BSI), Coimbatore as Chaetomorpha crassa, Caulerpa

racemosa, Caulerpa verticillata, and Caulerpa scalpelliformis.

3.2 Extraction using different Solvents

The finely crushed shade dried powdered (15 days) seaweeds were macerated with different solvent system like ethanol, methanol and aqueous systems (Table 1). The percentage yields of different extracts were calculated using the following formula:

$$\% \text{ of yield} = \frac{\text{Final weight of the dried extract}}{\text{Initial weight of the powder}} \times 100$$

Name of the macro algae	Yield of the extract (g)		
	Ethanol	Methanol	Aqueous
Chaetomorpha crassa	0.26	0.27	0.24
Caulerpa racemosa	0.23	0.31	0.23
Caulerpa verticillata	0.25	0.12	0.21
Caulerpa scalpelliformis	0.24	0.32	0.21

*(g) extract/2gram.

Table 1: Yield of the extract from different solvent systems.

3.3 Qualitative analysis of phytochemical Constituents

Phenolic acids present in marine macro algae known to contain mainly phenols and flavanoids, because of their efficient, biological and pharmacological importance, including anti-inflammatory, anti carcinogenic and anti atherosclerotic activities. Therefore, phytochemical analysis was performed to confirm the presence of phenols, flavanoids, alkaloids, tannins, saponin, glycosides and steroids from various seaweeds using different solvent systems. Among the various solvent system used, we observed ethanolic extracts exhibited maximum number of phytochemical constituents compared to methanolic and aqueous (Table 2).

3.4 Quantitation of Total Phenol content

We observed an increased total phenolic content by ethanolic extract of Caulerpa verticillata (87.24±0.009mg GAE/g) and Caulerpa scalpelliformis (69.36±0.01mg GAE/g) followed by C.racemosa (42.72±0.006mg GAE/g) and the lowest phenolic content in Chaetomorpha crassa (35.99±0.005mg GAE/g) (Figure 1). The phenolic content of C.verticlillata was statistically significant when compared with other algal extracts (p value= 0.05.) Apparently, the methanolic extracts, has shown to have less total phenolic content compared with ethanolic extracts. TPC of the (methanolic) extracts varied from 10.46±0.004 mg to 14.11±0.003mg GAE/g, in a descending order of C.verticillata (14.11±0.003mg GAE/g), C.crassa (11.58±0.01mg GAE/g), C.scalpelliformis (10.52±0.003mg GAE/g) and C.racemosa (10.46±0.004mg GAE/g). Aqueous extracts showed a very less phenolic content ranging from (2.66±0.002mg to 6.71±0.006mg GAE/g).

Algal Extract	Solvent system	Phenol	Flavanoid	Tannin	Saponin	Alkaloid	Steroid	Glycoside
Chaetomorpha crassa	Methanol	+	+	+	+	+	+	+
	Ethanol	+	+	+	+	+	+	+
	Aqueous	+	–	–	+	–	–	–
Caulerpa racemosa	Methanol	+	+	+	+	–	+	+
	Ethanol	+	+	+	+	+	+	+
	Aqueous	+	+	–	–	+	+	+
Caulerpa verticillata	Methanol	+	+	+	+	+	+	+
	Ethanol	+	+	+	+	+	+	+
	Aqueous	+	+	–	–	–	–	–
Caulerpa scalpelliformis	Methanol	+	+	+	+	+	+	+
	Ethanol	+	+	+	+	+	+	+
	Aqueous	+	–	+	+	–	–	–

Table 2: Qualitative analysis of Phytochemical constituents from different algal species by various solvent systems: Ethanol, Methanol and Aqueous

Similar kind of work was reported by Gazali et al., 2019, with ethanolic extract of C.crassa exhibited maximum total phenolic content of 1853.71 ± 67.02mg GAE/g and 99.50 ± 28.9mg GAE in ethyl acetate. Yin Yin Chia et al., 2015 also has documented that the methanolic and hexane extract of C.racemosa had shown a phenolic content of 0.75% and 0.004% respectively. This increase may be due to dilution of phenolic compounds per gram by extracted matter other than the phenolic compounds. The current study had shown that the solvent system can also have some intensity of effect on the extraction of bioactive compounds. For instance, Patricia Louise et al., 2017, has reported that Caulerpa lentifera has exhibited slightly polar and mostly nonpolar antioxidants since the extracts had shown high antioxidant activity in chloroform and acetone solvent system. These fluctuations may be because of the variations in the extraction process, collection site, temperature and time. Several literature studies have proved that there is a strong interrelationship between phenols and the antioxidant property of seaweeds (Namjooyan et al., 2007; Luo et al., 2010). Many literatures have already documented that there is relation between antioxidant studies and concentration of phenols and flavanoids content in Ulva prolifera (Cho et al., 2011). As per our literature survey, this report is first to document the presence of increased level of total phenolic content in C.verticillata and C.scalpelliformis, which highly contributes to its antioxidant property.

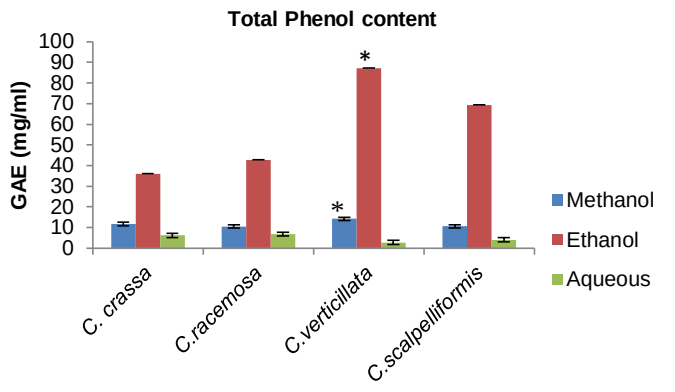

Figure1: Quantitative estimation of Total phenol content from different algal extracts using various solvent systems..

3.5 In vitro antioxidant activities

Extracts of marine seaweeds are known to have a variety of phytochemical constituents. Many authors have acknowledged that there is a positive correlation between the phytoconstituents and the capability of the seaweeds to scavenge the free radicals. Extensive studies done by Dorman and Peltoketo et al., 2003 have recognized that the increased total phenolic content contributed to a higher antioxidant activity. Contrastingly, some authors have also reported that there might be a low antioxidant activity due to the synergistic actions between the phenolic compounds of the seaweed extract and various other negligible phytoconstituents that might influence the long-term antioxidant response (Kulisic et al., 2004). However it is predominantly accepted that there is a considerable discussion among the researchers that drying process has also shown to influence the efficiency of the antioxidant potential (Cousins, Adelberg, Chen and Rieck 2007) as the drying process may decrease the competence of the extracts to reduce the free radicals. In the present study, 2 different types of assay were conducted to analyze the antioxidant activities of the extracts, since antioxidant activities of different types of substances involve different mechanism and no single assay is conclusively applicable to all of them.

3.5.1 DPPH radical scavenging activity

Phenols and flavanoids has the ability to scavenge the free radicals by donating hydrogen atoms, thus by the process of reduction which decolorizes DPPH to DPPH-H. The current study focuses on the determination of the antioxidant capacity of the selected marine macro algae. For this, the ethanolic extracts of all the algal samples were allowed to react with DPPH for 30 minutes and the absorbance was recorded at 517nm. We observed a varied level of radical scavenging activity ranging from 34.92±0.83 % to 46.32±0.96% at 1000µg/ml and the highest scavenging activity was recorded for C.racemosa (46.32±0.55%) and C.scalpelliformis (46.14±0.5%) followed by C.crassa (45.10±0.48) and the lowest scavenging activity in C.verticillata (34.92±0.614%) against the standard ascorbic acid (84.71±0.005%) (Figure 2). Previous literature has reported that, C.racemosa had highest antioxidant activity of 90.00±0 in hexane extract collected from Malaysian coast (Yin Yin Chia et al., 2015). A similar pattern of results was obtained by Manoj kumar et al., 2011 suggesting that methanolic extracts of C.racemosa collected from Veraval coast, Gujarat has showed significantly increased level of non-enzymatic antioxidants activity of DPPH (87.01±5.74%) with respect to its total phenolic content (61.69±2.64 mg GAE^{-1} extract) . Moreover, seaweed extracts collected from ocean of Kanyakumari have shown that the DPPH activity (methanolic extract) of C.racemosa and C scalpelliformis was found to be 34.1±0.83 % and 40.3±1.06 %, respectively (ShanthaSubitha et al., 2016).

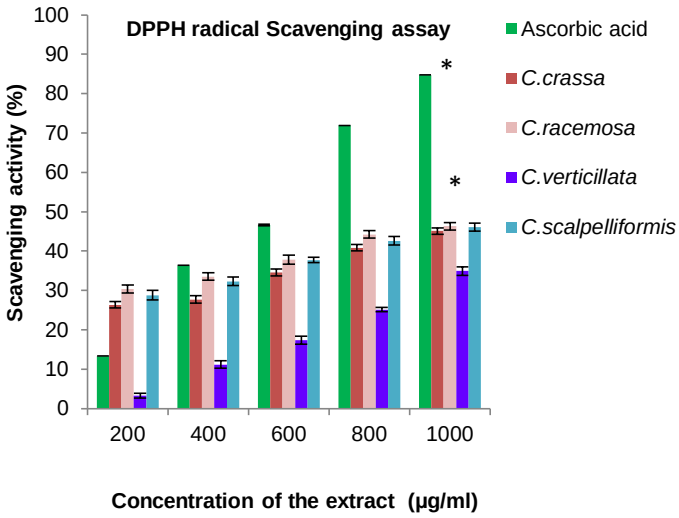

Figure 2: Percentage of DPPH scavenging activity from different algal extracts at various concentrations

We observed an increasing concentration of antioxidant activity in C.racemosa and C. Scalpelliformis compared to other algae, suggesting that the selected seaweeds have noticeable antioxidant potential to scavenge the free radicals. This large disparity in antioxidant activity of the green seaweeds may be due to the difference in the location of the collection sites of the seaweeds, drying procedures, solvent system, seasonal variations, temperature and mainly the contribution of the phytoconstituents of the extracts as reported by Patricia Lousie et al., 2017 and Zhongrui et al., 2012

3.4.2 FRAP assay (Ferric reducing antioxidant potential)

FRAP assay effectively determines the reducing property of the antioxidant compounds present in the algal extracts reacting with ferric tripyridyltriazine (Fe^{3+} -TPTZ) complex, resulting in the production of ferrous tripyridyltriazine (Fe^{2+} -TPTZ). The formation of the blue color complex (Fe^{2+} -TPTZ) at a low pH (3.6) determines the ferric reducing capacity of the extracts at 593nm (Benzie and Strain .1996). FRAP values of the studied algal extracts varied (10.07±0.007 to 61.83±0.01µM) with ascorbic acid as standard (160.98±0.088µM). The results (Figure 3) showed that the ethanolic extracts of C.verticillata (61.83±0.012µM) exhibit high ferric reducing activity followed by C.racemosa (49.34±0.004µM) and C.scalpelliformis (38.79±0.007µM). The lowest activity was observed in C.crassa (10.07±0.007µM). Similar results have been reported by Parthasarathy et al., 2014, that the highest FRAP value was observed in methanolic extract of C.racemosa (89±1.98µM) collected from Gulf of Mannar. Gazali et al., 2019 has reported that the ethanolic extract of C. Crassa displayed 62.71± 0.30µM, whereas in n-hexane extracts they observed no antioxidant

activity. Thus solvent system plays a vital role in determining the antioxidant potential of the algal extracts.

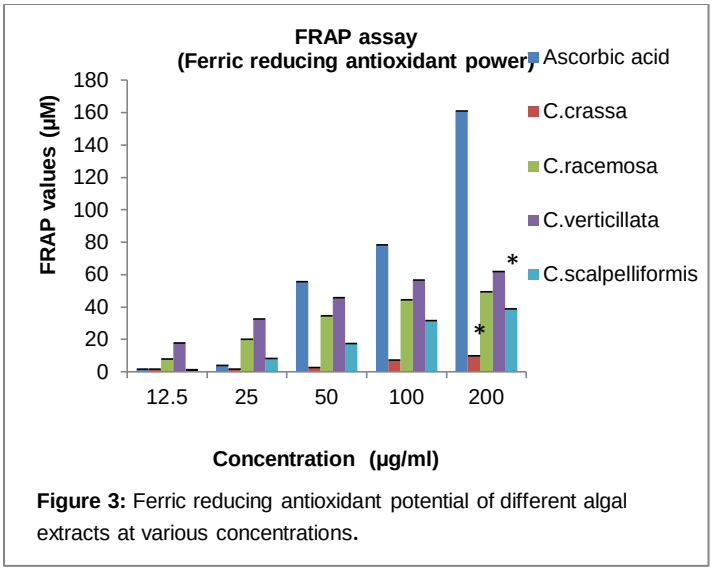

Figure 3: Ferric reducing antioxidant potential of different algal extracts at various concentrations.

Moreover, FRAP assay was carried out in acidic pH (3.6) to maintain the solubility of the iron. Previous studies have reported that reactions taking place at a low pH would minimize the efficiency of the ionization potential which would drive the electron transfer and maximizes the redox potential leading to a shift in the mechanism. Hence, the FRAP values are less with respect to the ionization potential of the algal extracts (Simic et al., 1994 and Hagerman et al., 1998).

3.5 Antiproliferative activity (MTT assay)

To determine the cell viability, HepG2 liver cancer cell line was used. The crude extracts was mixed with cancer cells at various concentrations ranging from 10µg/ml to 200µg/ml. (Figure 4) shows that, among the four algal extracts, C.scalpelliformis exhibits 78.52% of cell viability at 25µg/ml. This was in line with the report published by Joshi and Sudha et al., 2012, that the extracts of C.scalpelliformis has shown cytotoxicity activity against Hep2 cells in vitro with a CD50 of 250 µg/ml. C.racemosa and C.crassa did not showed any cytotoxic activities at various concentrations, whereas C.verticillata showed a cell viability of 85.69%(cytotoxicity is 14.31%) at 25µg/ml. In contrast, when there is an increase in the concentration, the viable cell count did not decrease. Though C.verticillata had very good antioxidant properties, we could not observe any significant anticancer activities. Even at various concentrations, C.racemosa and C.crassa do not exhibit cytotoxic property. Literature reviews shows that, C.racemosa (IC50 value of 30.17±0.5µg/ml) and C.crassa (IC 50 value of 179.88±1.6µg/ml) collected from SriLanka had shown cytotoxicity against HL-60 cancer cell line (Chaminda Lakmal et al., 2014). This could be due to the collection site of seaweeds, type of cancer cell line used; stress condition, tolerance to salinity, and more importantly the polyphenolic structural configuration might play an important role in cell cytotoxicity at a concentration dependant manner (Grigalius and Petrikaite et al., 2017).

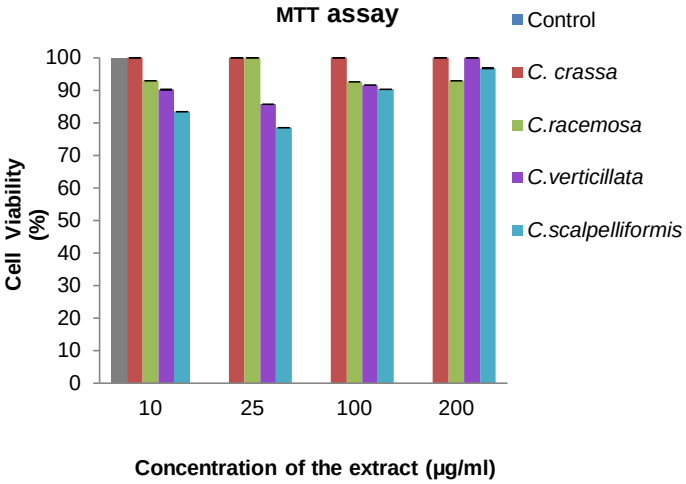

Figure 4: Percentage of cell viability using HepG2 cell lines against different algal extract.

Moreover, the results also demonstrated that percentage of cell viability is a concentration dependant, as the viable cells decrease at a particular concentration. This is the first report to observe anticancer properties (cytotoxic effects) on HepG2 cell lines with algal extracts of C.verticillata.

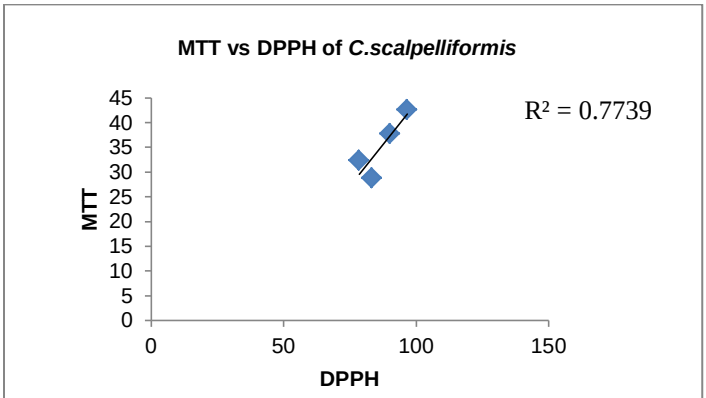

Figure 5a: Correlation of MTT and DPPH of C.scalpelliformis

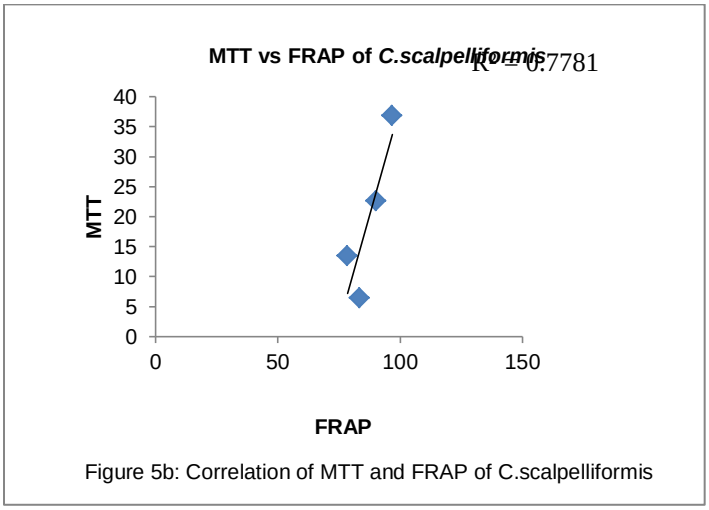

Figure 5b: Correlation of MTT and FRAP of C.scalpelliformis

Further the correlation coefficient (R^2) between the antioxidant activity from DPPH, FRAP and the cytotoxicity activity from MTT assay has proved to possess a good correlation (0.773 and 0.778) respectively (Figure 5a and 5b). Minimum correlation between DPPH and MTT indicated that not only phenols, flavanoids might also be involved in the antioxidant pathway. Further it was also observed that the cytotoxic property might be also due to the phytochemical constituents that directly involves in cell cycle pathway, cell signaling mechanisms and apoptosis (Kuttan et al., 2007 and Lamoral-Theys et al., 2010).

4. STATISTICAL ANALYSIS

All the assays were studied in triplicate and the data was graphically presented as the mean ± error of triplicates (n=3). ANOVA was performed using Graph Pad Prism. P values <0.005 were considered significant with confidence of 95%.

5. CONCLUSION

In the past few decades, marine environment has been represented as a vast resource that offers great opportunity for drug discovery. Present and previous literature, has well documented that marine macro algae is known for its rich source of structurally variegated bioactive substances displaying a wide variety of biological action. The novel chemicals produced from seaweeds differs from the secondary metabolites produced from the terrestrial plants due to their ability to withstand extreme diversity in temperature, pressure, increased salinity which are unique in their structure and functional aspects. Polyphenols derived from marine algae have attained the interest due to the wealthy source of major compounds like Polyphenols. These polyphenols are well characterized for their antioxidant activities that modulate the oxidative stress mechanism. Hence, the ethanolic algal sample was then carried out containing in vitro antioxidant assays like DPPH, FRAP and to confess the antioxidant effectiveness of the seaweeds. The antiproliferative effect of the algal extracts was determined by MTT assay to analyze the cell viability and cell proliferation. Contrary to our aforementioned analysis, C.verticillata was observed to exhibit a minimum anticancer property. The anticancer property of ethanolic extract of C.scalpelliformis was found to be 78.52% of cell viability at 25µg/ml. In line with the previous reports, the structural configuration of the polyphenols also plays a crucial role in the anticancer potential.

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