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Research Article

Comparative Analysis of Phytochemical and Antioxidative Properties of Different Solvent Extracts of *Codium tomentosum* Stackhouse for Therapeutic Application

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Abstract

Seaweeds are a phenomenal source of bioactive components in marine environments yet to be explored extensively. The study focused on estimating and identifying phytochemical components, secondary metabolites, and antioxidant efficiency of edible green seaweed *Codium tomentosum* (Chlorophyta). Qualitative phytochemical analysis of five solvent extracts revealed the presence of Phenol, Flavonoids, Terpenoids, Alkaloids, Tannins, Steroids, Carbohydrates, Glycosides, Amino acids and Proteins. Total phenol content (TPC) equivalent to GAE of five extracts estimated shown highest in chloroform and lowest in methanol - CE (194.53 ± 0.008 mg GAE/g) and ME (138.97 ± 0.007mg GAE/g). Total flavonoid content (TFC) quercetin equivalent (QE) was reported lowest in aqueous extract AE (59.982 ± 0.024 mg QE/g) and highest in chloroform – CE (213.07± 0.014 mg QE/g). The secondary metabolites profile of methanol extract analyzed by GC-MS revealed prominent components, Hexadecanoic acid, Flavones, Oleic acid, Dodecanoic acid, Pentadeconoic acid, and phenol derivative having a spectrum of bioactivity. *In vitro,* the Antioxidant activity of the five solvent extracts of *Codium tomentosum* was confirmed by DPPH and ABTS methods. It is evident from the present investigation that the edible green seaweed *Codium tomentosum* Stack House is a promising source of bioactive components that could be further explored for antimicrobial, anticancer, and antibiofilm properties through green synthesis of nanoparticles for pharmaceutical and nutraceutical applications.

Keywords: *Codium tomentosum,* phytochemical, GC-MS, anti-oxidative, edible seaweed

INTRODUCTION

Bioactive components isolated from natural sources are in high demand due to their various bioactivities such as antimicrobial, anti-oxidative, anti-cancer, anti-inflammatory, and anti-diabetic properties.[1-2] Living organisms produces Reactive oxygen species (ROS) during the normal metabolic process. Free radicals such as superoxide anion (O_2), hydroxyl radical (OH.), nitric oxide (NO) and hydrogen peroxide (H_2O_2) are various forms of activated oxygen that create oxidative stress in the cell. Oxidative stress damages the cellular mechanism and causes diseases like ulcerative colitis, cancer, atherosclerosis, cardiovascular diseases, ageing and rheumatoid arthritis.[3-4] Antioxidants minimize oxidative stress, thereby decreasing cellular damage by scavenging the free radicals or preventing radical formation. Natural antioxidants emerge as a better option for synthetic antioxidants, which pose poisonous and carcinogenic effects[5-6]. Hence antioxidants from harmless and easily available natural sources are expanding attention.

Marine is a huge natural resource hub with potential biota with various applications. Since ancient times in India, China and other countries, thousands of natural products were isolated and exploited from marine flora, microalgae, seaweed, and mangrove plants for biomass and medical applications.[7-9] Seaweed is a natural treasure with essential bioactive phytochemicals, polysaccharides, fibre, amino acids, ω-3 fatty acids, minerals, iodine and vitamins.[10-11]

Seaweeds are classified based on the pigment into Phaeophycean (fucoxanthin – Brown seaweed), Chlorophyta (chlorophyll a, chlorophyll b – Green seaweed), and Rhodophyta (Red seaweed- phycocyanin, and phycoerythrin).[12-13] Bioactive components of seaweeds possess various therapeutic significance, such as anti-inflammatory,[14] anti-diabetic,[15] anti-bacterial,[16] anti-viral, anti-fungal, anti-cancer,[17-18]and antioxidant properties.[1,19-20] In order to fight nutritional deficiencies, seaweeds are also used as a supplement in traditional food cultures [21] and for extracting and isolating bioactive substances to formulate nutraceutical supplements.[22] Phytochemical components as potential functional food intended to protect against metabolic deficiency and non-communicable diseases.[23,12] The present study concentrated on *Codium tomentosum* Stack House, with a wide biotechnological application [24] is an edible green seaweed that grows up to 30 cm long and belongs to the family *Codiaceae* in the Phylum *Chlorophyta*. The fronds have a

velvety texture and are firm, spongy, and covered in colourless hair visible when submerged [25]. The current study intends to determine the antioxidative activity and to identify the phytochemical compounds and the metabolites present in the five different solvents (Methanol, Chloroform, Hexane, Aqueous and Ethyl Acetate) extracts of *C. tomentosum*. Solvents of various polarity are employed to determine the best choice of solvent for the extraction process in seaweeds. Standard methods of Qualitative and quantitative phytochemical assay were carried out. The antioxidant potential of *Codium sp* was characterised by DPPH and ABTS biochemical methods by evaluating their 1,1-diphenyl 2 - picrylhydrazyl radical (DPPH) and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) radical cation scavenging abilities. The primary and secondary metabolites were identified by GC-MS analysis from high polar solvent extract (Methanol) of *Codium tomentosum.*

MATERIALS AND METHODS:

Sample collection and processing

Green Seaweed *Codium sp* was collected from the coastal region of Mandapam, Ramanadhapuram, Tamil Nadu. The collected Samples were thoroughly washed with seawater to remove the surface debris and further washed with distilled water. The cleansed seaweed was shade dried, powdered and stored for further extraction. The seaweed was authenticated as *Codium tomentosum* - Stack house (Fig.1a) by the botanist of the Botanical Survey of India, Howrah, India.

Figure (1a): *Codium tomentosum* Stackhouse

Figure (1b): Solvent extraction by Soxhlet

Extract preparation

Five solvents with different polarities, Hexane, Chloroform, Ethyl acetate, Methanol and Aqueous, were employed to prepare extracts in soxhlet at the proper temperature. The extracts were concentrated, dried and stored at 4ºC. (Fig.1b)

Characterization of Extracts

Qualitative analysis of Phytochemical

Qualitative Phytochemical analysis of all the extracts was carried out by standard methods for the following components - Alkaloids, Terpenoids, Steroids, Phenol, Flavonoid, Tannin, Carbohydrate, Saponin and Glycosides.[26-29]

Test for Alkaloids:

Concentrated 2 mL HCl was added to 2 mL algal extract. A few drops of Mayer's reagent were added. The appearance of a green or white precipitate indicates the presence of alkaloids.

Test for steroids:

To 0.5 mL of extract, 2 mL of chloroform and 1 ml sulphuric acid (H_2SO_4) was added. The appearance of a reddish-brown ring at the interface indicates the presence of Steroids.

Test for tannins:

5% ferric chloride was added to 1 mL of algal extract. The formation of a dark blue or greenish-black colour indicates the presence of tannins.

Test for Terpenoids:

Chloroform (2 mL) and concentrated Sulphuric acid were added to 0.5 mL of algal extract for terpenoid identification. The presence of terpenoids is confirmed by forming a reddish-brown hue at the contact.

Test for flavonoids:

2 mL of algal extract was mixed with 1 mL 2N sodium hydroxide (NaOH). The yellow colour formation confirms the presence of flavonoids.

Test for phenol:

Algal extract (1ml) was diluted with 2 mL of distilled water, and a few drops of 10% ferric chloride were added. The presence of phenols is identified by forming a blue or green colour.

Test for carbohydrate:

To the 2mL of seaweed extract, 5mL of Fehling's solution was added and heated to a boil. The formation of a yellow or brownish-red cuprous oxide precipitate indicated the presence of carbohydrates.

Test for glycosides:

Ammonium solution (10%) and 3 mL chloroform were added to 2 mL algal extract. The formation of pink colour identifies the presence of glycosides.

Test for saponins:

To the 2ml algal extract, an equal volume of distilled water was added and agitated for 15 minutes. The formation of foam indicates the presence of saponins.

Test for amino acids and proteins

To 1.0 ml of seaweed extract, 5-8 drops of 5 % sodium hydroxide solution were added, followed by two drops of 1 % copper sulphate. The formation of pink or purple colour confirmed the presence of amino acids and proteins.

Quantitative Phytochemical Analysis

Estimation of Total Phenol (TPC)

The Folin-Ciocalteau method was used to determine the total phenolic content of the methanol extract [30-33]. An aliquot of 0.1 ml of seaweed extract was mixed with 3 ml of distilled water, and then 0.5 ml of Folin-Ciocalteau reagent was added. To this, 20% sodium carbonate was added and mixed thoroughly. The tubes were incubated in a boiling water bath for 30 min and then cooled, and absorbance was measured at 760 nm. Total phenol concentration was estimated by a standard calibration curve using different concentrations of gallic acid (0.01- 0.1 mM), and the results were expressed as mg of gallic acid equivalents (GAEs) per g of extract.

Estimation of Total Flavonoid Content (TFC)

The aluminium chloride method estimated the TFC of Methanol extracts of seaweed.[34] 0.5 ml of 2% $AlCl_3$ in an ethanol solution was added to 0.5 ml of extract, after one hour of incubation at room temperature, the yellow colour developed. Absorbance was measured at 420 nm with a UV-visible spectrophotometer. A standard graph was prepared using quercetin, and the total flavonoid content was expressed in mean as quercetin equivalent (mg QE /g).

Gas chromatography–Mass Spectrometry (GC–MS)

GCMS analyzed the methanol extract of the *Codium sp* was Shimadzu-QP2010 Plus Model. The following chromatographic conditions were used: Helium was used as the carrier gas at a flow rate of 1.05 mL/min; the injector was operated at 250 °C, and column oven temperature was programmed at 45-280°C at a rate of 10°C/min in injection mode. The following MS conditions were used: an ionization voltage of 70 eV; ion source temperature of 200°C; interface temperature of 280°C; and a mass range of 40 to 700 m/z. The mass spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST 14s.lib (National Institute of Standards and Technology) library and the WILEY8.LIB library.

***In vitro* antioxidant assays**

$DPPH^{\cdot}$ radical scavenging activity

The antioxidant activity of various solvent extracts of *Codium* sp was measured based on the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical [35-36]. One mL of 0.1 mM DPPH solution in methanol was mixed with 1 mL of various seaweed extract concentrations (20 -120 µg/mL). The mixture was then allowed to stand for 30 minutes in the dark. Distilled water was used as the reference standard. One mL of methanol and 1 mL of DPPH solution were used as controls. The decrease in absorbance was measured using a UV-Vis spectrophotometer at 517 nm. The percentage of inhibition was calculated using the following formula:

$$\%\ of\ DPPH^{\cdot}\ radical\ inhibition = [Control - Sample/\ Control] \times 100$$

The IC50 concentration of extracts capable of reducing 50% of DPPH radical were calculated by linear regression using the concentration of samples and the percentage of the inhibition curve.

$ABTS^{\bullet+}$ Radical Scavenging assay

The antioxidant capacity was determined in terms of the ABTS●+ (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) radical cation scavenging activity following the procedure described by Delgado-Andrade.[37] ABTS●+ was obtained by reacting 7 mM ABTS stock solution with 2.45 mM potassium persulfate, and the mixture was left to stand in the dark at room temperature for 12-16 h before use. The ABTS solution (stable for 2 days) was diluted with 5 mM phosphate-buffered saline (pH 7.4) to an absorbance of 0.70±0.02 at 730 nm. After adding ethanol and ethanol extracts of varying concentrations (5-30µg/mL) to 1 mL of diluted ABTS●+ solution, the absorbance was measured at 734 nm after 10 min. The ABTS●+ radical-scavenging activity of the samples was expressed as follows:

$$\%\ of\ ABTS^{\cdot} + radical\ cation\ inhibition = [\ Control - Sample\ /\ Control\ ]\ x\ 100$$

The IC50 concentration of extracts capable of reducing 50% of ABTS radical was calculated by linear regression using the concentration of samples and the percentage of the inhibition curve. Concentration expressed in µg/mL equivalent to Ascorbic acid Standard.

RESULTS AND DISCUSSION

Marine life exhibits an abundance of distinct phytochemical constituents based on their physiological and environmental habitat. Seaweeds have been a good source of bioactive molecules for various applications. Red, Brown and Green seaweeds are recently explored for various environmental, medical [38] and nutraceutical applications [39]. The present study exposed the choice of solvents for extraction, various chemical components and secondary metabolites present in the *Codium tomentosum* with diverse potential.

Qualitative Analysis of Phytochemicals

Table 1 illustrates the results of *Codium tomentosum*'s phytochemical screening. Various metabolites were identified through the current screening of hexane, chloroform, ethyl acetate, methanol, and aqueous extracts. Alkaloids were present in all the extracts except chloroform. Phenol and flavonoids were positive in HE, ME, CE, and EAE, whereas Saponins were absent in chloroform and ethyl acetate extract. Terpenoids, steroids, carbohydrates, glycosides and proteins tested positive in all five solvent extracts [40-41] Among the five solvents, methanol was the best choice, as it was strongly positive for Phenol, flavonoids, tannins and steroids and positive for all other metabolites [42]. The Alkaloids, Phenol, Flavonoids, Terpenoids and Tannins were reported as potential therapeutical agents with antibacterial, antiviral, anti-inflammatory, antiulcer and antioxidant activity, [43-44]. The presence of carbohydrates, amino acids and proteins proves the nutritional value of edible seaweed *Codium tomentosum* [12,45,27]

Table 1: Qualitative analysis of Phytochemicals

Phytochemicals	Aqueous (AE)	Hexane (HE)	Methanol ME)	Ethyl Acetate (EAE)	Chloroform (CE)
Alkaloids	+	++	++	+	-
Phenols	-	+++	+++	++	+
Flavonoids	-	+++	++	++	+
Tannins	+	+	+++	+	-
Saponins	+	++	++	-	-
Terpenoids	+	++	++	+	++
Steroids	+	++	+++	++	++
Carbohydrates	++	++	++	+	+
Glycosides	+	++	++	+	+
Amino acids	++	++	++	+	+
Proteins	++	+++	++	++	+

+++ → Strongly positive, ++ → Moderately positive, + → Positive, - →Absent

Quantitative analysis of Phytochemicals

The quantitative analysis of Phenol and Flavonoid contents in the extracts of *Codium sp*. is shown in Table 2. Total phenol content was expressed as mg equivalent to gallic acid (GAE) per gram and mg/g quercetin equivalent (QE) for Flavonoid. Among the five extracts quantified, Chloroform extract reported highest TPC 194.53±0.008 mg GAE/g, and Methanol extract (138.97±0.007mg GAE/g) with the lowest [46]. While the total quantity of flavonoids reported in various extracts ranged from the lowest in aqueous extract (59.982±0.024 mg QE/g) to the highest in chloroform extract (213.07±0.014 mg QE/g).[47] The solvents employed in the current investigation for the extraction process - chloroform, hexane, ethyl acetate, and methanol were found to be effective in extracting phytochemicals. Flavonoids with antimicrobial, antioxidative, and spasmolytic activity and phenol compounds with antiviral, anti-inflammatory, and anticancer characteristics were reported by Aliyu *et al*[48]. The presence of flavonoids and phenol components implies that *Codium tomentosum* has therapeutic significance.

Table 2: Quantitative analysis of Total Flavonoid and Total Phenol content.

Component (µg/mg)	Methanol Extract	Chloroform Extract	Hexane Extract	Aqueous Extract	Ethyl Acetate Extract
Phenol	138.97±0.007	194.53±0.008	191.06±0.018	142.43±0.018	185.01±0.013
Flavonoid	154.28±0.003	213.07±0.014	205.90±0.022	59.982±0.024	197.85±0.038

Values are expressed as mean ± SD (n=3).

Gas chromatography–Mass Spectrometry (GC–MS)

In the present GCMS analysis, ten phytochemical components were identified from the methanol extract of *C.tomentosum*. Table 3 represents the profile of the components. The chromatogram is portrayed in Fig. 2. Major components identified were Dodecanoic acid, methyl ester; Flavone; 6.13-Pentacenedione, n-Hexadecanoic acid, Oleic acid, Phenol 2,-bis{1.1-dimethylethyl}-4 [{4-hydroxy-3,5 –dimethyl phenyl}methyl]. Most of the components were reported to have bioactivity. Fatty acids such as - Oleic acid, Hexadecanoic acid, Flavones and Methyl ester were reported with antioxidant activity.[42,49-51] Dodecanoic acid was reported to have potential antimicrobial and anti-inflammatory activity .[52,47]

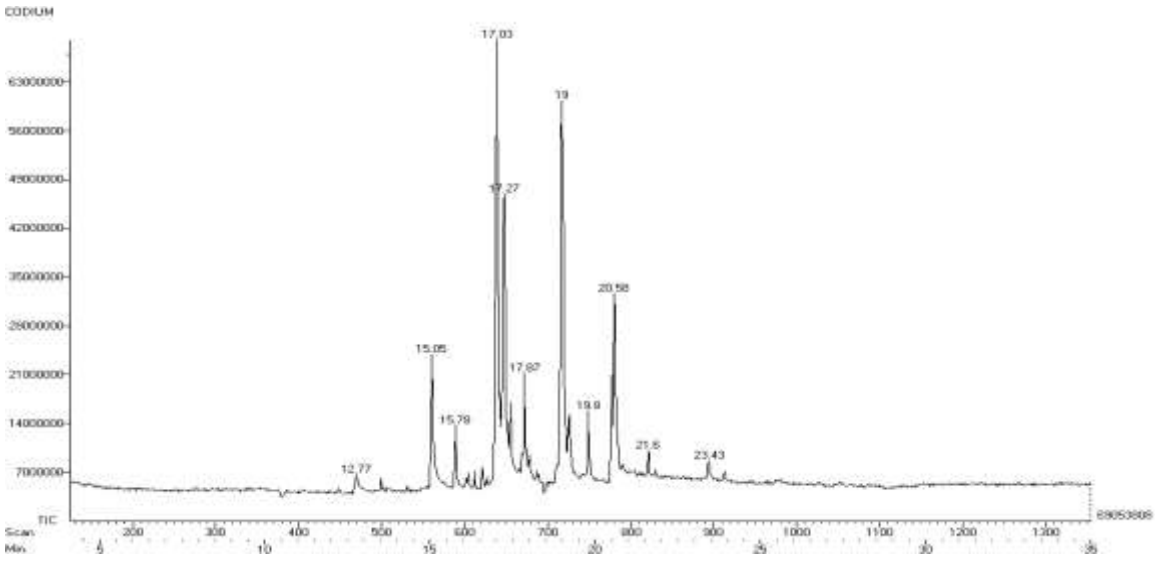
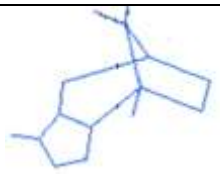
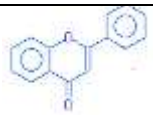
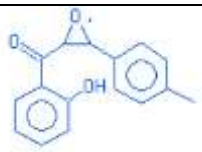
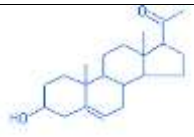
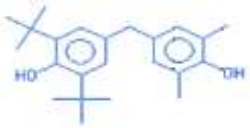
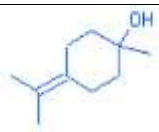
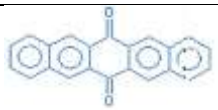
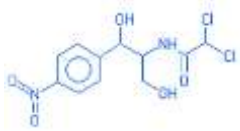


Figure 2: *Codium tomentosum* GCMS chromatogram

Table 3: GC-MS profile of Methanol extract of *Codium tomentosum*

S.No	Structure	Compound Name	RT	MW	MF
1.		4, 7 Methanoazulene,decahydro-1.4.9.9 tetramethyl	15.05	206.37 g/mol	$C_{15}H_{26}$
2.		Flavone	15.78	222.24 g/mol	$C_{15}H_{10}O_2$
3.		Dodecanoic acid ,2,8, dimethyl, methyl ester	17.03	242.4 g/mol	$C_{15}H_{30}O_2$
4.		Phenol 2- [[3-{4methyl phenyl}carbonyl]-	17.27	272.25g/mol	$C_{15}H_{12}O_5$
5.		n-Hexadecanoic acid	17.87	256.42 g/mol	$C_{16}H_{32}O_2$
6.		Pregn-5-en 20 one 3 hydroxy	20.58	1063.2g/mol	$C_{15}H_{82}O_{23}$
7.		Phenol 2,- bis{1.1-dimethylethyl}-4 [{4-hydroxy-3,5 – dimethylphenyl}methyl]-	23.43	340.5g/mol	$C_{23}H_{32}O_2$
8.		Cyclohexanol,1 methyl-4-{1-methylethylidene}-	12.77	182.26g/mol	$C_{11}H_{18}O_2$
9.		6.13-Pentacenedione	19.8	932.2 g/mol	$C_{44}H_{20}Br_4O_4$
10.		Chloramphenicol	21.6	323.13 g/mol	$C_{11}H_{12}Cl_2N_2O_5$
11.		Oleic acid	19	282.5 g/mol	$C_{18}H_{34}O_2$

3.4: *In vitro* Antioxidant assay

Radical Scavenging activity of five solvent extracts of *Codium tomentosum* was observed by DPPH and ABTS methods. The Percentage of Radical Scavenging ability data is presented in Table 4, and the IC 50 value is in Table 5. Corresponding graphs for DPPH and ABTS, % of RSA in Fig. 3(a),3(b) and IC 50 were depicted in Fig 4.

Table 4: *C. tomentosum* DPPH and ABTS % RSA

Test	Concentration	% Radical Scavenging Ability of *C.tomentosum*					
		ME	CE	HE	AE	EAE	STD
DPPH	20	15.13±2.49	24.58±2.57	20.46±2.65	14.78±2.66	16.71±2.65	12.07±0.11
	40	37.66±3.90	39.59±4.01	34.00±3.78	27.80±6.75	41.15±3.88	31.36±0.83
	60	51.62±3.95	51.89±4.73	48.97±5.38	49.73±6.62	60.88±1.35	46.85±1.29
	80	66.92±1.04	67.10±6.02	62.42±1.24	65.79±5.69	82.79±4.80	52.17±0.98
	100	78.91±4.33	79.26±3.96	82.82±4.58	76.90±3.59	89.23±3.17	59.73±1.65
	120	93.23±2.21	95.51±1.79	92.53±1.35	87.21±2.55	95.64±1.51	76.48±1.81
ABTS	20	10.58±2.65	15.62±4.16	20.46±5.50	10.43±2.47	13.05±2.06	12.07±0.11
	40	29.91±3.76	31.57±3.25	34.00±5.61	24.47±4.01	40.41±4.83	55.47±0.40
	60	55.41±0.75	45.09±5.48	48.97±3.36	46.03±1.53	61.82±2.21	79.19±0.57
	80	60.64±3.54	58.85±6.51	62.42±4.01	56.53±4.62	75.88±0.83	93.06±0.68
	100	78.91±4.66	79.26±4.25	82.82±5.85	71.27±6.74	88.88±0.97	96.71±0.70
	120	93.23±2.08	95.51±2.15	92.53±5.41	79.90±4.43	95.64±2.86	99.15±0.54

Result expressed Mean ± S.D the experiment was conducted in triplicates (n=3) value

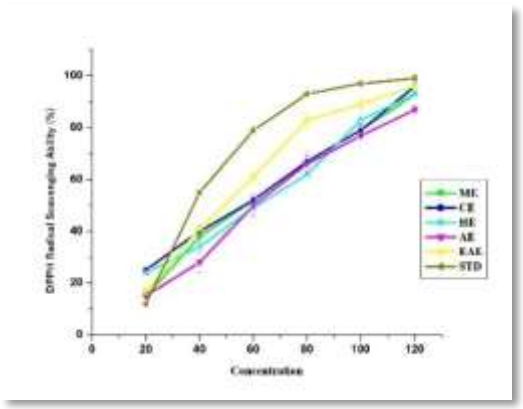


Figure 3(a): DPPH % RSA

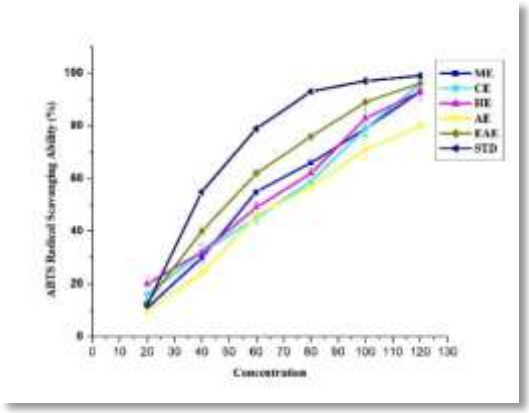


Figure 3(b): ABTS% RSA

Table 5: IC 50 value of various solvent extracts of *C.tomentosum*

Sample	IC 50 µg/mL	
	DPPH	ABTS^{+}
Methanol Extract	64.29	60.41
Chloroform Extract	84.06	56.16
Hexane Extract	60.90	60.75
Aqueous Extract	67.38	65.06
Ethyl Acetate Extract	54.77	52.01
Standard Vitamin C	3.79	2.11

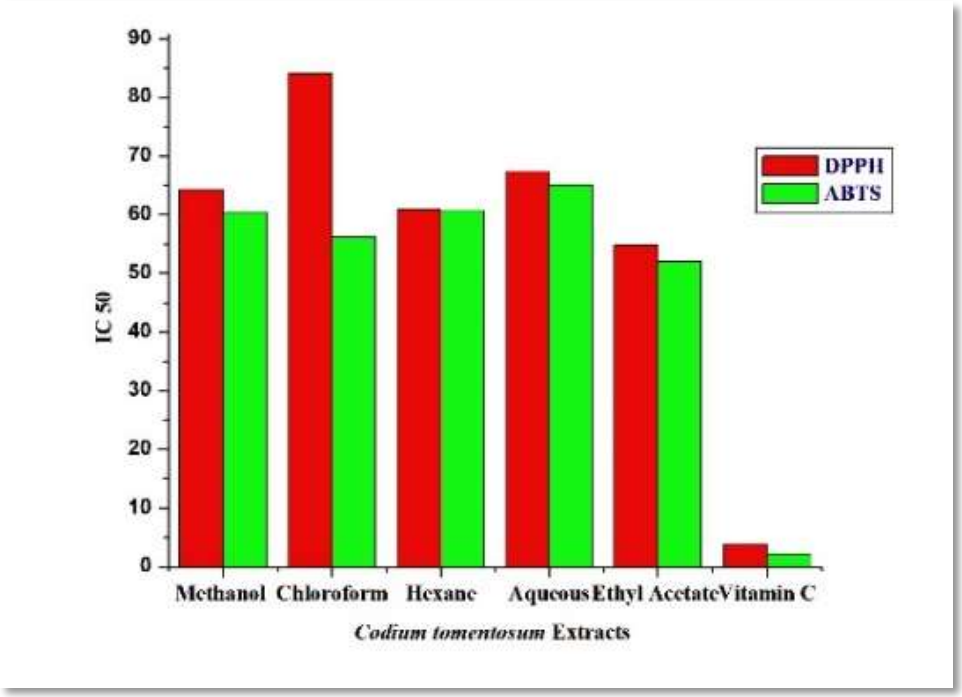


Figure 4: IC 50 of *C.tomentosum*

The presence of Phenolic components and flavonoids increases the antioxidant activity of the seaweed. Among the scavenging ability of all five extracts compared along with the standard Vitamin C (Ascorbic acid), Ethyl Acetate Exhibited IC 50 value at the lowest concentration of 54.77µg/mL for DPPH and 52.01 µg/mL for ABTS. All the extracts displayed radical scavenging ability, with slight differences in their potency. From the present analysis, Ethyl acetate, Hexane and Methanol extracts disclosed high antioxidant potency, followed by chloroform and Aqueous extracts. The presence of various quantities of phytochemicals in the different solvents supports the difference in the efficacy of antioxidant activity of *Codium tomentosum.*[41]

CONCLUSION

Seaweeds are a rich source of important biological components. Edible seaweeds are consumed raw or cooked for their nutritional value and pharmacological importance. The phytochemical analysis of *Codium tomentosum* indicated the presence of significant therapeutical components such as phenol and flavonoids with antioxidant, anti-microbial, and anti-inflammatory potency. Amongst the solvents employed, Methanol, Ethyl Acetate and Hexane displayed an extended range of phytocomponents and *in vitro* anti-oxidative activity. Separation, Purification, green synthesis of nanoparticles and their toxicity studies (*Codium tomentosum)* paves a promising application in medical and pharmaceutical industries in an eco-friendly approach.

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Conflicts of interest:

The authors declare no conflict of interest.

Authors Contribution:

Babini C K: Investigation, Methodology, Resources, analysis and Writing – original draft

Reena A: Conceptualization, Validation, and Supervision

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