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A Comparartive Analysis of the Anticoagulant Property of *Chaetomorpha Antennina* and *Ceratophyllum Submersum*

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Abstract *Marine green algae remain largely unexploited among the three main divisions of macroalgae (i.e., Chlorophyta, Phaeophyta, and Rhodophyta). Interest in utilizing green seaweeds as natural resources has recently increased because of their many active ingredients, particularly those that may be used for medical purposes. Green seaweeds have reported to contain lipid fractions, proteins, peptides, polysaccharide, carotenoids, phenolic compounds, alkaloids, thallus, holdfast, mucilaginous, and whole plants. Among all these active ingredients, polysaccharides are the components most intensively investigated for medical purposes. Thus there are several studies on the anticoagulant properties of polysaccharides isolated from seaweeds. The present research is a Comparative Analysis of the Anticoagulant Property of Chaetomorpha antennina and Chaetomorpha submersum.*

Keywords : *Marine Green Algae, Comparative Analysis, Anticoagulant Property, Chaetomorpha antennina and Chaetomorpha submersum.*

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I. Introduction

Research on the analysis of blood anticoagulant properties of marine seaweeds reveals the usage of sulphated polysaccharides derived from these algae as an alternative basis for the creation of novel anticoagulant drugs (**Dr. B. Bharathiraja *et.al.,* 2016)**. Unfractionated heparins and low molecular weight heparins are only sulphated polysaccharides currently used as anticoagulant drugs. The seaweed used in sulphated polysaccharides production have been known to possess a higher anticoagulant activity similar to heparin (**Harsha Kharal *et.al.,* 2012)**.

Some of the Polysaccharides, such as S-rhamnans, show their action by extending the clotting time via the intrinsic and extrinsic pathways whereas some polysaccharides from green seaweeds also show potent anticoagulant properties but their mechanisms of action are associated not only to a direct increase in the clotting time (APTT assays) by inhibiting the contact activation pathway (intrinsic pathway), but also by inhibiting the heparin cofactor II-mediated action of thrombin thus showing a potent antithrombotic bioactivity. Research has revelaed that a higher content of fucose and sulphate groups coincided with higher anticoagulant activities of sulphated polysaccharide fractions (**Hafiz Ansar Rasul Suleria *et. al.,* 2015, Hugo Alexandre Oliveira Rocha, 2012, Kambiz Jahanbina *et.al.,* 2010** and **M. R. Sahaa *et.al.,* 2010).**

Carbohydrate polymers of marine green algae have recently been exploited for various applications (**M. S. Leelavathi** and **Prasad M. P, 2015)**. Green algal polysaccharides have emerged as rich and important sources of bioactive natural compounds with a wide range of physiological and biological activities including immunomodulation, anti-inflammation, antioxidant, anticoagulant, and antitumor (**M. Shanmugam *et. al.,* 2001)**. Sulphated polysaccharides (SPs) of green seaweeds, are chemically and physicochemically different from those of land plants, and may have special physiological effects on the human body. SPs are associated with the surface of animal cells and are involved in biological activities, such as cell recognition, cell adhesion, and regulation of receptor functions, which are of great interest in medicine (**Manoj Saravana Guru *et.al.,* 2013)**.

Anticoagulants are especially used in medication to prevent the clotting of blood. Many oral coagulants are available these days. Various anticoagulant substances are also used in blood sampling equipment (**Maria Filomena de Jesus Raposo *et.al.,* 2013)**. Since the start of 21st century numerous new agents have been recognized and introduced, and referred as the directly acting oral anticoagulants (**Meenakshi Bhattachaerjee, 2016).** A study on the anti-coagulant property of the purified polysaccharides from *seaweeds* involved Hot water extraction and ethanol in the extraction process (**Mitali Priyadharshini Pati *et.al.,* 2016)**. The purification ion exchange and HPLC were used.

II. Materials And Methodology

Collection, Processing and Extraction of Seaweeds

Chaetomorpha antennina and *Ceratophyllum submersum* were collected from the shores of Royapuram fishing harbour (N4beach) in Chennai. The samples were manually collected; epiphytes and debris were removed by washing in running tap water and washed again with distilled water. The samples were then allowed to shade dry for 7 days at room temperature and were finely powdered using an electric blender. The materials required for the extraction process are *Chaetomorpha antennina* and *Ceratophyllum submersum*, Solvent (Methanol) 500ml and Conical flask (500 ml). 10gms of the dried Green algae and aquatic plant were extracted separately in 100ml of Methanol (1: 10 ratio) for 3 days in a separate conical flask. The solvent were filtered using a muslin cloth or filter paper. The filtrates were stored in screw capped container for further analysis.

Extraction of Crude Polysaccharides (Silva *et al)*

The materials required for the extraction of Crude Polysaccharides are dried powdered sample, Acetone, 0.25M Sodium chloride (Nacl), Sodium hydroxide (NaOH), Trypsin, Filter paper or cheese cloth and centrifuge tubes. 10g of powder sample was incubated overnight with acetone to remove lipid and pigments. The residue was then dissolved in 5 volumes of 0.25M NaCl, and the pH was monitored periodically and adjusted to 8 using NaOH. 10mg of trypsin was added to the content for proteolysis and incubated for 24 hours. After incubation, the content was filtered through cheese cloth or filter paper. The filtrate was precipitated using ice cold acetone under gentle agitation at 4°C. The precipitate formed was centrifuged at 10,000rpm for 20 minutes. The total polysaccharide extract was dried under vacuum. Extracted polysaccharide was re-suspended in distilled water and was used for further analysis.

Purification of Polysaccharides

Column Chromatography and Dialysis

The materials required for Column Chromatography and Dialysis are Crude polysaccharides, DEAE Cellulose column (3×45cm), Sodium chloride (0-3M), Dialysis bag and Distilled water. 50mg of crude polysaccharides was dissolved in 10ml of distilled water. It was applied to a DEAE cellulose column pre equilibrated with water and eluted in NaCl gradient (0-3M) until no carbohydrate was detected. Each fraction was assayed for carbohydrate content by phenol sulphuric acid method. The carbohydrate-positive fractions were pooled together and dialyzed (MWCO 14,000) for 24 hours against distilled water.

CHEMICAL ANALYSIS

ESTIMATION OF CARBOHYDRATES (PHENOL-SULPHURIC ACID- Dubois *et al.*,)

The materials required for the Estimation of Carbohydrates by Phenol – Sulphuric acid method are Polysaccharides, 5% Phenol, 96% Sulphuric acid, 2.5N Hydrochloric acid, Sodium carbonate, Glucose (standard), Stock- 100mg glucose dissolved in 100 ml of distilled water and Standard- 10ml of stock was made upto 100ml. 100mg of the sample was weighed into the boiling tubes. They were hydrolysed by keeping in boiling water bath for 3 hrs with 5ml of 2.5N Hydrochloric acid and was cooled to room temperature. The solution was neutralized with solid sodium carbonate until the effervescence ceased. It was made to a volume of 100ml and was centrifuged. The standard in ranging concentration (0.2ml-1ml) was pipette into a series of test tubes.0.2ml of the extract was pipette in 2 separate test tubes. The volume was made up to 1ml in each tube with distilled water. 1ml of phenol solution was added to each tube followed by 5ml of 96% Sulphuric acid and was shaken well. After 10 minutes the contents in the tubes were shaken and were placed in a water bath at 25°-30° C for 20 minutes. A blank with 1 ml of distilled water was set. The colour was read at 490nm and the amount of total carbohydrates was calculated using the standard graph.

ESTIMATION OF PROTEIN (LOWRY'S METHOD)

Protein was estimated using the Lowry's Method. The materials required are

- Fiolin-ciocalteau reagent
- Reagent A- 20% sodium carbonate in 0.1N Sodium hydroxide
- Reagent B- 0.5% copper sulphate in 1% potassium sodium tartarate
- Reagent C- Alkaline copper solution (50ml of A and 1ml of B reagents).
- Stock solution- 50mg of Bovine serum albumin dissolved in distilled water and made upto 50ml in standard flask.
- Standard solution- 10ml of the stock solution was diluted to 50ml with distilled water in standard flask. 1.0ml of this solution contains200µg of protein
- Polysaccharides

The standards in ranging concentration (0.2ml-1ml) were transferred into a series of test tubes. 0.2ml of sample extract was also transferred into two other test tubes. The volume was made upto 1.0ml in all the test tubes. 5ml

alkaline copper solution was added to each tube including the blank. It was mixed well and was allowed to stand for10mins. 0.5ml of Fiolin's-ciocalteau reagent was added**.** It was mixed well and was incubated at room temperature in the dark for 30 minutes till Blue colour developed. The Absorbance was read at 620nm.

ANTI-THROMBOPLASTIC ACTIVITY

The materials required for Antothromboplastic Activity are Aquatic weed extracts, Polysaccharides, 5 Glass slides, Sterile lancet, Cotton and Ethanol, The finger was wiped with ethanol and was pierced with a lancet and two drops of blood was placed in all the 5 slides.One slide was labeled as control. The Weed extract and polysaccharides were added to the slides. The clotting time was noted carefully and tabulated.

III. Results And Discussion

COLLECTION, PROCESSING AND EXTRACTION OF SEAWEEDS

10gms of the dried Green algae and aquatic plant were extracted separately and was placed in 100ml of Methanol (1: 10 ratio) for 3 days in a separate conical flask. The solvent were filtered using a muslin cloth or filter paper. The filtrates were stored in screw capped container for further analysis **(Figures 1 & 2**).

Figure 1 : *Chaetomorpha antennina*

Figure 2 : *Ceratophyllum submersum*

EXTRACTION OF CRUDE POLYSACCHARIDES (Silva *et al)*

The total polysaccharides extract were dried under vacuum. Extracted polysaccharides were re-suspended in distilled water and were used for further analysis (**Figures 3& 4**).

Figure 3 : *Chaetomsorpha* extract

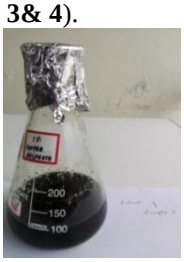


Figure 4 : *Ceratophyllum* extract

EXTRACTION OF CRUDE POLYSACCHARIDES

Extraction resulted by yielding 0.5g of green solid crude polysaccharides from 10g of *Chaetomorpha antennina* and 0.4g of brownish green crude polysaccharides from 10g of *Ceratophyllum submersum* **(Figures 5 & 6).**

Figure 5 : Polysaccharides after centrifuge

Figure 6 : Dry crude polysaccharides

COLUMN CHROMATOGRAPHY AND DIALYSIS

A few grams of crude polysaccharides were dissolved in 10ml of distilled water. From that 3ml of diluted samples were added to DEAE- Cellulose Column and were eluted with different gradients of NaCl (0-3M). Different fractions which contain polysaccharides were separated based on their ionic character at different molarity. 50ml of partially purified polysaccharides from *Chaetomorpha antennina* and 35ml of partially purified polysaccharides *Ceratophyllum submersum* were collected. The partially purified polysaccharides were subjected to dialysis. 26ml of purified polysaccharide from *Chaetomorpha antennina* and 17ml of purified polysaccharide from *Ceratophyllum submersum* were obtained **(Figures 7 - 10).**

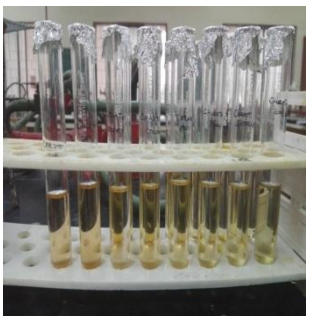



Figure 7 **Figure 8** **Figure 9** **Figure 10**

Figure 7 : Column Chromatography of *Chaetomorpha antennina*
Figure 8 : Partially purified Polysaccharides of *Chaetomorpha antennina*
Figure 9 : Column Chromatography of *Ceratophyllum submersum*
Figure 10 : Partially purified Polysaccharides of *Ceratophyllum submersum*

CHEMICAL ANALYSIS
ESTIMATION OF CARBOHYDRATES
GLUCOSE (STANDARD)

By phenol sulphuric acid method, 64mg/ml of carbohydrates in *Chaetomorpha antennina* and 68mg/ml of carbohydrates in *Ceratophyllum submersum* were estimated. Chemical composition of the purified polysaccharide from *Chaetomorpha antennina* and *Ceratophyllum submersum* were determined as carbohydrate content **(Tables 1 – 3** and **Graphs 1 & 2)**.

SAMPLE	O.D (490nm)	Concentration of Glucose (mg/ml)
C.antennina	0.14	64

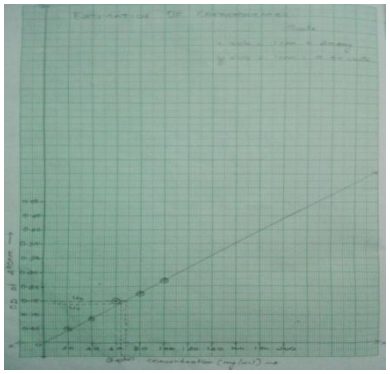
Table 1 : Total Carbohydrate Content of *C. antennina*

SAMPLE	O.D (490nm)	Concentration of Glucose (mg/ml)
C.submersum	0.15	68

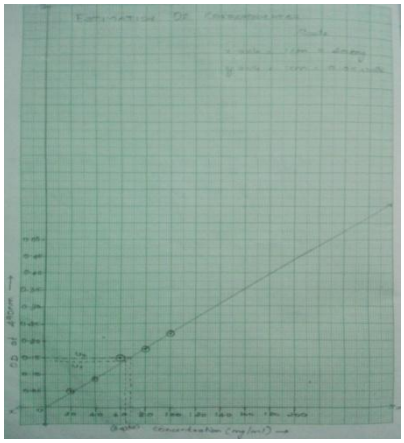
Table 2: Total Carbohydrate Content of *Ceratophyllum submersum*

S.NO	CONCENTRATION OF GLUCOSE (mg/ml)	O.D (490nm)
1	20	0.05
2	40	0.09
3	60	0.15
4	80	0.18
5	100	0.22

Table 3 : **Results for Glucose Standard Curve**



Graph 1 : Chemical Analysis for Carbohydrates of *Chaetomorpha antennina*



Graph 2 : Chemical Analysis for Carbohydrates of *Ceratophyllum submersum*

ESTIMATION OF PROTEINS

BOVINE SERUM ALBUMIN- BSA (STANDARD)

By Lowry's method, 4mg/ml of protein content was estimated both in *C.antennina* and *Ceratophyllum submersum* **(Tables 4 - 6).** In the chemical composition of purified polysaccharides only a small concentration of protein were present.

SAMPLE	O.D (620nm)	Concentration of protein (mg/ml)
C.antennina	0.03	4

Table 4 : Total Protein Content of *C.antennina*

SAMPLE	O.D (620nm)	Concentration of protein (mg/ml)
Ceratophyllum submersum	0.03	4

Table 5 : Total Protein Content of *Ceratophyllum submersum*

S.NO	CONCENTRATION OF BSA (mg/ml)	O.D (620nm)
1	20	0.10
2	40	0.27
3	60	0.43
4	80	0.56
5	100	0.70

Table 6: Results for Bovine Serum Albumin Standard Curve

FT-IR SPECTRUM for *Chaetomorpha* Crude Extract

The FTIR spectrum for the *Chaetomorpha* extract was analysed (**Figure 11**). The absorbance band were in the region of 3437cm^{-1} corresponds to the hydroxyl stretching vibration of the polysaccharides and that at 2923cm^{-1} corresponds to a weak C-H bonds. The intense peak at 1636cm^{-1}were equivalent to that of galactans. The region at 1415cm^{-1} indicates the carboxylic acid. The peaks around 1324cm-1 are the skeleton of galactans. The most important band were found at 1253^{-1}cm which indicated sulphatic groups (S=O). The most important band was found at 1028.06cm^{-1}.The band at 825cm^{-1} shows the mannuronic units.

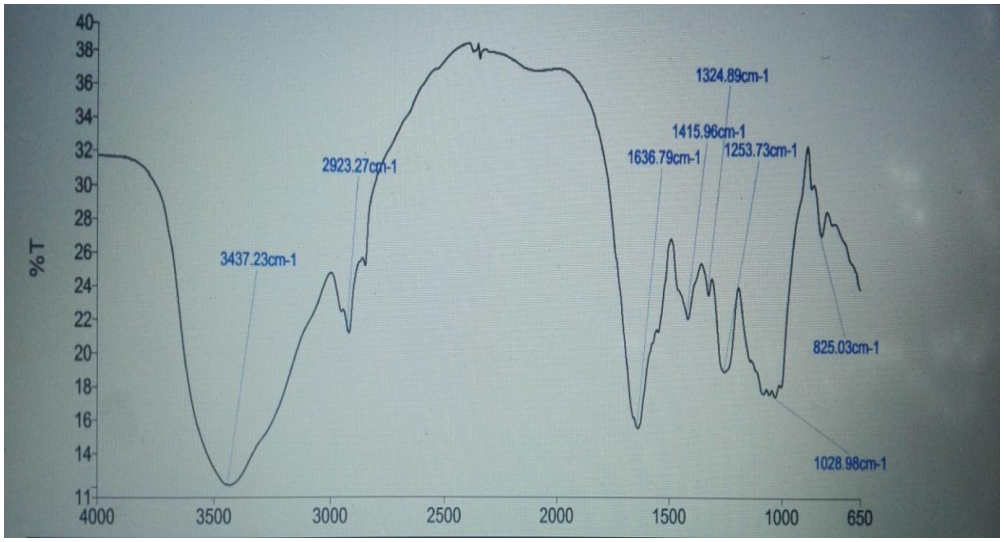


Figure 11 : FT-IR Image of *Chaetomorpha* Crude Extract

FT-IR SPECTRUM for *Ceratophyllum* Crude Extract

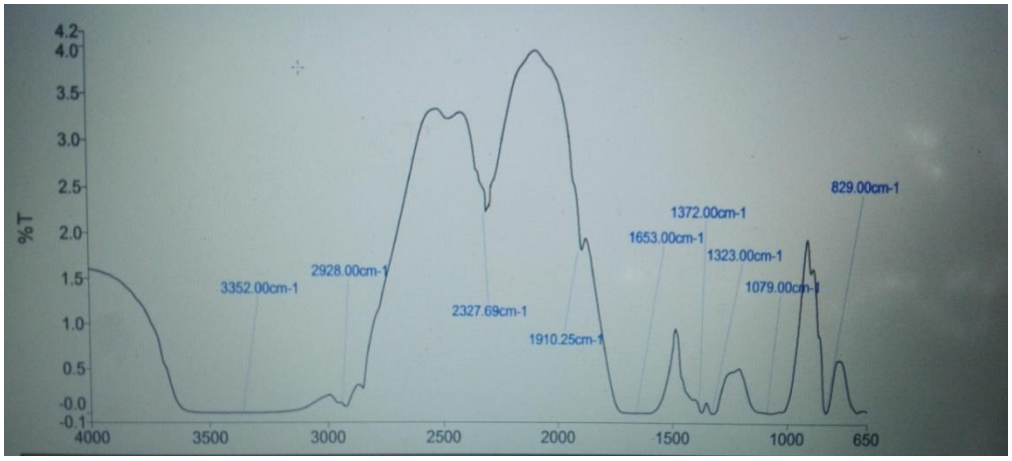


Figure 12 : FT-IR image of *Ceratophyllum* Crude Extract

The FTIR spectrum for the *Ceratophyllum* extract was analysed (**Figure 12**). The absorbance band in the region of 3352cm^{-1} corresponds to the hydroxyl stretching vibration of the polysaccharides and that at 2928cm^{-1} corresponds to a weak C-H bonds. The region at 2327cm^{-1}are equivalent to the alkyl group. The range at 1910cm^{-1} indicates the carbonyl group. The peak around 1653cm^{-1} are the C=H bonds. The band found at 1372cm^{-1} indicated carboxylic acid. The band at 1323cm^{-1} shows the galactan units. The most important band in the region 1079cm^{-1} was indicated as carbohydrates.

FTIR SPECTRUM for Polysaccharide of *Chaetomorpha*

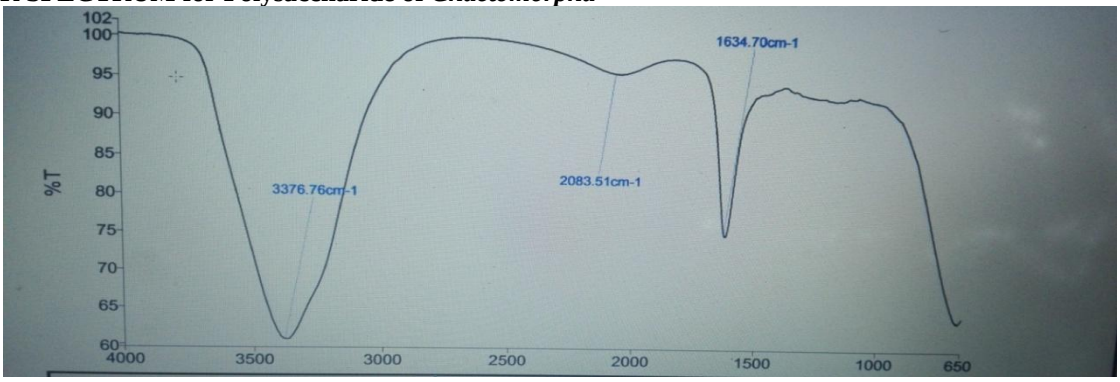


Figure 13 : FT-IR Image for *Chaetomorpha* Polysaccharide

The FTIR spectrum for the polysaccharide was analysed (**Figure 13**). The intense band at the region of 3376cm^{-1} indicated the hydroxyl group. The vibration at the region of 2083cm^{-1} shows the alkenes groups (C=C). The narrow steep range at 1634cm^{-1} represents the galactans.

FTIR SPECTRUM for Polysaccharide of *Ceratophyllum*

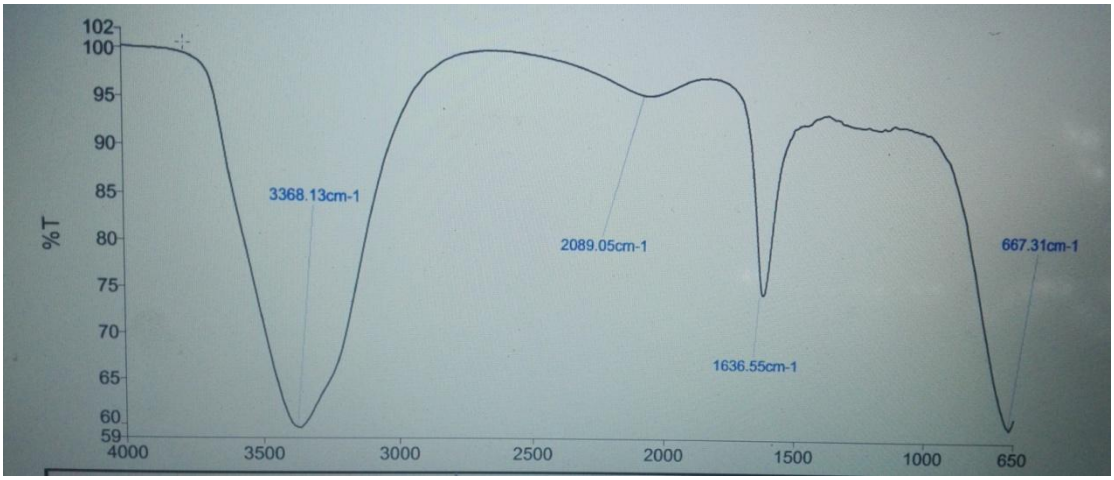


Figure 14 : FT-IR Image for *Ceratophyllum* polysaccharide

The FTIR spectrum for the polysaccharide of *Ceratophyllum* was analysed (**Figure 14**). The maximum absorbance at the region of 3368cm^{-1} was indicated as hydroxyl group stretching vibration of polysaccharides. The mild vibration at region of 2089cm^{-1} represents alkenes (C=C). The band at the region of 1636cm^{-1} indicates carboxylate O-CO bonds. The intense peak at the region of 667cm^{-1} represents the sulphate ester. The polysaccharide samples show a maximum absorption peak at 2900cm$^{-1.}$ Many intense peaks represents C=O, C-H, carboxylic bond, mannuronic unit, galactans and OH bonds which are evident to show there is presence of carbohydrates.

ANTITHROMBOPLASTIC ACTIVITY

To a drop of blood, 5µl of *Chaetomorpha antennina* and *Ceratophyllum submersum* extracts and extracted polysaccharides were added **(Figures 15 & 16)**. The aquatic weeds showed anticoagulant activity but the purified polysaccharides of both the samples showed maximum anticoagulant activity. The control coagulated within 10 minutes **(Tables 7 & 8).**

S.NO	SAMPLES	CLOTTING TIME (in minutes)
1	*Control*	8
2	*Chaetomorpha* extract	20
3	*Chaetomorpha polysaccharide*	32

Table 7 : Anti-coagulant activity

S.NO	SAMPLES	CLOTTING TIME (in minutes)
1	*Control*	8
2	*Ceratophyllum extract*	25
3	*Ceratophyllum polysaccharide*	35

Table 8 : Anti- coagulant Activity

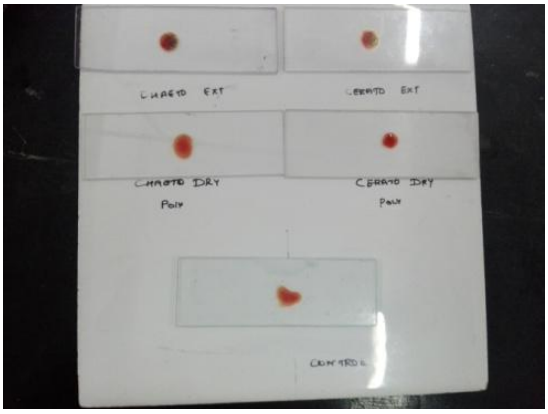


Figure 15: Anticoagulant activity in *Chaetomorpha* extract and its Polysaccharides

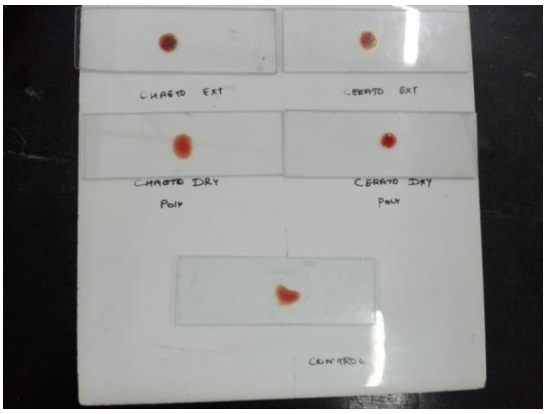


Figure 16 : Anticoagulant activity in *Ceratophyllum extract* and its polysaccharides

IV. Conclusion

There are several studies on the anticoagulant properties of polysaccharides isolated from seaweeds. Research has shown that sulphated polysaccharides from green seaweeds to possess anticoagulant properties. Some polysaccharides from green seaweeds besides showing potent anticoagulant properties are also associated not only to a direct increase in the clotting time (APTT assays) by inhibiting the contact activation pathway (intrinsic pathway), but also by inhibiting the heparin cofactor II-mediated action of thrombin thus showing a potent antithrombotic bioactivity. Carbohydrate polymers of marine green algae have recently been exploited for various applications and green algal polysaccharides have emerged as rich and important sources of bioactive natural compounds with a wide range of physiological and biological activities including immunomodulation, anti-inflammation, antioxidant, anticoagulant, and antitumor. Thus the present Comparative Study of the Antico-agulant Property of *Chaetomorpha antennina* and *Ceratophyllum submersum* paves way for tapping its potential usage and contribution to the field of Medical Science in the future.

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