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ANTIFOULING EFFECTS OF *GRACILARIA EDULIS* TO SCREEN THE BIOCHEMICAL AND CYTOTOXICITY ASSAY IN MALLIPATTINAM HARBOUR

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ABSTRACT

Ship operators have faced the problem of biofouling, or the adhesion and growth of organisms on submerged, man-made surfaces, for at least two millennia. Barnacles and other sessile marine invertebrates contribute to biofouling, which raises the frictional resistance of a ship's hull and increases the power and fuel needed to maintain speed. More than a century ago, scientists and engineers realized that a deeper understanding of the biology of the organisms involved particularly with regard to larval settlement and metamorphosis as well as adhesives and adhesion would be necessary to solve the biofouling problem. Using barnacles has been a valuable tool for this research. One strategy for studying innovative non-toxic anti-fouling materials used in this study is to take advantage of some marine organisms' intrinsic ability to manufacture chemical defense compounds in order to maintain an epibiont-free surface. In this study *Gracilaria edulis* was intensely analyzed for its biochemical constituents like protein and lipid content along with the cytotoxicity analysis. These newly developed materials can be helpful probes to further our understanding of these processes, even though basic research on topics like the nature of the interaction between organismal adhesives and the substrate or the ability of settling larvae to perceive surface cues has not had a significant impact on the development of most current biofouling control technologies.

Keywords: Barnacles; anti-fouling; Chemical compound; Biochemical; toxicity; *Garcilaria edulis*

INTRODUCTION

A significant group among the fouling communities found in the oceans around the world are barnacles. Due to control procedures and cleaning initiatives, the settlement of marine creatures on ships and maritime infrastructure, including barnacles, bivalves, tube worms, and ascidians, results in significant financial losses. Coatings are typically used on surfaces to prevent organisms from attaching. However, a worldwide prohibition on TBT compounds for marine uses was introduced due to ecological concerns including the bioaccumulation of harmful compounds in non-target organisms, such as fish. Following the global prohibition on TBT, numerous environmentally acceptable anti-fouling techniques have been implemented, and the hunt for a suitable replacement is still ongoing. Fouling-release coatings are a significant alternative that are currently the subject of intense investigation. These coatings rely on the development of weak connections between the surface and marine organisms. (Almeida *et.al.,* 2014)

Seaweed offers a wide range of therapeutic possibilities both internally and externally. Seaweed is an extensive profile source of secondary metabolites. More than 600 secondary metabolites have been isolated from marine algae (Faulkner, 1986). Although a majority of these (about 60%) are terpenes, some fatty acids are also common (20%) with nitrogenous compounds. Many of these compounds are bioactive and have been extensively studied using bioassays and pharmacological assays. In this study *Gracilaria edulis* has been used to study biological activities and the toxicology studies.

In order to overcome the biofouling problems, antifouling paints for marine structures have been developed. Antifouling paints based on tributyltin and other organotin compounds as the active agents pose a serious threat to the marine environment. (Furman, 2010)

A detailed study of the attachment mechanisms of the many species involved in biofouling was necessary for the construction of foul-release coatings. Every fouling creature, including bacteria, produces chemicals that are sticky and may aid in the organisms' ability to settle on surfaces. The adhesive mechanism of *Mytilus edulis*, the blue mussel, has been thoroughly investigated. The majority of adhesives found in marine creatures are mostly composed of a high number of proteins. However, because it is hard to obtain the necessary quantities and because they cure quickly, the adhesive mechanisms of barnacles are not much researched.

*Amphibalanus amphitrite (Balanus amphitrite)*is a predominant species of barnacle found in tropical and subtropical seas' intertidal and subtidal fouling ecosystems. They are frequently observed on submerged surfaces and adhering to the shells of gastropods and mollusks. The current study aims to compare commonly used tests using an ecotoxicological test using organisms to ascertain the susceptibility of larvae to metals. (Cuculescu *et.al.,* 2015)

MATERIALS AND METHODS

Collection and Maintenance of Adults

The barnacle (*Balanus Amphitrite*) adults were gathered and brought to the laboratory in a bucket with seawater together with our various substratum in mallipattinam Harbour**(10.2775° N,**

79.3188° E). They were identified by using the guidelines of fouling organisms and instruction regarding the docking report (1978). Adults in the lab were housed in an aerated tank after being washed by spraying seawater over them. (Beutler, 2015)

Sample preparation

Following the grinding of the samples, a 3 ml solution of TCA (10%) in acetone and 2 ME (0.07%) was homogenized. The entire protein was precipitated at -20°C for an entire night. The precipitate underwent vortexing and was centrifuged for 15 minutes at 4°C and 13,000 rpm. The resulting pellet was rinsed three times with acetone that had been enhanced with one tablet of complete EDTA-free protease inhibitor, two ME (0.07%), and two EDTA (2 mM). 500 µl of chilled wash buffer was added for each washing, vortexed quickly, then centrifuged for 15 minutes at 4°C at 13,000 rpm. The last rinse was done with 100% acetone that had been refrigerated beforehand. An air-dried pellet was stored at -80°C for a whole night in order to eliminate any last traces of acetone. Next, the pellet was dissolved in buffer used for rehydration.(Ivankovic *et.al.,* 2012)

Collection and Preservation of Macro-algae

Seaweed was collected during the lowest tide of chart datum from the seaweed infested locations along the southeast coast of India, Mallipattinam Harbour, Thanjavur district,Tamilnadu, India. The live and healthy macro algal sample was collected by handpicking method at a depth of 1-2m.Immediately after collection the surface of seaweed were washed in fresh sea water to eliminate the epiphytes, extraneous matter course sand and other calcareous impurities. The collected macro algae samples were transported to laboratory in polythene bags under ice at 20^{o}c.

Seaweed sample preparation (*Gracilaria edulis*)

The collected seaweeds were cut in to small pieces, shade dried for two weeks and later the dried seaweed was coarsely ground and cleaned three times in an ultra-sonicator bath to remove the free salts and other debris. These seaweeds were then blotted dry using filter paper and were air dried. One gram of powdered seaweed was added to 100 ml of de-ionized water,was heated, and was maintained at 600^{θ}C for 20 minutes. The dried aqueous extract powder was used for biochemical and toxicity studies.(Kappus, 2016)

Isolation of cement from the *Balanus Amphitrite*

A thin micro-needle was used to separate the glands in order to isolate the cement from the barnacle. With a few adjustments, the techniques provided by Kamino *et al.,* 2000 were used for the cement's additional analysis. For later usage, the cement was often kept in storage at -20^{o}C right away. The cement was then suspended in a solution of 10 mM sodium phosphate buffer containing 6 mM guanidine hydrochloride. The blend underwent a one-hour, 20,000×g centrifugation at 20°C. After being re-suspended in the same solution, the precipitate was centrifuged once more. The resultant precipitate was again suspended in Tris-HCL buffer that contained 20mM EDTA and 7M guanidine hydrochloride. It was then reduced for one hour at 60°C using 0.5M dithiothreitol (DTT). Following the reduction, 2-mercaptoethanol and mono-iodoacetic acid (a 2.5-fold addition to DTT) were added to the mixture. After centrifuging the

mixture, the supernatant was separated and saved for additional examination. (Medeiros *et.al.*, 2015)

Analysis of Biochemical Composition of cement

Using established procedures, the biochemical composition, including the amounts of protein, lipids, and carbohydrates, was examined. This cement's protein content was measured using Bradford's (2016) technique. Estimates of lipids were made using the Folch technique. reduction of sugar estimated using Benedict's technique. (Keen *et.al.,* 2016)

PROTEIN ESTIMATION USING BRADFORD'S METHOD

M. Bradford created the Bradford method that is currently in use the most. It is based on the finding that when Coomassie Brilliant Blue G-250 dye attaches to a protein in an acidic solution, its wavelength shifts from 465 nm to 595 nm. The dye is in the cationic form and has a maximum absorbance of about 470 nm when it is not bound. The protein's hydrophobic and basic amino acids interact with the dye. The color of the dye varies from brown to blue to darker blue hues as the concentration of protein increases. Therefore, there is a linear relationship between absorbance and the sample's total protein concentration over a small range; that is, the dye-protein complex's light absorbance at 595 nm is proportionate to the amount of protein bound (within a limited range).(Mirlean *et.al.,* 2013)

LIPID ESTIMATION BY VANILLIN METHOD

Every 100 g sample of either fresh or frozen tissue is homogenized using a 100 ml chloroform and 200 ml methanol combination in a Waring Blender for two minutes. Following a 30-second blending period, 100 ml of chloroform and 100 ml of distilled water are added to the mixture, and the blending process is repeated for an additional 30-seconds. With a small amount of suction, the homogenate is filtered through Whatman No. 1 filter paper using a Coors No. 3 Buchner funnel. In order to guarantee the most possible solvent recovery, pressure is applied with the bottom of a beaker as the residue starts to dry out during the typically quick filtration process. After letting the filtrate settle completely and become clear for a few minutes, the alcoholic layer is aspirated out of the 500 ml graduated cylinder and the volume of the chloroform layer which must be at least 150 ml is measured. To guarantee that the top layer is completely removed, a tiny amount of the chloroform layer is also removed. The refined lipid is present in the chloroform layer.(Niyogi *et.al.,* 2011)

ESTIMATION OF REDUCING SUGAR BY BENEDICT'S METHOD

Approximately 5 milliliters of Benedict's reagent were poured into a sanitized conical flask. To get the necessary alkalinity, about 600 mg of anhydrous sodium carbonate was added, along with a few pieces of porcelain, and heated to boiling over a medium flame. In a burette, standard glucose solution is taken. Benedict's solution is continually brought to a boil before adding drops of glucose solution (1 drop per second) until the last vestige of blue color is gone. The titrations are repeated until concordant readings are reached after noting the amount of glucose rundown. Distilled water was used to make up to 100 milliliters of the specified unknown sugar solution in a standard flask. The Benedict's reagent was then titrated as previously, and the burette was then filled with an unidentified sugar solution. After noting the

depletion of the sugar solution volume, titrations are repeated to obtain concordant values.(Keepler, 2019)

Toxicity analysis

The toxicity analysis of the test sample that was provided was carried out using barnacles. In short, five batches of five barnacles and the control were gathered. The barnacles (GE) were treated with varying doses of the sample (500 g/ml, 250 g/ml, 100 g/ml, 50 g/ml, and 10 g/ml). The number of dead barnacles was counted after a 24-hour exposure period, and the average of five replicates was used to determine the percentage of mortality. The following formula was developed using Graph Pad Prism software and used to determine the fatality percentage. (USA). (Lau *et.al.,* 2013)

RESULTS

The samples were identified by their morphology, which included surface roughness, pigmentation, and colony forming units. There were obvious bacterial colonies on top of the two-layer plate's second layer. These colonies were thought to be possible enemies of the fungus because of the obvious halo surrounding them. For additional research, microorganisms from colonies with a clear halo (wider than 2 mm) were purified.*Balanus Amphitrite*dropping had the highest count in bacterialand fungal count while contaminated soil had the lowestcount in bacterial and fungalcount before amendment. The figure 1 illustrates maximum colonies were observed in wood (180/CFU) when compared to fibre, iron and concrete.(Manduzio *et.al.,* 2014)

Figure 1: Enumeration of fungal organism from Test sample (Barnacles)

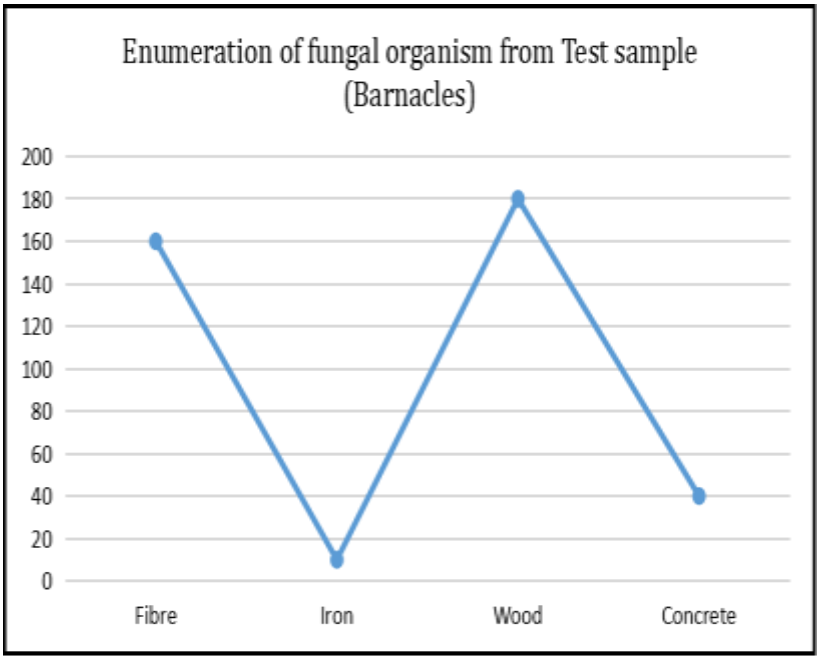
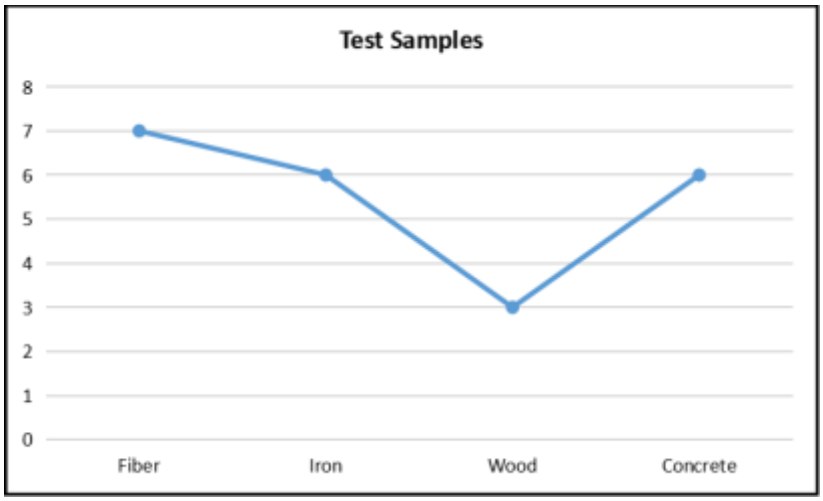


Fig 2: Test Samples



The total fiber content was present in the extract sample Control was found to be 3.967 mg/ml.The total iron content was present in the extract sample10 µg/ml was found to be 4.603mg/ml.The total wood content was present in the extract sample 50 µg/ml was found to be 4.357mg/ml.The total Concrete content was present in the extract sample 100 µg/ml was found to be 4.323mg/ml.(Matthiensen *et.al.*, 2010)

PROTEIN ESTIMATION

These secondary metabolites are also responsible for the defense mechanism showed by barnacles against various disease-causing organisms. The total protein content of the aqueous extract was found to be 300µg/mL at O.D 660nm using BSA as a standard curved. The extract showed good quantity of protein content, which help the plant to carry its various enzymatic as well as structural and functional use. (Regoli *et.al.*, 2012)

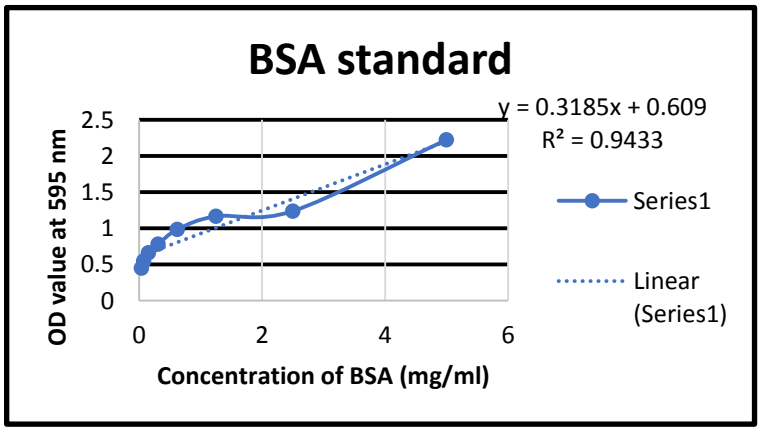


Figure 2: BSA Standard

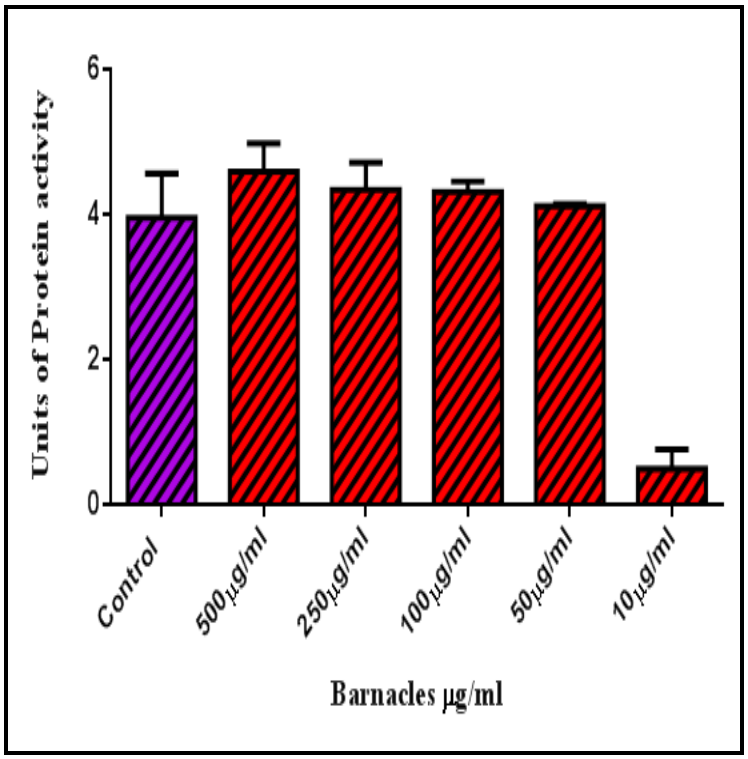


Figure 3: Protein Activity

LIPID ESTIMATION

The total lipid content was present in the extract sample Barnacles Control was found to be 0.942mg/ml.The total lipid content was present in the extract sample Barnacles -250µg/ml was found to be 0.248mg/ml.The total lipid content was present in the extract sample Barnacles -100µg/ml was found to be 0.928 mg/ml.The total lipid content was present in the extract sample Barnacles -50µg/ml was found to be 1.186mg/ml.The total lipid content was present in the extract sample Barnacles -10 µg/ml was found to be 2.16mg/ml.(Rosa *et.al.*, 2015)

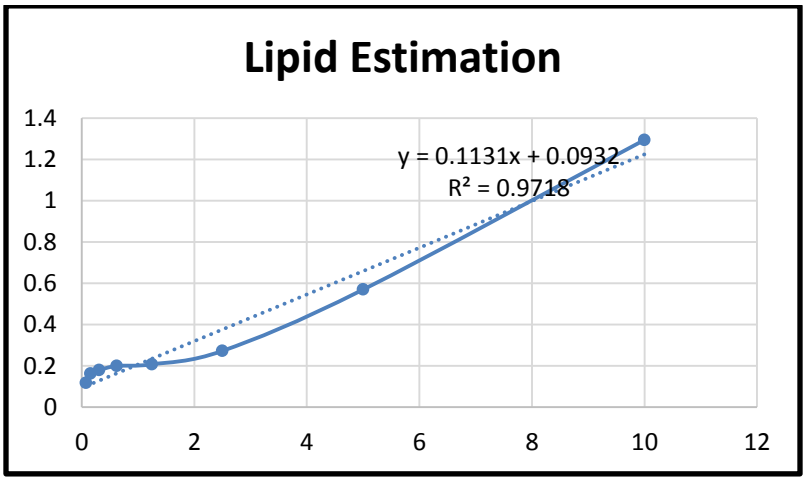


Figure 4: Lipid estimation

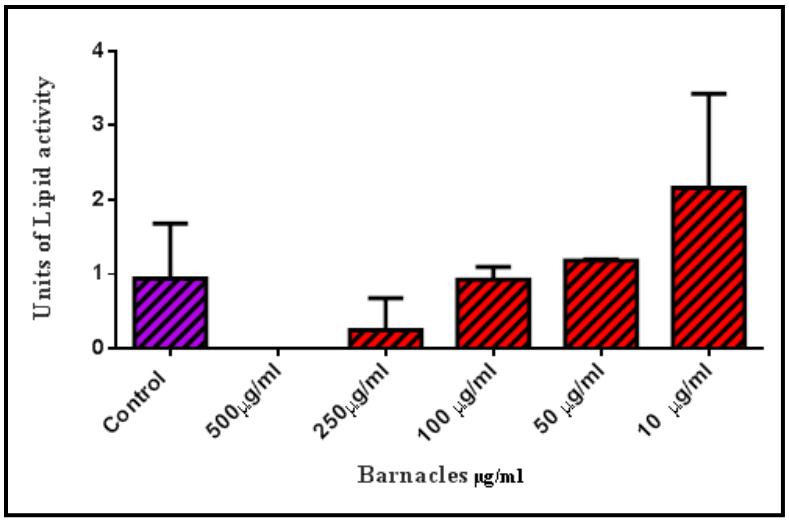


Figure 5: Lipid Activity

GLUCOSE ESTIMATION

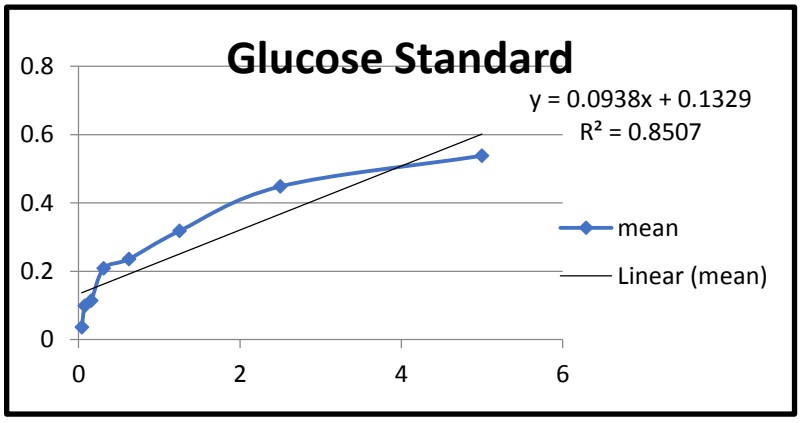


Figure 6: Glucose Standard

The total Glucose content was present in the extract sample Control was found to be 1.555 mg/. The total Glucose content was present in the extract sample10 µg/ml was found to be 2.225mg/ml.The total Glucose content was present in the extract sample 50 µg/ml was found to be 1.559mg/ml.The total Glucose content was present in the extract sample 100 µg/ml was found to be 1.148mg/ml.The total Glucose content was present in the extract sample 250 µg/ml was found to be 0.544mg/ml.The total Glucose content was present in the extract sample 500 µg/ml was found to be 0.233mg/ml.(Sastry, 2019)

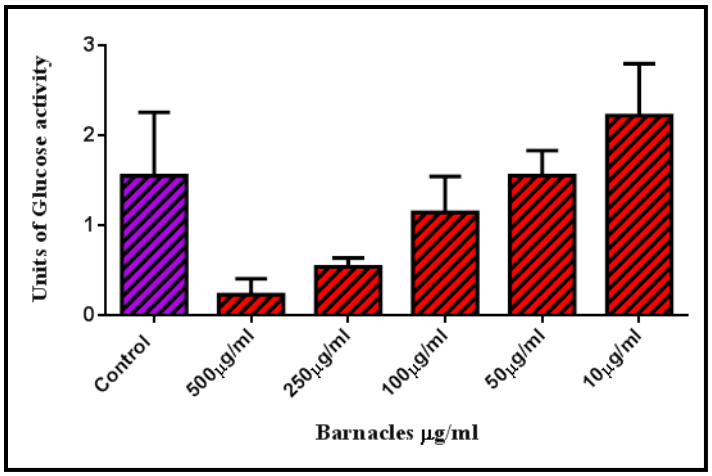


Figure 7: Glucose Activity

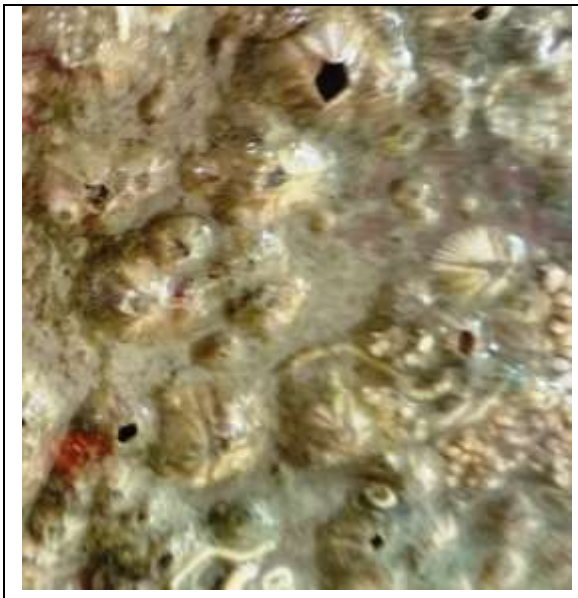
Toxicity studies

It is necessary to study the response of biogenic toxicity against non-targeted species. When *G.edulis* is cultured in laboratory conditions, natural death could be possible. Samples also had a minimal lethal effect on *G.edulis*. During the first hour of observation, 85 % of the populations were alive; gradually, the survival rates in both control and test systems were almost equal. This study indicates that the *G.edulis* is to sudden initial shock when a foreign material is added to the system. The optimal results were observed at 100 µg/ml in which almost 80% lethality was observed.(Sheehan *et.al.,* 2019)

Table 1: Toxicity studies

Name of the sample	Different concentration of drug in triplicates											
	500 µg/ml		250 µg/ml		100 µg/ml		50 µg/ml		10 µg/ml		Control	
	D	L	D	L	D	L	D	L	D	L	D	L
GE	5	0	4	1	2	3	1	4	0	5	0	5
	5	0	3	2	3	2	0	5	0	5	0	5
	5	0	4	1	2	3	1	4	0	5	0	5

Percentage of mortality



500 µg/ml

250 µg/ml

100 µg/ml

50 µg/ml

10 µg/ml

Control

Figure 8 – Visualization Percentage of mortality

10µg/ml 50µg/ml 100µg/ml 250µg/ml 500µg/ml

Name of the sample	Different concentration of drug in triplicates					
	500 µg/ml	**250 µg/ml**	**100 µg/ml**	**50 µg/ml**	**10 µg/ml**	**Control**
GE	100	80	40	20	0	0
	100	60	60	0	0	0
	100	100	40	20	0	0
Mean	100	80	46.66	13.33	0	0

DISCUSSION

The first attempt to locate antibacterial activity in marine organisms was initiated around 1950s. Though the occurrence of unusual natural products from marine invertebrates and their associated bacteria has been widely reported, their relative biological function has not been studied much. In the marine environment, most of the microorganisms form associations with marine eukaryotes. These kinds of associations may provide nutrients or dietary cofactors to the host or perhaps molecules that protect the host from 'infection' by other eukaryotes.The total viable bacterial count varied from 3 x 106 to a lowest of 3 x 103 CFU/cm2/g. A total of 80 colonies were isolated from seaweeds represented by 32 (40%) from Ulva lactuca, 11 (14%) from *Chaetomorpha linoids*, 22 (27%) from *Gracilaria edulis* and 15 (19%) from *Enteromorpha compressa*. Incase of biochemical analysis. (Wallaert *et.al.,* 2013)

Although there is enormous literature on the effect of sulphate group for reducing gel strength, this statement will not hold good in this case. It may be presumed that the accumulation of storage polysaccharides and the sulphate content increased as the plant gets matured. During the initial stage of estimation (72 days of culture period), the galactose content was very high and might not have converted to the storage polysaccharides. As the plant gets matured, the polysaccharides content increased with respect to total galactose. While comparing the gel strength with other species like Gelidiella, the lower gel strength of *G.edulis* might have been contributed by the sulphate group with agar.(Niyogi *et.al.*, 2021)

CONCLUSION.

The current study assessed biochemical indicators in the cosmopolitan barnacle; the findings will be helpful in developing methods for future bio-monitoring initiatives including these microbes. Barnacles from contaminated locations had more activity, but this reaction was only noticeable in the winter. It was demonstrated that the seasonality effect on those responses was an undesirable aspect of using this species of biomonitor. Therefore the present work is successful in identifying potential candidate seaweed from the south east coast of india, *Gracilaria edulis* which can be exploited for the management of fouling organisms. The toxicological evaluation and biochemical process of the seaweed *Gracilaria edulis* brings forth the fact that they are safe at minimum levels and can be used economically. Seaweed has to be used as nature's wealth and harnessed in the right way to promote future health.

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