SCREENING OF ANTI-MICROBIAL ACTIVITIES OF SOME MARINE MACRO AND MICRO ALGAE

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CERTIFICATE

This is to certify that the work entitled, "SCREENING OF ANTI-MICROBIAL ACTIVITIES OF SOME MARINE MACRO AND MICRO ALGAE" submitted by VIPUL SHARMA and ANKIT MALLA in fulfillment for the award of degree of Bachelor of Technology in BIOTECHNOLGY of Jaypee University of Information Technology has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

Roma Sarkar 29.05.09

Dr. Roma Sarkar

Associate Professor

Biotechnology

JUIT

ACKNOWLEDGEMENT

Any assignment puts to litmus test of an individual's knowledge, credibility or experience and thus sole efforts of an individual are not sufficient to accomplish the desired work. Successful completion of a project involves interest and efforts of many people and so this becomes obligatory on my part to record thanks to them.

Therefore, first of all we would like to thank our guide and mentor Dr. ROMA SARKAR, for her guidance, help and constant encouragement throughout this training. Working under her was an enriching experience.

We would like to thank Dr. M.KASIM, CMFRI, Chennai, for providing us with the samples. Without his help it wouldn't have been possible for us to carry out our objective.

We express our heartfelt thanks to our Head of the Department DR. R.S CHAUHAN for providing us with the opportunities of doing this final year project.

Next in equivalence, we would like to thank Ms. MAMTA BHATIA, for the encouragement and her constant interest in the activities of our project right from its inception.

We are also thankful to all my seniors and to the lab staffs for their guidance and friendly cooperation.

DATE: 29.05.09

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ABSTRACT

Over the last few decades, there have been emerging evidences which substantiate the therapeutic as well as nutritional values of many marine natural products. Of particular interest is the algal flora as potential source of medicines. Several of the compounds isolated from marine macro and micro algae exhibit biological activity. The present investigation aimed at screening some marine algae from Indian coast (Rameswaram Mandapam region) for anti-bacterial activity against some known human pathogens. The macro algae screened were *Gracilaria crassa, Hypnea valentiae, Hypnea musciformis, Acanthophora, Stoechospermum marginatum, Ceramium sp, Gigartina sp, Gelidiella acerosa, Sargassum tenerrimum, Padina gymnospora, Turbinaria ornata, Gracilaria edulis, Gracilaria corticata, Gracilaria conifera, Hydroclathrus ciathratus, Kappaphycus acvarezii, Var. Cylindrica* and *Hormophysa triquetra.* Apart from these some micro algae were also screened, which are *Dicrateria, Isochrysis, Paclova sp., Tetraselmis, Chaetoceros, Chlorella* and *Nanochloropsis.* They were tested against *Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Salmonella typhi* using broth dilution and agar disc/well diffusion methods. *Chlorella* showed appreciable activity against all test bacteria used, *Bacillus subtilis, E.coli, Salmonella typhi* and *Staphylococcus aureus. Gracilaria conifera* also gave positive results against *Bacillus subtilis. Gracilaria crassa* was also found effective against *E.coli. Sargassum tenerrimum* also showed inhibition for *Salmonella typhi.* Among the test algae *Chlorella* which is also valued for its nutritional qualities was found unique, exhibiting anti-microbial activity against all test organisms and thus merits attention as a broad spectrum antibiotic.

OBJECTIVE

- **A comparative evaluation of anti-bacterial activity of some marine macro and micro algae.**

LITERATURE REVIEW

3.1 ALGAE (1)

Algae (*sing*. Alga) are a large and diverse group of simple, typically autotrophic organisms, which may be unicellular or multicellular. The largest and most complex marine forms are called seaweeds which have the photosynthesizing capacity. However they lack the many distinct organs characteristic of land plants.

Algae are found in enormously diverse habitats, like moist places, tree trunks, flower pots, ocean depths, highest peaks, hot springs to ice cold atmosphere. Some thrive in still water (Eg: *Zygnema, Oedogonium*) , some others in running water (Eg: *Cladophora*), while some are marine (most brown and red algae).

Algae in general show three types of reproduction. Vegetative reproduction which is by fragmentation or fission. Asexual reproduction which is under favourable conditions. Various types of asexual spores formed (zoospores, aplanospores, hypnospores, etc.) . Sexual reproduction which is under unfavourable conditions and gametes are formed.

There are 11 classes of algae :

- Chlorophyceae (green algae)
- Xanthophyceae (yellow green algae)
- Chrysophyceae (orange)
- Bacillariophyceae (yellow or golden brown)
- Cryptophyceae (nearly brown)

- Dinophyceae (dark yellow)
- Chloromonadineae (bright green)
- Euglenineae (pure green)
- Phaeophyceae (brown)
- Rhodophyceae (red)
- Myxophyceae (blue-green)

3.1.1 MARINE ALGAE

Marine algae is a major group with diverse kind of species of mainly multicellular forms. The largest and most complex marine forms are called seaweeds. Seaweeds grow mostly in shallow marine water, under 100 metres (330 ft); however some have been recorded to a depth of 360 metres (1,200 ft). Presence of both micro and macro algae in marine water is observed.

The two largest group of marine algae are:

1. *Rhodophyceae* (red algae)
2. *Phaeophyceae* (brown algae)

	Brown algae	Red algae
Pigments	Yellow fucoxanthin	Red phycoerythrin and blue phycocyanin
Reserved food material	Mannitol , laminarin and fats	Floridean starch
Structure	Simple filamentous to bulky parenchymatous forms	Simple filamentous to attaining considerable complexity
Reproduction	Sexual (ranges from isogamous to oogamous)	Sexual (oogamous)
Occurrence	All plants but few are marine	Few are fresh water,others are marine

3.2 Anti-bacterial activity

Although the marine flora of algae is considerably rich with diverse kinds of species, however prospecting the resources for biotechnological use, particularly in drug discovery, is relatively a recent approach. Seaweeds, specially the brown and the red algae are expected to contain bioactive compounds (**2**) . Lipophilic solvent extracts from marine algae have been investigated as a source of substances with pharmacological implications (**3**) . Marasneh et al.(1995) (**4**), have shown anti-bacterial activity in organic solvent extract of six species of marine algae against multi drug resistant bacteria. Sastry et al.(1998) (**5**) also reported anti-bacterial activity in the organic solvent extracts of seaweeds against Gram positive and Gram negative pathogenic bacteria.

Extensive chemical investigations of extracts from marine organisms have led to the discovery of a variety of secondary metabolites with antimicrobial activities against human pathogens (Rinehart et al. 1981(**6**); Reichelt and Borowitzka 1984(**7**). While chemically mediated disease resistance is well documented among terrestrial plants (Ingham 1972 ,1973 (**8,9**); Hammerschmidt 1999(**10**)) , little is known about the antimicrobial functions of secondary metabolites produced by marine plants. However, a high number of novel bio-products with useful and sometimes unique pharmacological properties have been described and some of them are in late stage of clinical trials (**2**) .

3.3 Metabolites Identified (11)

Extensive work has been done on secondary metabolites of marine algae(**12**). The work carried out on *laurencia* species(**13**). Blue – Green algae(**14**) and dinoflagellates(**15**) have been reviewed. Reports are available dealing with amino acid from marine algae(**16**), guanidine derivatives(**17**), phenolic substances(**18**), carotenoids(**19**), diterpenoids(**20**), indoles(**21**), bioactive polymers(**22**), and halogenated compounds(**23,24**).

Some of the metabolites identified are brominated phenols and brominated oxygen heterocyclics from *Polysiphonia lanosa* and *laurencia sp.*(**25-29**). Isomers of Kainic acid have been isolated from alga *Digenea*(**30,31**). Laminine, a choline like basic amino acid has been isolated from a number of marine algae(**32,33**) like *Laminaria angustata*. The other amino acids isolated from this source were L-lysine, L-arginine, ethanolamine and choline. The presence of sterols in algae was first established by Heilbron et al(**34**) and later by Tsuda et al(**35**). The sterols from marine algae are reported to be non-toxic and have the ability to reduce blood cholesterol level. They are also reported to reduce the tendency to form a fatty liver and excessive fat deposition in the heart(**36**).

The present study aims at screening both macro and micro marine algae from Indian coast (Rameswaram and Mandapam coastal region) for the purpose of a comparative evaluation for their antimicrobial properties in order to identify the species promising as potential therapeutics.

MATERIALS AND METHODS

4.1 Test bacteria considered

Gram positive Bacteria :

- *Bacillus subtilis*
- *Staphylococcus aureus*

Gram negative Bacteria :

- *Escherichia coli*
- *Salmonella typhi*

4.2 Algal Materials

Materials were procured from CMFRI (Central Marine Fisheries Research Institute), which were collected from Rameswaram and Mandapam coastal region, Tamil Nadu.

4.2.1 Algal species of interest :

MACRO ALGAE:

(RED ALGAE)

1. *Gracilaria crassa*
2. *Gracilaria edulis*
3. *Gracilaria corticata*
4. *Gracilaria conifera*
5. *Hypnea valentiae*
6. *Hypnea musciformis*
7. *Acanthophora spicifera*
8. *Kappaphycus acvarezii*
9. *Ceramium sp.*
10. *Gigartina sp.*
11. *Gelidiella acerosa*

(BROWN ALGAE)

12. *Sargassum tenerrimum*
13. *Sargassum myriocystum*
14. *Padina gymnospora*
15. *Turbinaria ornata*
16. *Hydroclathrus ciathratus*

17. *Stoechospermum marginatum*

18. *Var. cylindrica*

19. *Hormophyra triquetra*

MICRO ALGAE:

1. *Dicrateria*

2. *Isochrysis*

3. *Paclova sp.*

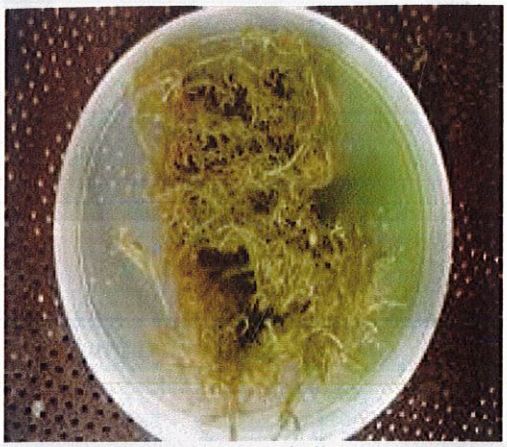
4. *Tetraselmis*

5. *Chaetoceros*

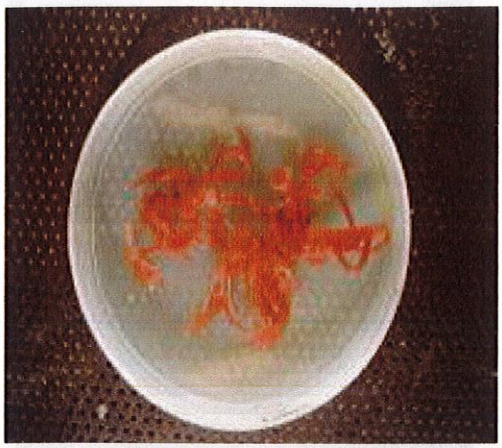
6. *Chlorella*

7. *Nanochloropsis*

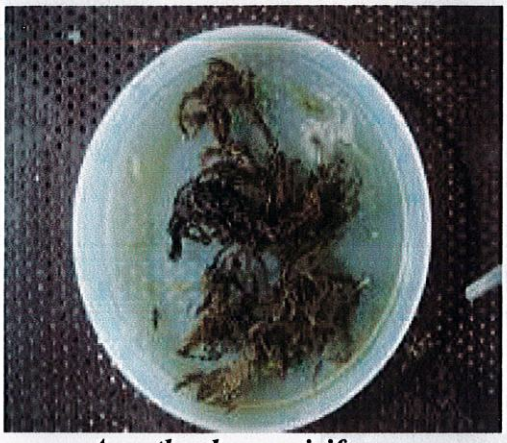
ALGAL SPECIES UNDER STUDY



Gracilaria edulis

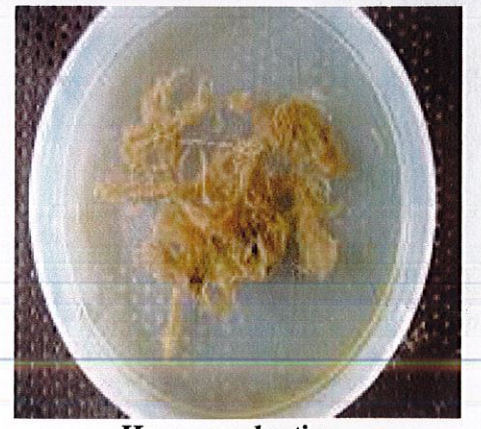


Kappaphycus alvarezii

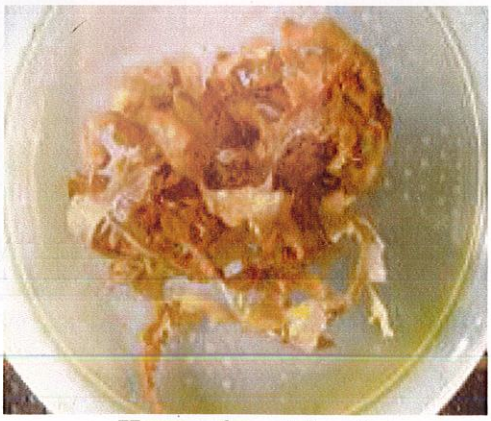


Acanthophora spicifera

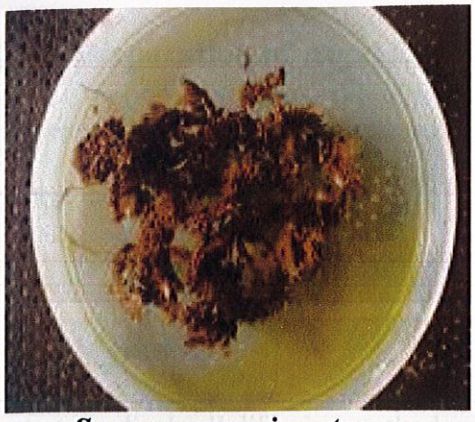
Sargassum tenerrimum



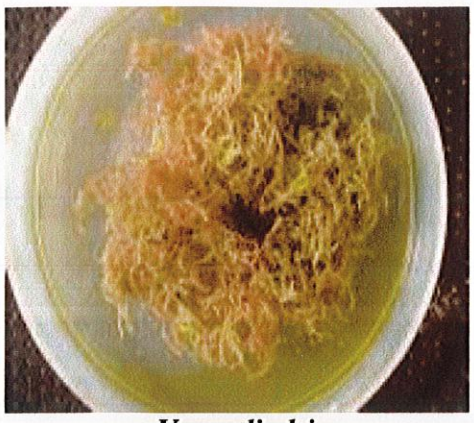
Hypnea valentiae



Hormophyra triquetra

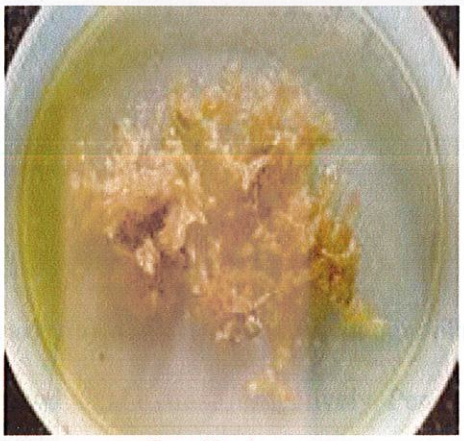


Sargassum myriocystum



Var. cylindrica

Gracilaria corticata



Gracilaria crassa

Hypnea musciformis

4.3 Preparation of Algal Extract

Preparation of algal extract was done in 100% methanol.

(Ref. Mitra *et. al* 2007)(**37**)

4.4 Methods

1. Agar disc/well diffusion method.

2. Broth dilution method.

4.5 Procedure

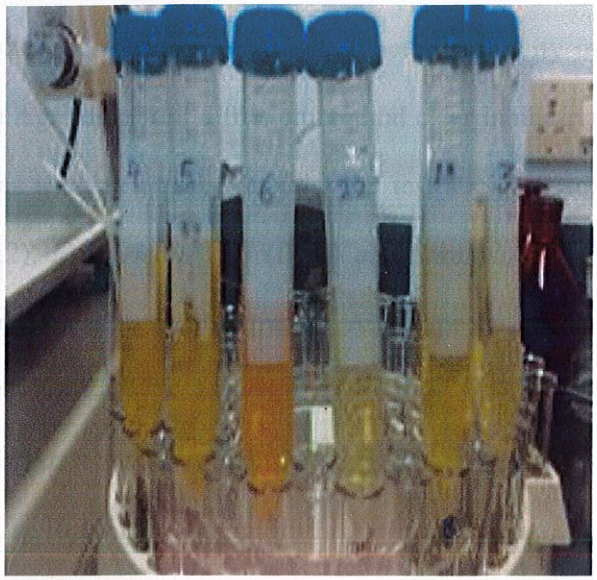
4.5.1 Agar disc/well diffusion method (38)

Pure culture of bacteria was prepared and lawn (100µl bacteria) was made on nutrient agar plates and 3 punctures were done for distilled water, methanol, and algal extract respectively. Penicillin was taken as positive control. Autoclaved Whatman paper discs were dipped in algal extract for ½ hour and then placed on nutrient agar plates with lawn. Subsequently, observations were made to check the formation of inhibition zone.

4.5.2 Broth dilution method

A modified version of a bacterial growth inhibition assay described by Kubanek et al (2003)(**39**) was used to assess the antibacterial effects of the algal extracts. Pure culture of bacteria was prepared and 100 µl was inoculated in nutrient broth with increasing amount of algal extract, methanol and distilled water. Penicillin was taken as positive control. Change in turbidity (as a mark of bacterial growth) was observed.

ALGAL EXTRACTS



BROTH DILUTION METHOD

RESULTS & DISCUSSION

In the present analysis, some of the algal species were found to have antibacterial activity in varying degree against the test bacteria used. In Agar disc/well diffusion method clear inhibition zones were observed for penicillin (which was used as control) against the test strains used, *Bacillus subtilis* (fig.1), *Salmonella typhi* (fig.2), *Escherichia coli* (fig.3) and *Staphylococcus aureus (fig.4).* The same method was followed using the algal extracts. Algal species which showed inhibition zones against the test strains are *Gelidiella acerosa* (fig.5), *Hydroclathrus ciathratus* (fig.6), *Padina gymnospora (fig.7), Sargassum tenerrimum(fig.8,11), Stoechospermum marginatum* (fig.9) ,*Gracilaria crassa* (fig.10,11), *Kappaphycus acvarezii* (fig.12), *Stoechospermum marginatum (fig.12), Gracilaria edulis (fig.13), Gracilaria conifera* (fig.13), *Isochrysis* (fig.14), *Chlorella* (fig.15), *Chaetoceros* (fig.16), *Nanochloropsis* (fig.16), *Gracilaria corticata* (fig.17), *Dicrateria* (fig.17), *Paclova* (fig.18), *Var. cylindrica* (fig.19) and *Hormophysa triquetra* (fig.19). A comparative evaluation for all algal samples used against the test bacteria are presented in Table 1 and Table 2. Diameters of inhibition zone against test bacteria for all algal species are indicated in Table 3. The algal species under study which did not show any antibacterial activity against any of the test bacteria are *Hypnea valentiae, Ceramium sp., Gigartina sp., Turbinaria ornata, Acanthophora spicifera, Var. Cylindrica, Hormophysa triquetra, Tetraselmis.* The experiments for all the test algal species against the individual test organisms were repeated three times and the results were found consistent.

In Broth dilution method there was a decrease in bacterial growth with the increasing amount of algal extract, which was noticed by change in turbidity and by measuring the O.D at 595nm. Methanol showed a slight decrease in growth of bacteria, while distilled water obviously didn't

have any effect. *Chlorella* was found effective against *Bacillus subtilis* (Table 4) and *Staphylococcus aureus* (Table 7). *Sargassum tenerrimum* showed activity against *Salmonella typhi* (Table 5) and *Gracilaria crassa* against *E.coli* (Table 6).

The results show the presence of appreciable anti-microbial activity in most of the algal samples screened. Disc diffusion methods are used extensively to investigate the antibacterial activity. These assays are based on the use of discs as reservoirs containing solutions of the substances to be examined. In case of solutions with low activity, however, a large concentration or volume is needed. Since discs have limited capacity, therefore holes or punctures are preferably used **(40)**. Since agar disc/well diffusion technique does not always give reliable results as because some of the anti-bacterial macro compounds are not able to diffuse through gel pores, therefore Broth Dilution Test was conducted. Moreover this is a much more sensitive test and gives a better and reliable result than the disc diffusion test. The potency of the anti-bacterial compounds in the extract is appreciable considering the fact that it has shown activity against a growing log phase culture. This agent may result in the bacterial death rather than just inhibiting bacterial growth as is evident from the cellular debris that were found at the bottom of the test tubes.

The present studies reaffirm the antibacterial potential of some of the marine macro and micro algae. Among them *Chlorella* stands most promising by exhibiting maximum activity against all test bacteria used, *Bacillus subtilis, E.coli, Salmonella typhi* and *Staphylococcus aureus. Gracilaria conifera* also gave positive results against *Bacillus subtilis. Gracilaria crassa* was also found effective against *E.coli*. *Sargassum tenerrimum* also showed inhibition for *Salmonella typhi.* Sargassum has already been reported for its anti-microbial activity. Sieburth and Conover

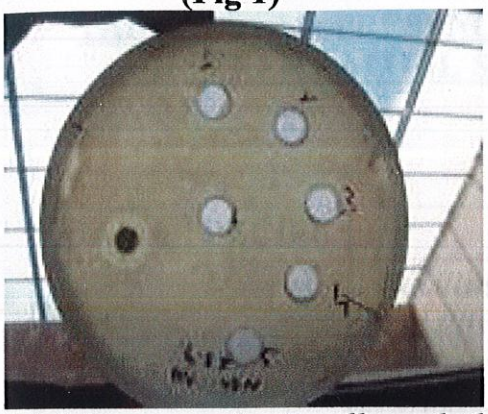
(1965) demonstrated that phlorotannins from Sargassum was the bio-active agent (**41**). Pratt *et al.*(1944) were the first to isolate an antibacterial substance from *Chlorella.* A mixture of fatty acids, named Chlorellin, exhibited inhibitory activity against both Gram positive and Gram negative bacteria (**42**). Chlorella has also been found to be nutritionally valuable being as a rich source of omega 3 fatty acids (**43**).

The present analysis showed that there is a decrease in antimicrobial activity when algal samples were stored over a time (1 year) in comparison to freshly preserved samples (1 month). Earlier reports also demonstrated that the method of storage can significantly alter the composition of a crude extract (Cronin et al. 1995). (**44**). The production of the secondary bio-active metabolites are often influenced by seasonal variation (**45,46**).

For further research it is essential to isolate and characterize the secondary metabolite(s) responsible for the antimicrobial activity shown by the test samples which is expected to yield a more definite information as to the antimicrobial potentials of the promising algal species.

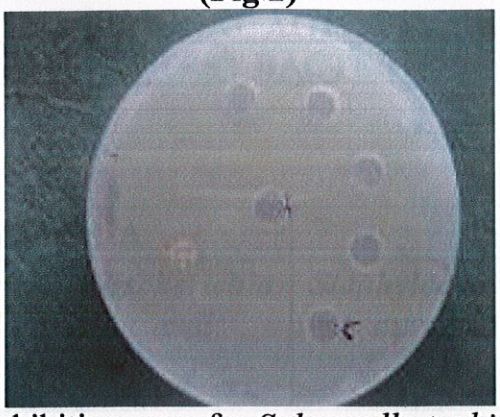
PENICILLIN RESULT

(Fig 1)



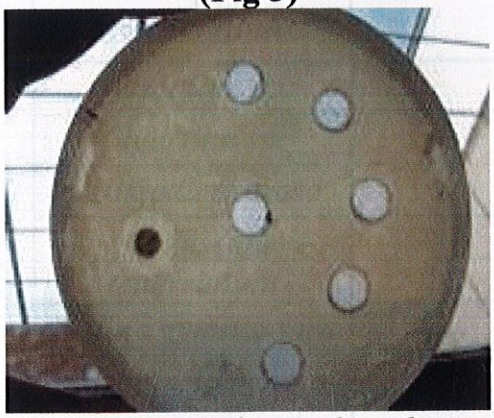
Inhibition zone for *Bacillus subtilis*
(12mm)

(Fig 2)



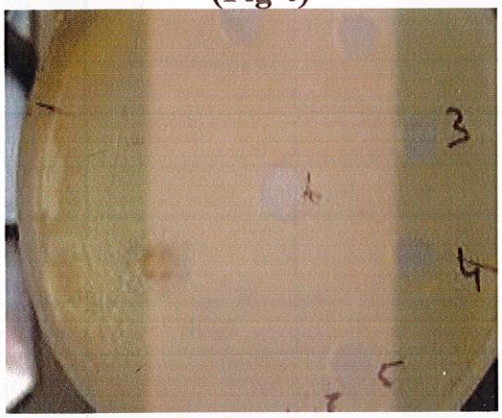
Inhibition zone for *Salmonella typhi*
(10mm)

(Fig 3)



Inhibition zone for *Escherichia coli*
(14mm)

(Fig 4)



Inhibition zone for *Staphylococcus aureus*
(11mm)

ALGAL SAMPLES RESULT

TABLE 1 and 2: PRESENCE OF ANTI-BACTERIAL ACTIVITY IN DIFFERENT ALGAL SPECIES AGAINST TEST BACTERIA

(Table 1)

ALGAL SPECIES	TEST BACTERIA			
	Bacillus subtilis	*Salmonella typhi*	*Escherichia coli*	*Staphylococcus aureus*
Gracilaria crassa		+	+	
Hypnea valentiae				
Hypnea musciformis	+			+
Acanthophora				+
Stoechospermum marginatum		+	+	
Ceramium sp.				
Gigartina sp.				
Gelidiella acerosa	+	+		+
Sargassum tenerrimum		+	+	
Padina gymnospora		+	+	
Turbinaria ornata				
Hydroclathrus ciathratus	+	+		+

'+' implies presence of antibacterial activity.

(Table 2)

ALGAL SPECIES	TEST BACTERIA			
	Bacillus subtilis	*Salmonella typhi*	*Escherichia coli*	*Staphylococcus aureus*
Gracilaria edulis		+	+	
Acanthophora spicifera				
Kappaphycus acvarezii		+	+	
Hypnea valentiae				
Sargassum tenerrimum		+	+	
Gracilaria corticata				+
Sargassum myriocystum		+	+	
Hypnea musciformis	+			+
Gracilaria crassa		+	+	
Gracilaria conifera	+	+		+
Var. Cylindrica				
Hormophysa triquetra				
Dicrateria		+		
Isochrysis	+		+	
Paclova sp.	+			+
Tetraselmis				
Chaetoceros			+	
Chlorella	+	+	+	+
Nanochloropsis	+	+		

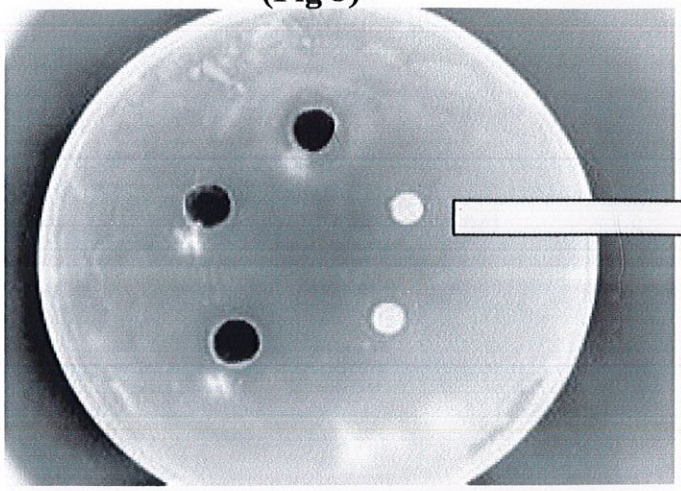
'+' implies presence of antibacterial activity.

DIAMETER OF INHIBITION ZONE AGAINST TEST BACTERIA

(Table 3)

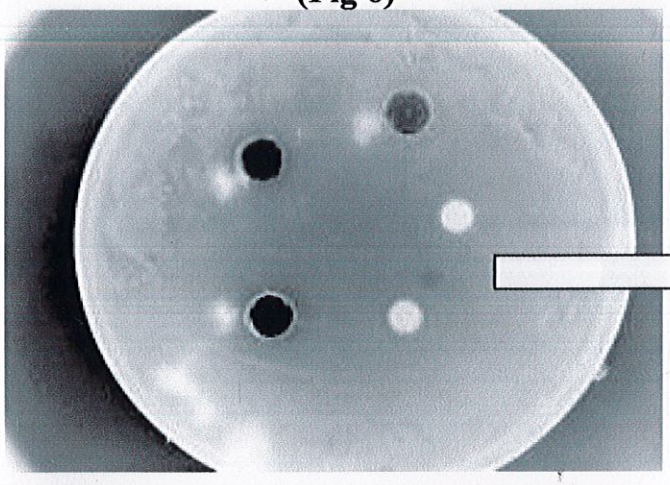
ALGAL SPECIES	DIAMETER OF INHIBITION ZONE (in mm) AGAINST FOLLOWING TEST BACTERIA			
	Bacillus subtilis	*Salmonella typhi*	*Escherichia coli*	*Staphylococcus aureus*
Gracilaria edulis		6	7	
Sargassum tenerrimum		8	8	
Sargassum myriocystum		7	8	
Hypnea musciformis	6			6
Gracilaria crassa		6	7	
Gracilaria conifera	7	6		6
Isochrysis	7		7	
Paclova sp.	7			6
Chaetoceros			8	
Chlorella	8	10	10	9
Nanochloropsis	7	7		
OLD SAMPLES				
Gracilaria crassa		4	5	
Hypnea musciformis	4			4
Stoechospermum marginatum		5	5	
Gelidiella acerosa	5	4		
Sargassum tenerrimum		6	6	
Padina gymnospora	4			5
Hydroclathrus ciathratus			4	4

(Fig 5)



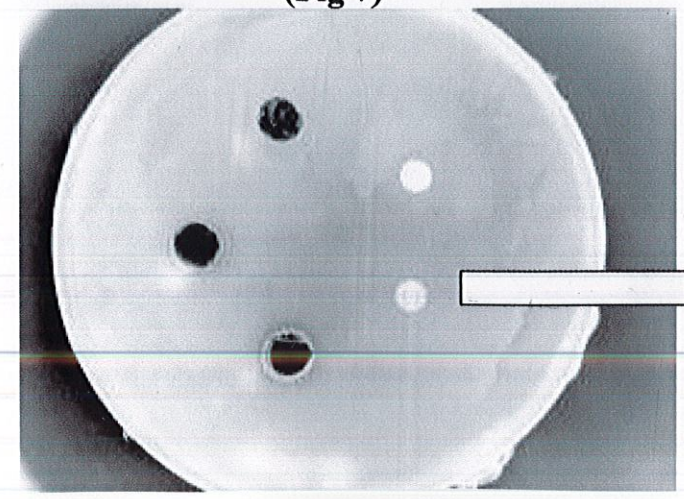
Inhibition zone seen using *Gelidiella acerosa*

(Fig 6)



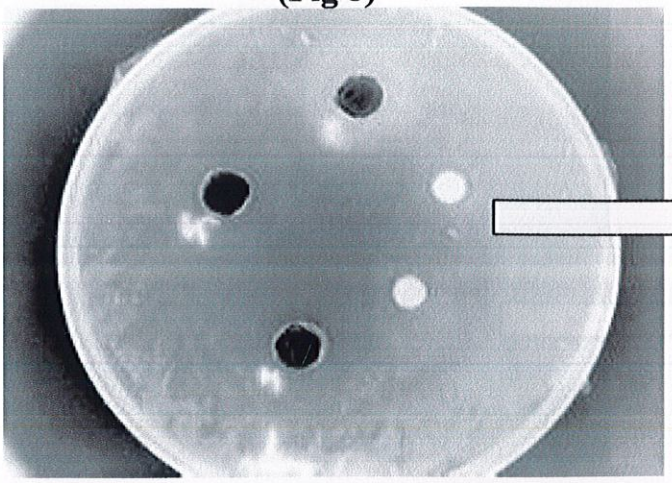
Inhibition zone seen using *Hydroclathrus ciathratus*

(Fig 7)



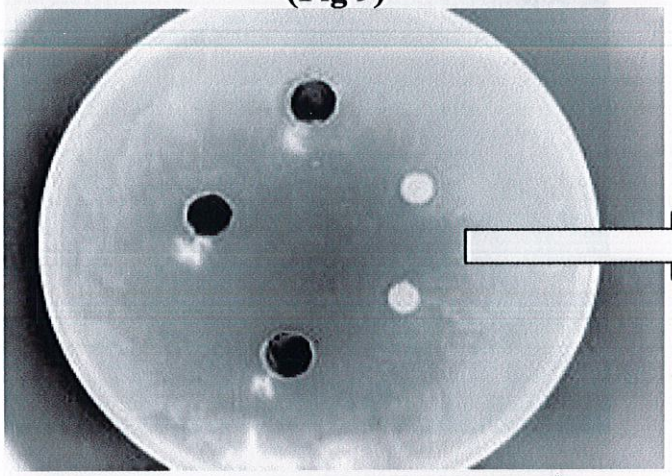
Inhibition zone seen using *Padina gymnospora*

(Fig 8)



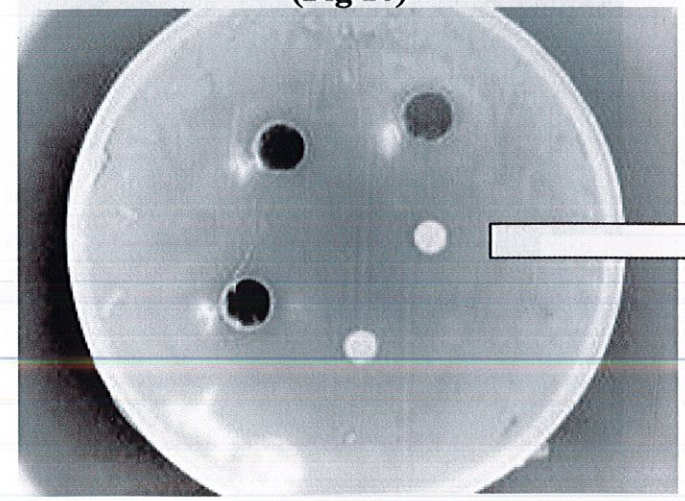
Inhibition zone seen using *Sargassum tenerrimum*

(Fig 9)



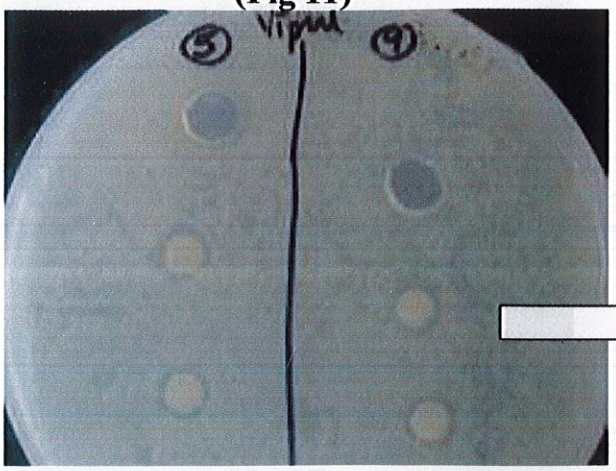
Inhibition zone seen using *Stoechospermum marginatum*

(Fig 10)



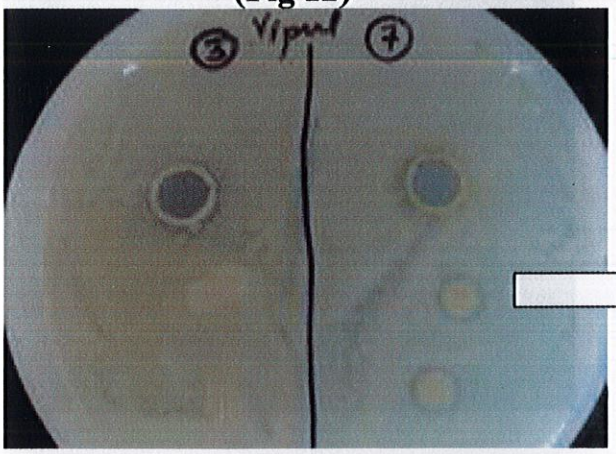
Inhibition zone seen using *Gracilaria crassa*

(Fig 11)



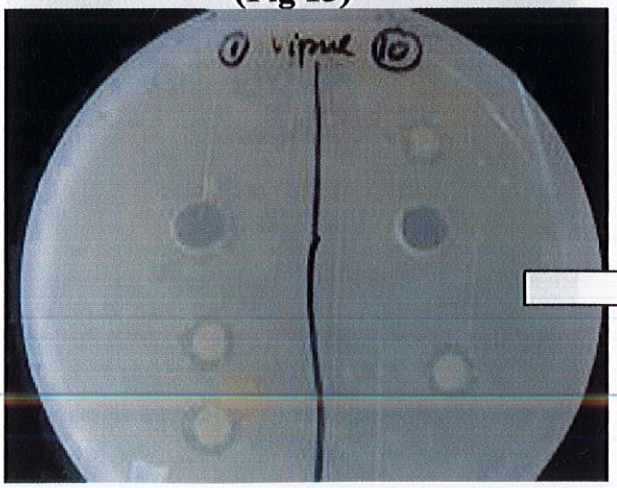
Inhibition zone seen using *Gracilaria crassa(9)* , *sargassum sp.(5)*

(Fig 12)



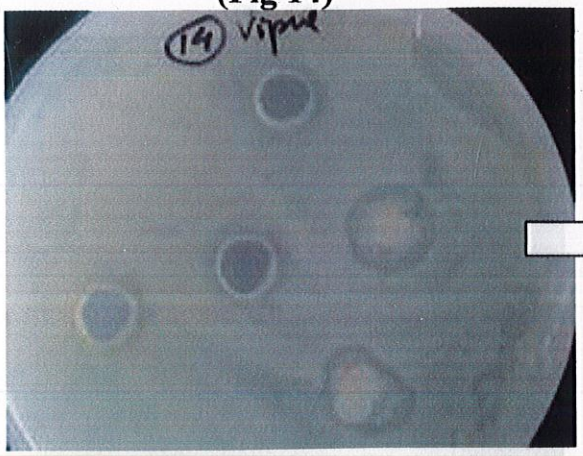
Inhibition zone seen using *kappaphycus Acvarezii(3)* , *sargassum myriocystum(7)*

(Fig 13)



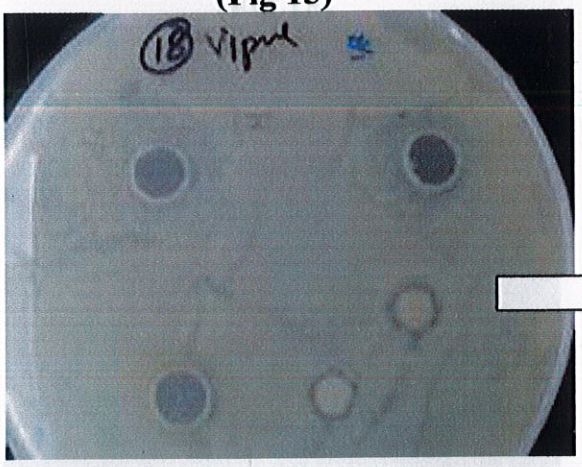
Inhibition zone seen using *Gracilaria edulis(1)*, *Gracilaria conifera(10)*

(Fig 14)



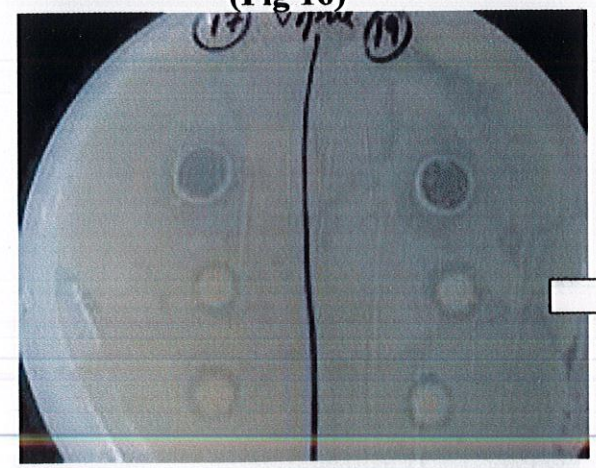
Inhibition zone seen using *Isochrysis(14)*.

(Fig 15)



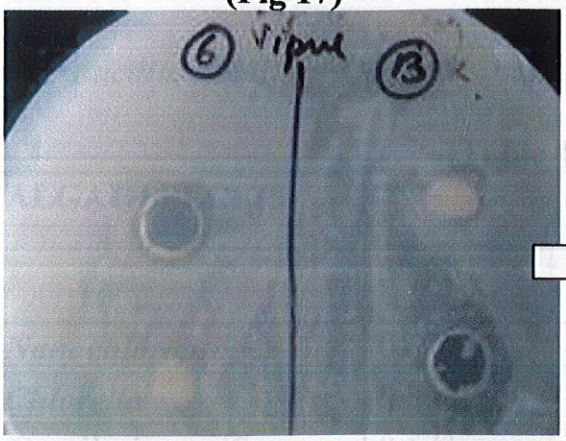
Inhibition zone seen using *Chlorella(18)*.

(Fig 16)



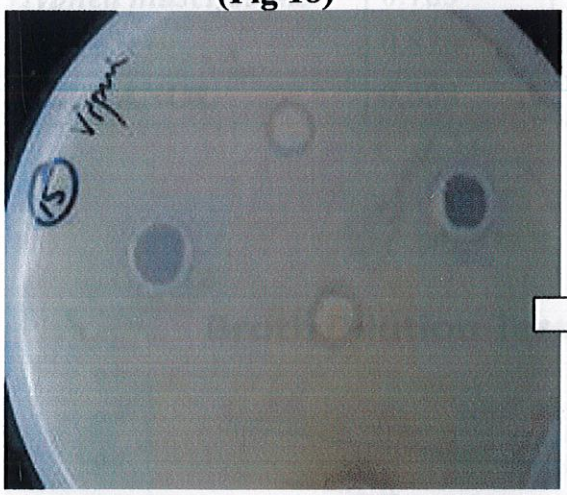
Inhibition zone seen using *Chaetoceros(17), Nanochloropsis(19)*

(Fig 17)



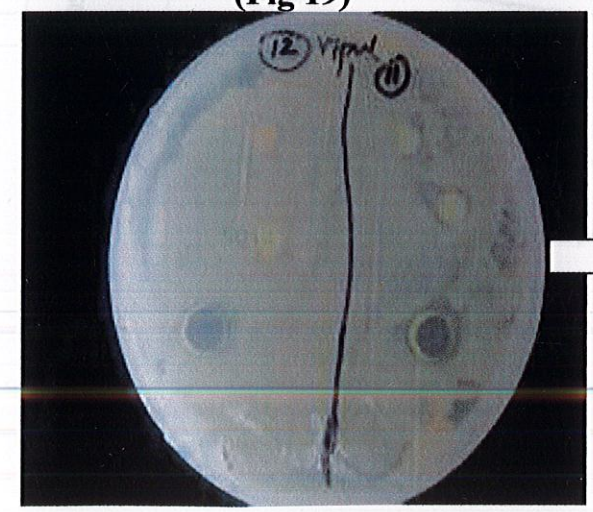
Inhibition zone seen using *Gracilaria corticata(6), Dicrateria(13).*

(Fig 18)



Inhibition zone seen using *Pavlova(15).*

(Fig 19)



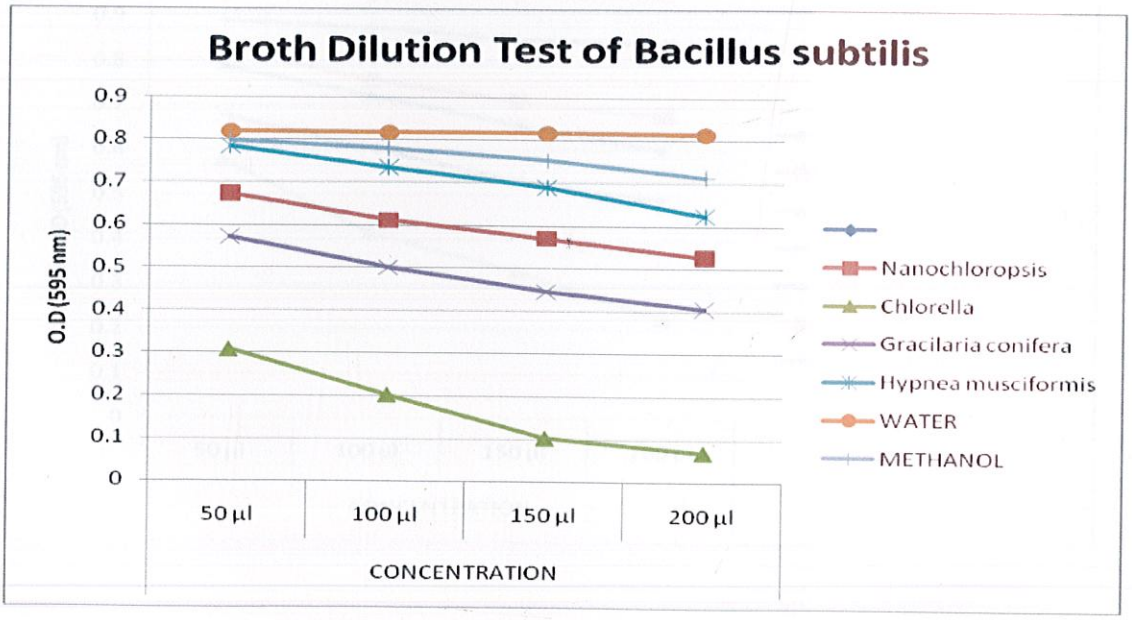
Inhibition zone seen using *Hormophysa triquetra(12), Var.cylindrica(11).*

BROTH DILUTION TEST WITH ALGAL EXTRACTS

For *Bacillus subtilis* (Control – 0.820)

(Table 4)

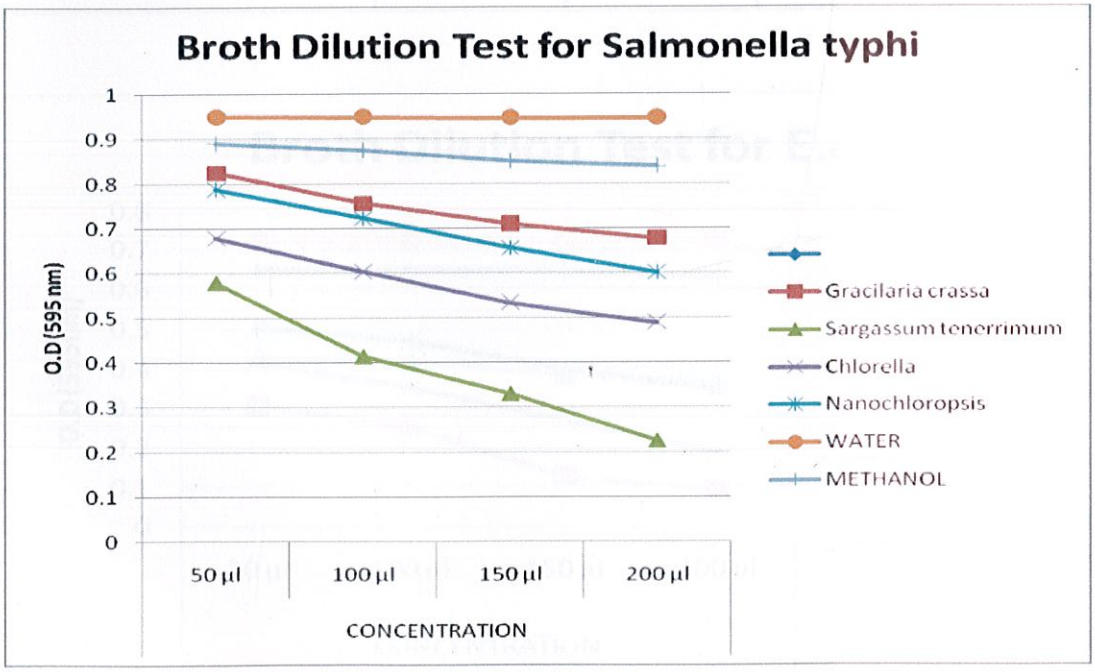
ALGAL SPECIES	CONCENTRATION			
	50 µl	100 µl	150 µl	200 µl
Nanochloropsis	0.675	0.612	0.571	0.527
Chlorella	0.308	0.203	0.103	0.070
Gracilaria conifera	0.573	0.500	0.445	0.405
Hypnea musciformis	0.785	0.735	0.690	0.623
WATER	0.820	0.818	0.818	0.816
METHANOL	0.608	0.590	0.575	0.570



For *Salmonella typhi* (Control – 0.950)

(Table 5)

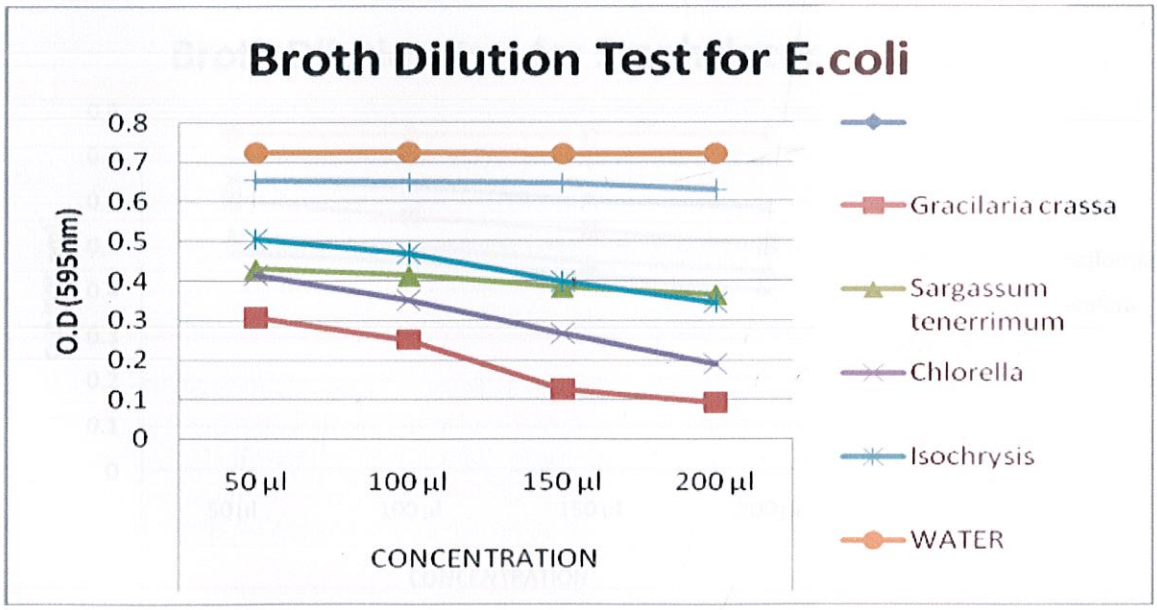
ALGAL SPECIES	CONCENTRATION			
	50 µl	100 µl	150 µl	200 µl
Gracilaria crassa	0.825	0.756	0.710	0.678
Sargassum tenerrimum	0.580	0.414	0.330	0.225
Chlorella	0.680	0.604	0.534	0.489
Nanochloropsis	0.789	0.723	0.656	0.601
WATER	0.950	0.949	0.947	0.947
METHANOL	0.890	0.875	0.850	0.839



For *E.coli* (Control – 0.624)

(Table 6)

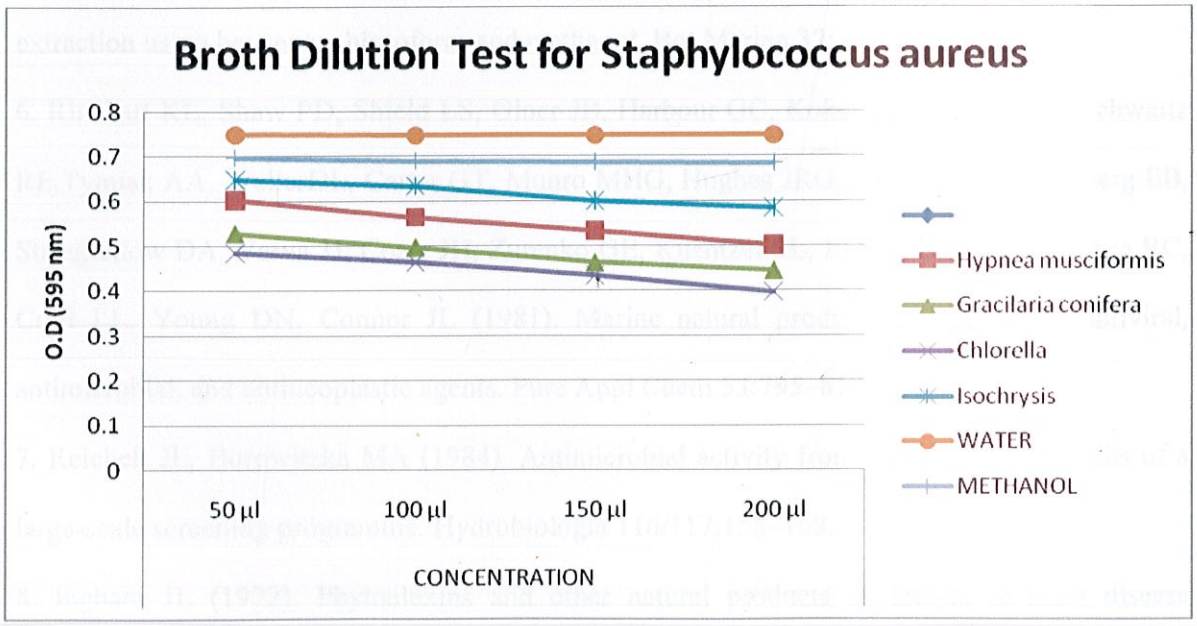
ALGAL SPECIES	CONCENTRATION			
	50 µl	**100 µl**	**150 µl**	**200 µl**
Gracilaria crassa	0.306	0.250	0.125	0.090
Sargassum tenerrimum	0.430	0.412	0.383	0.364
Chlorella	0.414	0.350	0.267	0.189
Isochrysis	0.505	0.467	0.398	0.343
WATER	0.724	0.724	0.721	0.720
METHANOL	0.654	0.649	0.647	0.630



For *Staphylococcus aureus* (Control – 0.750)

(Table 7)

ALGAL SPECIES	CONCENTRATION			
	50 µl	**100 µl**	**150 µl**	**200 µl**
Hypnea musciformis	0.604	0.565	0.534	0.502
Gracilaria conifera	0.530	0.498	0.463	0.445
Chlorella	0.487	0.466	0.435	0.398
Isochrysis	0.650	0.635	0.601	0.585
WATER	0.750	0.748	0.747	0.747
METHANOL	0.698	0.690	0.687	0.685



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