"In-vitro antibacterial and antifungal activity and preliminary phytochemical screening of *Kappaphycus alvarezii* and standardization of herbal product formulation"

TO BE SUBMITTED AS MAJOR PROJECT IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF **M.TECH**

SUBMITTED BY

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2K15/BME/09

UNDER THE SUPERVISION OF

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CERTIFICATE

This is to certify that the dissertation entitled: ***"In-vitro* antibacterial and antifungal activity and preliminary phytochemical screening of *Kappaphycus alvarezii* and standardization of herbal product formulation"** by Shivli Banerjee (2K15/BME/09) in the partial fulfilment of the requirements for the reward of the degree of Masters of Engineering, Delhi Technological University (Formerly Delhi College of Engineering) is an authentic record of the candidate's own work carried out by her under our guidance. The information and data enclosed in this thesis is original and has not been submitted elsewhere for honouring of any other degree.

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CERTIFICATE

This is to certify that **Ms. Shivli Banerjee,** D/o **Mr. G. C. Banerjee,** student of M.Tech Biomedical Engineering, Final year, Enrollment No – 2k15/BME/09, from **"Delhi Technological University, Rohini, New Delhi"** has successfully completed her project of Six months, from 1st January 2017 to 30th June 2017, in our Biotechnology Division at Noida Campus with **A+ Grade** on the topic: **"In vitro antibacterial and anti fungal activity and preliminary phytochemical screening of *Kappaphycus alvarezii* and standardization of herbal product formulation.",** this work has not be submitted anywhere for the award of degree.

We wish for her bright future.

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ABSTRACT

Phytochemical analysis and antimicrobial activity of different solvent extracts of *Kappaphycus alvarezii* were investigated. *Kappaphycus alvarezii* synonymously known as red algae is one of the most important sources used in herbal medicine for its anti-inflammatory, diuretic, choleretic and haemostatic properties. We have isolated different bacterial and fungal strains from soil landfill. The bacterial strains were characterized using biochemical tests and gram staining method while fungal strains were identified on the basis of morphological characteristics. Phytochemical screening and antimicrobial activity of different extracts of red algae were tested against different bacterial and fungal strains which are *Shigella flexneri, Aspergillus niger, Penicillium species*, etc. using the disc diffusion assay. Phytochemical analysis of the red algae revealed the presence of flavonoids, saponins, phytosterols, terpenoids, phenolic and tannin. The quantitative analysis of aqueous extracts of red algae was evaluated. We have checked the antimicrobial activity of red algae, extracted from different solvent against different pathogenic bacterial and fungal strains. Cow urine extract and benzene extract was found to be very effective against majority of fungal strains similarly cow urine extract and acetone extract was found to be very effective against bacterial strain. Minimum inhibitory concentration was also calculated for different solvent extracts of red algae. We have also performed the thin layer chromatography of red algae sample which resulted in the presence of metabolic compounds like alkaloids and triterpinoids .The preliminary studies on *Kappaphycus alvarezii* extracts exhibited their antimicrobial potential which could be exploited further for cosmetic applications. In order to identify the cosmetic potential of *Kappaphycus alvarezii* physical, biological as well as chemical parameters are evaluated. Hence, this study would lead to the development of biologically active compounds which can be employed in the formulation of cosmetic product.

Table of Contents

1. INTRODUCTION

In microbiology, minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial (like an antifungal, antibiotic or bacteriostatic) drug that will inhibit the visible growth of a microorganism after overnight incubation. After a pure culture is isolated in the form of plates of solid growth medium or diluted into broth medium MICs can be calculated. For example, in case of liquid broth dilution in order to identify the MIC equal amount of bacteria are cultured in the wells containing liquid broth with progressively lower concentrations of the drug. The concentration of last well in which no bacterial growth is seen and the next well containing lower dose which has bacterial growth is checked and then value of MIC is determined between these both values. There are many commercial methods to determine MIC values.

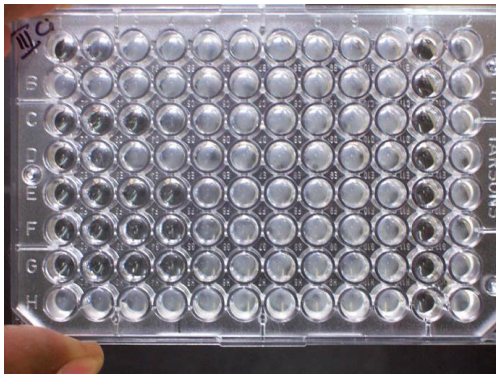


Fig 1: Showing Minimum Inhibitory Concentration

In order to identify the lowest concentration of the antibiotic that is responsible for inhibiting the bacterial growth, an identical amount of bacteria is introduced into the liquid media containing progressively lower concentrations of the drug. (Here, the dilution series of the drug is set up from left to right: for example, well E_1 might contain 100 units of drug; E_2, 50 units; E_3, 25 units; E_4, 12.5 units; etc.). Due to the turbidity occurrence in well E_5 because of bacterial growth and the media present in well E_4 is not distinguishable from clear media, this indicates that the minimum inhibitory concentration is between the drug concentrations in wells E_4 and E_5.[1]

Thin layer chromatography (TLC) is chromatographic technique, which is based on a multistage distribution process, a process in order to separate mixture.[2-4]. Chromatography

basically is a method in which compounds are separated are distributed into two different phases, one is stationary phase (a suitable absorbent like silica gel or aluminium oxide etc. which is coated onto a sheet of glass, plastic or aluminium foil) and a mobile phase (solvent or solvent mixture) which moves in a definite direction and when the samples are applied on the plate,[5-7] the compounds are separated because in the capillary action of solvent along with the solute different analyte ascend the TLC plate at different rates.

The sum of all of the chemical reactions that take place in an organism is called metabolism. Molecules like carbon and nitrogen are abundant in nature and are required essentially for the development of cells in plants. The molecules, which are essential for plant growth, are called primary metabolites (proteins, nucleic acids, and carbohydrates, lipids etc.). Unlike animals, however, most plants divert a significant proportion of assimilated carbon and energy to the synthesis of organic molecules that may have no obvious role in normal cell function. These molecules are known as secondary metabolites. These protect plants against being consumed by herbivores and against being attacked by microbial pathogens. Example like nicotine, cocaine etc belong to alkaloid family and are obtained from tobacco and cocoa plant majorly. Similarly anthocyanin and catechin are from flavonoids class which are present in almost all plants.

2. LITERATURE REVIEW

Microalgae appearing as individual cells or filaments and can grow under all types of environmental conditions. Being the most important member in blue biotechnology they require small amounts of nitrogen and phosphorus sources and carbon dioxide as base nutrient. Microalgae depend on sunlight for their energy requirements. They have profound number of bioactive compounds which have numerous valuable applications in pharmaceutical industry for herbal drug formulations. They possess many essential properties like antibacterial, antialgal and antiviral against pathogens present in marine water or in food. Their extracts (majorly aqueous which are cell free) have been tested for food and feed formulation to make an attempt for the use of antimicrobial components in developing therapeutical doses of regular antibiotics. 19 marine algal species (6 Chlorophyta, 8 Phaeophyta and 5 Rhodophyta) were collected from the western coast of Libya and their crude methanolic and aqueous extracts were evaluated for antibacterial activity against four gram-positive and four gram-negative pathogenic bacteria. These extracts showed a significant antibacterial activity against Gram positive (*Staphylococcus aureus, Bacillus subtilis, Bacillus* spp., and *Staphylococcus epidermidis*) as well as gram negative bacteria (*Escherichia coli, Salmonella typhi, Klebsiella* spp., and *Pseudomonas aeruginosa*). Both the aqueous and methanolic extracts of the above mentioned 19 marine algae displayed different variants of antimicrobial activities against different bacteria, in some cases methanolic extracts showed higher degree of anti-bacterial activity than aqueous extracts.

Among the erstwhile eukaryotic algae groups, [8] Rhodophyta or red algae is one of the oldest and largest group with about 5,000–6,000 species [9] and are mostly multicellular. It has been reported as many as 10,000 species;[10] more precisely it is indicated that there are about 4,000 species in about 600 genera (3,738 marine species in 546 genera and 10 orders (plus the unclassifiable); 164 freshwater species in 30 genera in eight orders).[11] The red algae group form a different group due to some peculiar characteristics like they have flagella and centrioles absent cells, with pigments like phycobiliproteins (cause of their red color), and external endoplasmic reticulum without chloroplasts and consisting of unstacked thylakoids. [10]. Laver and dulse (*Palmaria palmata*) are two examples of red algae and they form a traditional part of Asian and European cuisines. They are also used to make products such as agar, carrageenans and other food additives. [11]

In early seventies and eighties some investigation about the exploration of rare algae (thirty species were found) that displayed the immuno modulatory activity in humans revealed enhanced immune system's regulatory response and also had antiviral properties which was the most promising part of the discovery. Current research on Rhodophyta reported that these algae have the power to control as well as reduce both Candida and Herpes Simplex Virus populations. Therefore scientists predicted this algal population to exhibit various antiviral and antibacterial features against numerous pathogens.

Till now only the nutritional and medicinal properties of ocean vegetables have been mentioned or discovered by scientists. Huge population have taken advantages of ocean vegetables in their healthy diet as they contain minerals and vitamins of land plants in large amounts that are necessary for healthy diet and proper metabolism. [12] These days researchers are busy establishing a link between the numerous nutrient rich properties of red algae and their impact on our immune systems.

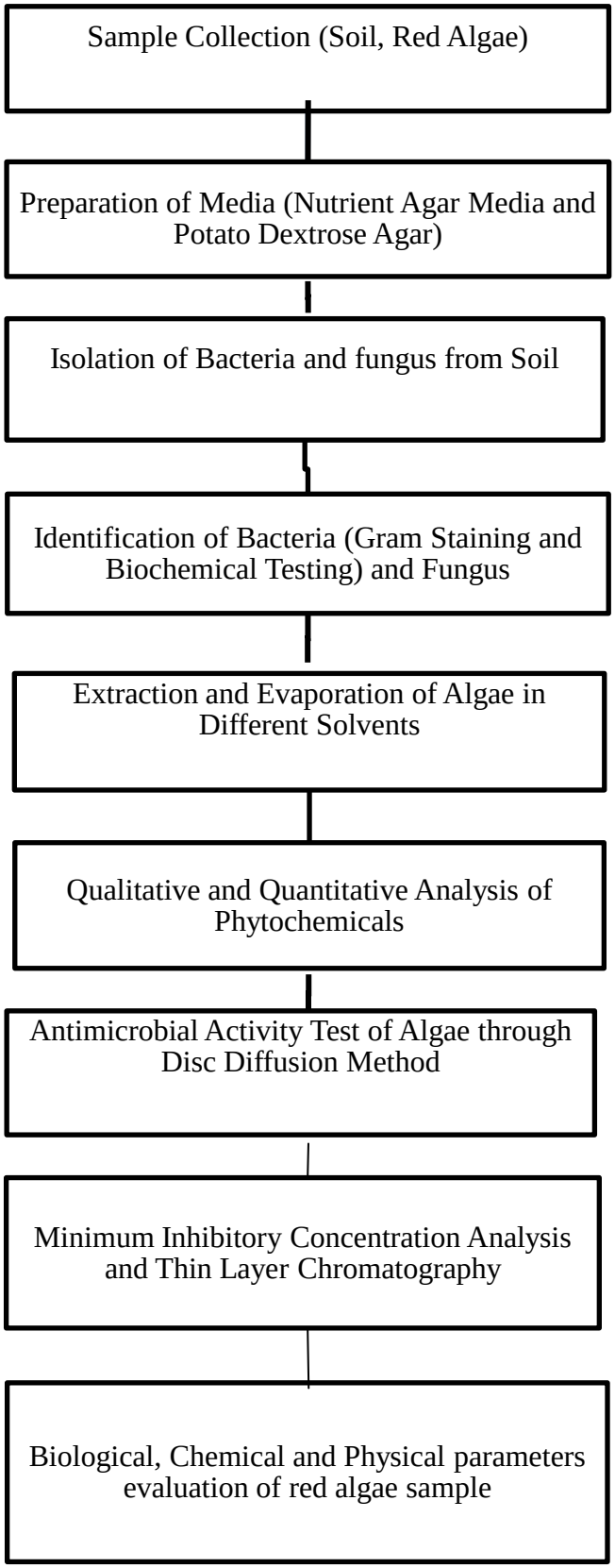
Marine algae, the *Kappaphycus alvarezii* synonymously known as red algae is one of the most important sources, used in a variety of commercial applications as gelling, thickening, and stabilizing agents, especially food products such as frozen desserts, chocolate milk, instant products, yogurt, jellies, and in sauce preparation. *Kappaphycus alvarezii* is a tough, fleshy, firm marine algae ("seaweed") up to 6 feet in length. Kappaphycus has been used in herbal medicine for its anti-inflammatory, diuretic, choleretic and haemostatic properties and has been approved for food use in South eastern Asia in Japan, Korea, and Taiwan. [13] At present, there is a trend to use functional foods that provide health benefits by reducing the risk of chronic diseases, enhancing the ability to manage them and promoting better performance, consequently improving the quality of life.

Since algae is rich in minerals, vitamins, bioactive substances, proteins, lipids and polyphenols as well as they have antibacterial, antiviral and antifungal properties they can be used as sources to extract bioactive compounds and have the potential to be taken as supplements. [14] In India, spices are commonly used, therefore for the preparation of spices in order to enhance the nutritional quality because of the presence of ash, the protein and crude fiber, the high amount of Vitamin E and the small amounts of niacin and Vitamin B2, *K. alvarezii* powder has been used as an ingredient and as a raw material. It has been reported that Kappa carrageenan derivatives from *Kappaphycus striatum*, majorly the sulphated

derivative at a high dose, manifest enhanced antitumor activity in comparison with carrageenan oligosaccharides and promoted immunostimulatory activity.

Natural sulphated polysaccharides are responsible for affecting the early stages of carcinogenesis which is the stage prior to the appearance of an invasive malignant tumor. [15] For the therapy or cure of malignant tumors natural sulphated polysaccharides can be used as a drug carrier and as a prophylactic with low toxicity, which decreases the side effects of cytostatic drugs. Evaluation for antimicrobial activity was done with the aqueous extract of Kappaphycus alvarezii and Minimum inhibitory concentration was determined against human pathogens. Due to inhibition of hyaluronidase enzyme Kappaphycus alvarezii possesses significant anti-inflammatory activity. Therefore, it was worthwhile to review its anti inflammatory property to give an overview of its status to scientist both modern and ancient.

3. MATERIALS AND METHODS



3.1 Quantitative Analysis of Phytochemicals

Preparation of Reagents –

1. For 20ml of reagent required in tannin estimation test-

0.1M $FeCl_3$	- 0.32g
0.008M Potassium ferrocyanide	- 0.0676g
Water	- 19.818ml
HCl	- 181.8µl

2. For Carbohydrate estimation in the sample reagents required-
   a. Na-K tartarate- Dissolve 5.625g of Na-K tartarate in 9.375ml of distilled water.
   b. 3, 5-DNS reagent- Dissolve 0.1875g DNS reagent in 3.75ml of NaOH.
   c. 2M NaOH- Dissolve 10g of NaOH in 125ml of distilled water.
   d. Final DNS reagent- Prepare fresh by mixing reagent a and reagent b and make up the volume to 18.75ml with water.
   e. Standard Sugar Solution- 0.1g of dextrose is dissolved in 10ml of distilled water.

3. For phenol estimation in the sample reagents required-
   a. Gallic acid standard solution- Dissolve 0.05g of gallic acid in 50ml of distilled water.
   b. Sodium Carbonate- Dissolve 1.125g of sodium carbonate in 15ml of distilled water.
   c. 0.2N Fc reagent- For 20 ml we add 2ml of 2N Fc reagent in 18ml of water.

Quantitative Analysis of Alkaloid-

1. Take 5g of red algae sample in 250ml beaker.
2. Add 20ml acetic acid followed by 180ml of ethanol and cover the beaker properly.
3. Leave it to allow it to stand for four hours.
4. Then filtration is done and extract is concentrated in water bath to one-fourth of its original volume.
5. Concentrated sodium hydroxide is added drop wise to the extract that is filtered until precipitation occurs.
6. Then after the entire solution settles down, the precipitate is collected and washed with dilute sodium hydroxide.
7. It is then again allowed to filter.
8. The residue which is obtained is alkaloid which is dried and weighed.

Alkaloid content = (weight of alkaloid/ weight of sample)*100

Quantitative Analysis of Tannin-

1. Take 500mg of red algae sample in 500ml flask and add 50ml distilled water to it.
2. Keep the beaker at shaker and allow it to shake for an hour to mix the contents properly.
3. Then filter the solution after shaking it for an hour in a 50ml beaker.
4. 5ml of filtrate is pipetted out in a test tube and 2ml of solution (ten -fold diluted 0.1M $FeCl_3$ in 0.1N HCl and 0.008M potassium ferrocyanide) is added.
5. Absorbance is taken within 10 minutes at 605nm.

Quantitative Analysis of Flavonoids-

1. Take 1g of red algae sample and extract it repeatedly with 10 ml of 80% aqueous methanol at room temperature.
2. Solution is filtered with the help of whatman paper.
3. The filtrate is transferred into a petridish and it was left to evaporate to dryness over water bath.
4. It is weighed until weight becomes constant after intervals of drying.

Quantitative Analysis of Carbohydrate-

1. For estimating the carbohydrate content take 500µl of sample (0.5g in 1ml) solution and add 2.5ml of distilled water.
2. Add 2ml of 5% DNS reagent to the above mixture and mix well.
3. Measure the absorbance at 540nm against blank.
4. Then compare it with standard solution of dextrose.
5. To prepare the blank 3ml of distilled water is added to 2ml of 5% DNS.

Quantitative Analysis of Phenol-

1. Take 50µl sample solution and add 2.5ml of Fc reagent mixture to it.
2. Add 450µl of distilled water to the above mixture.
3. Mix the solution and incubate at room temperature for 5 minutes in dark.
4. After this add 2ml of 7.5% sodium carbonate and incubate at room temperature for 90 minutes.
5. Measure the absorbance at 765nm. (Gallic acid dilutions were used as standard solution).

3.2 Preparation of media for MIC

3.2.1 Preparation of NAM (Nutrient Agar Media)

NAM (Nutrient Agar Media) (200ml):

To 200 ml of distilled water, add

A. Peptone - 1.0gm
B. Beef Extract - 0.6gm
C. NaCl - 1.0gm
D. Agar - 3.0gm

It was shaken well until the solutes have dissolved and the pH is adjusted to 6.8 and the volume was made up to 200ml with distilled water and agar was added and is kept for autoclaving. Pour nutrient agar into all the Petri dishes and leave plates and allow the agar to solidify.

3.2.2 Preparation of PDA (Potato dextrose Agar)

PDA (Potato Dextrose Agar) (200 ml):

To 200ml of distilled water, add

A. Dextrose - 4.0gm
B. Potato Starch - 0.6gm
C. Agar - 3.0gm

It was shaken well until the solutes have dissolved and the pH is adjusted to 5.6 and the volume was made up to 200ml with distilled water and agar is added and is kept for autoclaving. Pour the media into each Petri dish and leave plates to dry until the agar has solidified.

Protocol for MIC-

1. The disc diffusion method is used to screen for antimicrobial activities and the same is done for MIC.
2. The plates are prepared by pouring 25ml of media (NAM for bacterial strains and PDA for fungal strains).
3. After 15 minutes, a sterile cotton swab is dipped into the bacterial and fungal broths and is spread uniformly on respective agar plates.
4. Then sterile Whatman number one filter papers (6mm diameter) are placed on the spread-surface of agar plates and 3µl of extract (dissolved at 200mg/ml) is spotted on each of the filter papers.
5. Number 1 disc is always referred to as stock solution prepared of algae extract while the number 2, 3, 4 etc. discs showed further dilutions of stock prepared.
6. After this, the plates are incubated at 37°C for 24 hours (for bacteria) and at 30°C for 5-7 days (for fungi). Subsequently, the inhibition zones formed around the discs are measured in millimeters.

3.3 Thin Layer Chromatography

TLC is used to determine the Rf value of the alkaloids and triterpinoids in the sample.

1. Take silica gel coated sheets (Merck sheets) for TLC.
2. Length of the sheet to be cut is 10 cm and the breadth depends upon the number of samples to be loaded.
3. Each sheet for alkaloids and triterpenoids the breadth to be cut is 2cm.
4. The respective methanolic extracts are loaded (5µl each) and then the sheets are allowed to dry.
5. The loaded plate is then placed in a tank containing the respective running solvent for each bioactive compound with caution such that the loaded sample does not dip in the solvent system.
6. The set up is left undisturbed and the solvent is allowed to move upright (capillary action) till 9.5 cms.
7. The plates are then removed and allowed to dry and the spots are visualized accordingly.

$$\text{Rf value} = \frac{\text{Distance from start to centre of substance spot}}{\text{Distance from spot to solvent front}}$$

Table no. 1: Running solvents of different bioactive compounds

Compounds	Running solvents	Respective ratio
Alkaloids	Benzene:Methanol	8:2
Triterpenoids	Acetic acid:Water	1:3

Table no.2: Spot visualization of different bioactive compounds

Compounds	Spot Visualization
Alkaloids	Visible as yellow or orange florescent spots under UV
Triterpenoids	Visible as yellow or orange florescent spots under UV

3.4 Evaluation of physical parameters

Swelling Index

1. Take 1g of red algae sample and add 25ml of water to it in a measuring cylinder.
2. Shake at each 10 minutes interval for an hour.
3. Leave it for three hours at room temperature.
4. Measure the volume occupied by the sample.

Foaming Index

1. Take 1g of red algae sample and add 100 ml of water in a beaker.
2. Boil it moderately for 30 minutes and allow it to cool.
3. Then filter it in a 100ml volumetric flask and make up the volume of the filtrate till 100 ml with water to dilute the filtrate.
4. Take 10 test tubes and pipette out 1ml, 2ml, 3 ml till 10 ml of diluted filtrate in corresponding test tubes and make up the volume to 10ml in each test tube.
5. Shake each test tube for 15 seconds.
6. Leave all the test tubes for 15 minutes.
7. Measure the height of the foam formed.

pH evaluation

1. Dissolve 0.5g of red algae sample and 50ml of distilled water to it in a beaker.
2. Incubate it for an hour.
3. Filter the entire solution into another beaker and measure the pH through pH meter.

Moisture content percentage calculation

1. Record the weight of the empty aluminium foil without the red algae sample in it).
2. Weigh 2g of sample in the same aluminium foil and incubate the same at 105°C for 3 hours.
3. After 3 hours weigh the same until and record the readings till the weight becomes constant at each interval of weighing.

Moisture content % = (Weight of the sample taken- final weight)*100

Determination of foreign matter

1. Weigh accurately 10g to 50 g of red algae sample and spread it out in a thin layer.
2. Inspect the sample with the unaided eye or with the use of a 6X lens and separate the foreign organic matter manually as completely as possible.
3. Weigh and determine the percentage of foreign organic matter from the weight of the sample taken.

Extractive values:

Extractive values of crude drugs helps to determine the type and nature of the constituents present in it as well as gives the idea of the constituents present as it is the only method to determine the existence of a constituent in a crude drug.

Determination of extractive values:

1. Alcohol soluble extractive values-

a. Take 0.625g of powdered red algae sample, with 12.5ml of methanol in a closed flask for 24 hours, shaking frequently during 6 hours and allowing it to stand for 18 hours.
b. Filter rapidly taking precautions against loss of alcohol.
c. Evaporate 25% alcohol soluble extractive with reference to the sample.

2. Water soluble extractive values-

a. Take 0.625g of powdered red algae sample with 25% of chloroform (3.125ml) and 75% water (9.375ml) in a closed flask for 24 hours, shaking frequently during 6 hours and allowing it to stand for 18 hours.
b. Filter rapidly taking precautions against loss of alcohol.
c. Evaporate 25% water soluble extractive with reference to the sample.

Volatile Oil content determination

1. The distillation method (Clevenger apparatus) is used to determine the volatile oil content.
2. Take 10g red algae sample and the sample is placed in a distillation flask with 90ml water and 1ml glycerin and is connected to the receiver.
3. The connection is further done with the condenser.

4. On distillation, condensation of oil and water takes place and the volatile oil which collects in the graduated receiver on top of the layer of water is measured.

3.5 Evaluation of Biological parameters

These are the some specific tests to confirm the presence of microbes present in the algae sample that otherwise can be pathogenic and these are required to be eliminated from the sample through repeated filtration or dilution in order to proceed for the development of a herbal product through the red algae sample.

Preparation of Media-

Fluid Lactose Medium

Meat Extract - 0.03g

Gelatin peptone - 0.05g

Lactose monohydrate - 0.05g

Water - 10ml

pH after sterilization through autoclaving - 6.7-7.1

Fluid Enterobacteria Enrichment Broth Mossel Medium

Gelatin Peptone - 0.1g

Glucose - 0.05g

Bile Salts - 0.2g

Monobasic potassium phosphate - 0.02g

Disodium hydrogen phosphate - 0.08g

Brilliant green - 0.00015g

Water - 10 ml

pH after boiling the components at 100°C for 30 minutes – 7.0-7.4

VRB (Violet/Red/Bile) Agar with glucose

Yeast extract - 0.075g

Gelatin Peptone - 0.175g

Bile Salts - 0.0375g

Lactose monohydrate - 0.25g

Sodium Chloride - 0.125g

Glucose - 0.25g

Neutral Red - 0.00075g

Crystal violet - 0.00005g

Agar - 0.375g

Water -25ml

pH after boiling (boil to effect solution and do not autoclave) - 7.2-7.6

Fluid EC Medium

Peptone - 0.2g

Lactose monohydrate - 0.05g

Bile Salts - 0.015g

Dipotassium hydrogen phosphate - 0.04g

Monobasic potassium phosphate - 0.015g

Sodium Chloride - 0.05g

Water - 10ml

pH after sterilization through autoclaving - 6.8-7.0

EMB(Eosin-Methylene Blue) Agar Medium

Gelatin Peptone - 0.25g

Dipotassium hydrogen phosphate - 0.05g

Lactose monohydrate - 0.25g

Agar - 0.375g

Eosin - 0.01g

Methylene Blue - 0.001625g

Water - 25ml

pH after sterilization (through autoclaving) - 6.9-7.3

Fluid Rappaport Medium

Soyabean peptone - 0.05g

Sodium Chloride - 0.08g

Monobasic potassium chloride - 0.016g

Malachite green - 0.0012g

Magnesium chloride hexahydrate - 0.4g

Water - 10ml

Dissolve malachite green and magnesium chloride hexahydrate, and the remaining solids separately in the water, and sterilize by heating in an autoclave. For the use, mix both the solutions after sterilization.

pH - 5.4-5.8

Brilliant Green Agar Medium

Peptone - 0.25g

Yeast extract - 0.075g

Sodium Chloride - 0.125g

Lactose monohydrate - 0.25g

Sucrose - 0.25g

Phenol Red - 0.002g

Brilliant green - 0.0003125g

Agar - 0.5g

Water - 25ml

pH after sterilization through autoclaving - 6.7-7.1

XLD (Xylose-Lysine-Deoxycholate) Agar Medium

$_{D}$-Xylose - 0.0875g

$_{L}$-Lysine monohydrochloride - 0.125g

Lactose monohydrate - 0.1875g

Sucrose - 0.1875g

Sodium chloride - 0.125g

Yeast extract - 0.075g

Phenol Red - 0.002g

Sodium deoxycholate - 0.0625g

Sodium thiosulfate pentahydrate - 0.17g

Ammonium iron(III) citrate - 0.02g

Agar - 0.3375g

Water - 25ml

pH after boiling (do not autoclave) - 7.2 - 7.6

Fluid Soyabean-Casein Digest medium

Casein peptone - 0.17g

Soyabean peptone - 0.03g

Sodium chloride - 0.05g

Dipotassium hydrogen phosphate - 0.025g

Glucose - 0.025g

Water - 10ml

pH after sterilization through autoclaving - 7.1-7.5

Mannitol Salt Agar Medium

Casein peptone - 0.125g

Animal tissue peptone - 0.125g

Meat extract - 0.025g

D-Mannitol - 0.25g

Sodium chloride - 1.875g

Phenol Red - 0.00625g

Agar - 0.375g

Water - 25ml

pH after sterilization through autoclaving - 7.2 - 7.6

Reinforced Clostridium Medium

Beef extract - 0.1g

Peptone - 0.1g

Yeast extract - 0.03g

Soluble starch - 0.01g

Glucose monohydrate - 0.05g

Cysteine hydrochloride - 0.005g

Sodium chloride - 0.05g

Sodium acetate - 0.03g

Agar - 0.005g

Water - 10ml

pH after autoclaving - 6.6-7.0

Columbia Agar Medium

Columbia agar - 1.1g

Water - 25ml

pH after autoclaving - 7.3±0.2

Cetrimide Agar Medium

Gelatin Peptone - 0.5g

$MgCl_{2.}6H_2O$ - 0.075g

Potassium Sulfate - 0.25g

Cetrimide - 0.075g

Glycerin - 0.25g

Agar - 0.34g

Water - 25ml

pH before autoclaving - 7.2±0.2

Czapek Dox Broth

Sucrose - 0.3g

Sodium nitrate - 0.03g

Dipotassium phosphate - 0.01g

MgSO4 - 0.005g

Potassium Chloride - 0.005g

Ferrous Sulfate - 0.0001

Agar - 0.15g

Water - 10ml

pH before autoclaving - 7.3±0.2

Czapek Yeast Dextrose Agar Medium

Sucrose - 0.75g

Yeast Extract - 0.125g

Dipotassium hydrogen phosphate	- 0.025g
Sodium Nitrate	- 0.0075g
Potassium Chloride	- 0.00125g
Magnesium Sulfate	- 0.00125g
Ferrous Sulfate	- 0.000025g
Copper Sulfate	- 0.0000125g
Zinc Sulfate	- 0.000025g
Agar	- 0.375g
Water	- 25ml
pH before autoclaving	- 7.3±0.2

Sabouraud Glucose Agar Medium with Antibiotics

Peptone (animal tissue and casein)	- 0.25g
Glucose	- 1g
Agar	- 0.375g
Water	- 25ml
pH before autoclaving	- 5.4-5.8
Chloremphenicol	- 250µl

Protocols for Detection of Microbial Contamination

1. Detection of *Enterobacteria*-

a. Take 1g of red algae sample and add 9ml of Fluid Lactose Medium to it to form a solution.
b. Transfer 1ml of the above solution to 9ml of Enrichment Broth Mossel medium in a fresh test tube and incubate it for 24 hours at 37°C.
c. After 24 hours, mix gently the test tube and dip a sterile cotton swab into it and spread it on the entire surface of VRB agar medium plate.
d. Allow it to dry in sterile conditions and then incubate it for 24 hours at 37°C.
e. Result- If red colonies are found then the sample may be contaminated with *Enterobacteria*.

2. Detection of *E.coli*-

a. Take 1g of red algae sample and add 9ml of Fluid Lactose Medium to it to form a solution.
b. Transfer 1ml of the above solution to a fermentation tube with 9ml of EC Broth and incubate it for 24 hours at 45°C in a water bath.
c. If gas bubbles do not appear then it is reported that the sample is free from *E.coli.*
d. After 24 hours, if gas bubbles appear then mix gently the test tube and dip a sterile cotton swab into it and spread it on the entire surface of EMB agar medium plate.
e. Allow it to dry in sterile conditions and then incubate it for 24 hours at 35°C.
f. Result- If metallic sheen with blue black colonies are found under transmitted light then the sample may be contaminated with *E.coli.*

3. Detection of *Salmonella*-

a. Take 1g of red algae sample and add 9ml of Fluid Lactose Medium to it to form a solution.
b. Incubate it at 30°C to 35°C for 24-72 hours and examine the medium for growth.
c. If growth appears then transfer 1ml of the above solution to a test tube containing 9ml of Rappaport Medium and incubate it for 12 to 24 hours.
d. After 24 hours, if growth appears then mix gently the test tube and dip a sterile cotton swab into it and spread it on the entire surface of Brilliant Green Agar medium plate as well as on XLD Agar Medium plate.
e. Allow it to dry in sterile conditions and then incubate it for 24 to 48 hours at 30°C to 35°C.
f. Result- If small transparent colorless or opaque pink or white colonies appear on Brilliant Green Agar Medium plate and Red with or without black center colonies appear on XLD Agar Medium plate then the sample may be contaminated with *Salmonella.*

4. Detection of *Staphylococcus aureus*-

a. Take 1g of red algae sample and add 9ml of Fluid Soyabean Casein Digest Medium to it to form a solution.
b. Incubate it at 30°C to 35°C for 24-48 hours and examine the medium for growth.

c. If growth appears then transfer 1ml of the above solution to a test tube containing 9ml of Fluid Soyabean Casein Digest Medium with 7.5% sodium chloride and incubate it for 24 hours for examination of growth.
d. After 24 hours, if growth appears then mix gently the test tube and dip a sterile cotton swab into it and spread it on the entire surface of Mannitol Salt Agar Medium plate.
e. Allow it to dry in sterile conditions and then incubate it for 24 to 48 hours at 30°C to 35°C.
f. Result- If yellow colonies surrounded by yellow zone appear on Mannitol Salt Agar Medium plate then the sample may be contaminated with *Staphylococcus aureus*.

5. Detection of *Pseudomonas*-

a. Take 1g of red algae sample and add 9ml of Fluid Soyabean Casein Digest Medium to it to form a solution.
b. Incubate it at 30°C to 35°C for 24-48 hours and examine the medium for growth.
c. After 24 hours, if growth appears then mix gently the test tube and dip a sterile cotton swab into it and spread it on the entire surface of Cetrimide Agar Medium plate.
d. Allow it to dry in sterile conditions and then incubate it for 24 to 48 hours at 30°C to 35°C.
e. Result- Growth of colonies indicates the presence of *Pseudomonas.*

6. Detection of *Clostridia*-

a. Take 1g of red algae sample and add 9ml of Reinforced Clostridium Medium to it to form a solution.
b. Incubate it at 30°C to 35°C for 24-48 hours and examine the medium for growth.
c. After 24 hours, if growth appears then mix gently the test tube and dip a sterile cotton swab into it and spread it on the entire surface of Columbia Agar Medium plate.
d. Allow it to dry in sterile conditions and then incubate it for 24 to 48 hours at 30°C to 35°C.

e. Result- Rod shaped white colored colonies appear if the sample is contaminated by *Clostridia.*

7. Detection of *Aspergillus brasiliensis*-

a. Take 1g of red algae sample and add 9ml of Czapek Dox Broth to it to form a solution.
b. Incubate it at 30°C to 35°C for 24-48 hours and examine the medium for growth.
c. After 24 hours, if growth appears then mix gently the test tube and dip a sterile cotton swab into it and spread it on the entire surface of Czapek Yeast Dextrose Agar Medium plate.
d. Allow it to dry in sterile conditions and then incubate it for 24 to 48 hours at 30°C to 35°C.
a. Result- Appearance of white with black spores at centre colonies indicate the presence of *Aspergillus brasiliensis.*

8. Detection of *Candida glabrata*-

b. Take 1g of red algae sample and add 9ml of distilled water to it to form a solution.
c. Then mix gently the test tube and pipette out 10µl of solution from it and spread it on the entire surface of Sabourand Glucose Agar Medium plate with antibiotic.
d. Allow it to dry in sterile conditions and then incubate it for 24 to 48 hours at 30°C to 35°C.
e. Result- Appearance of pink, glossy, small and rounded colonies indicate the presence of *Candida glabrata.*

Treatment of microbial contaminated sample-

1. Take 1g of *K.alvarezii* sample and dissolve in 5ml of autoclave water.
2. Mix the solution gently and use 5ml sterile syringe and autoclave cotton for filtration.
3. In LAF, open the syringe and put a layer of cotton inside it.
4. After this, pour 3ml solution in the syringe and press the syringe plunger gently to push sample through the cotton.
5. One drop of filtrate sample passes through a needle is spread on specific media. Incubate the media and observe the result.

4. RESULTS AND DISCUSSIONS

4.1 Quantitative analysis of phytochemicals

Quantitative analysis of phytochemicals present in *Kappaphycus alvarezii* is done which shows the following results-

Phytochemical	Total Composition
Alkaloid	6%
Flavonoids	42%
Phenol	1.4976 mg/ml
Carbohydrate	0.29018 mg/ml
Tannin	0.053 mg/ml

Fig 2: Table showing quantity of phytochemicals present in red algae

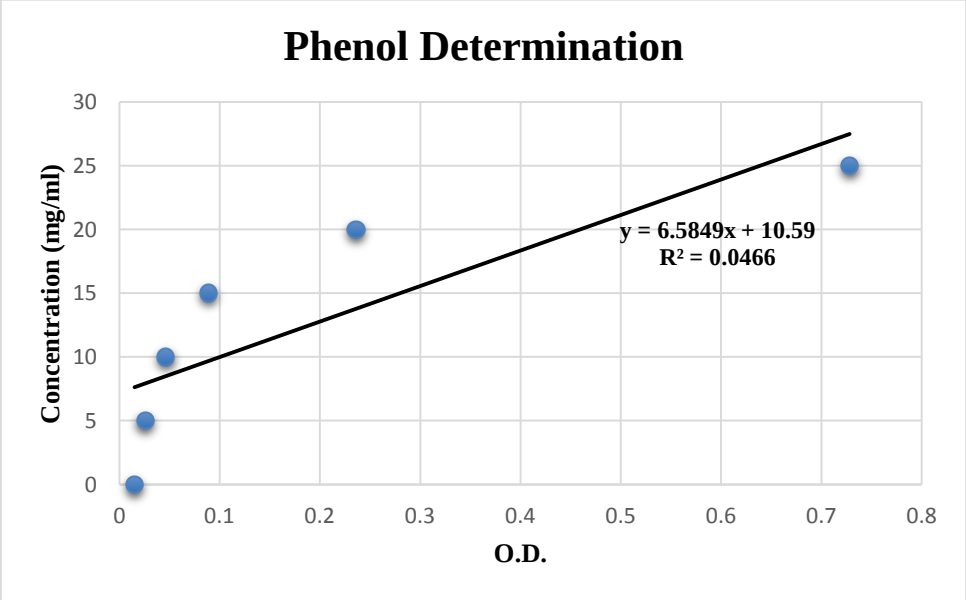


Fig 3: Graph showing phenol content in red algae sample

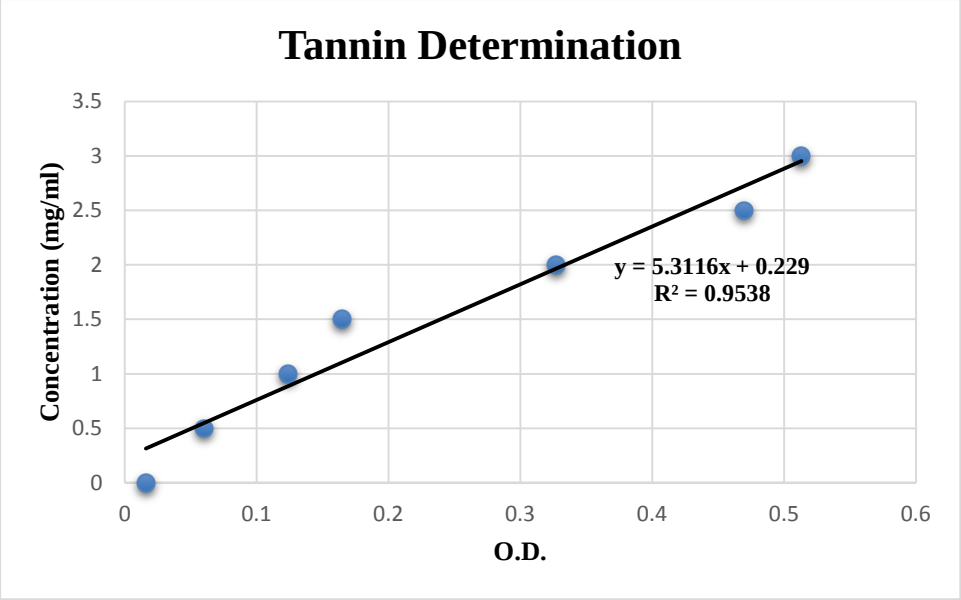


Fig 4: Graph showing tannin content in red algae sample

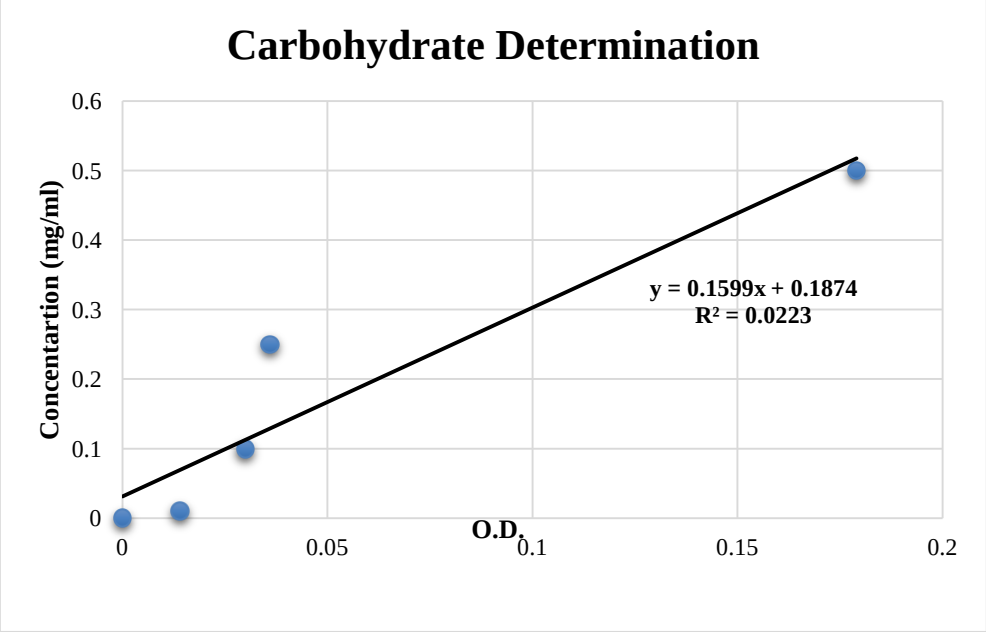


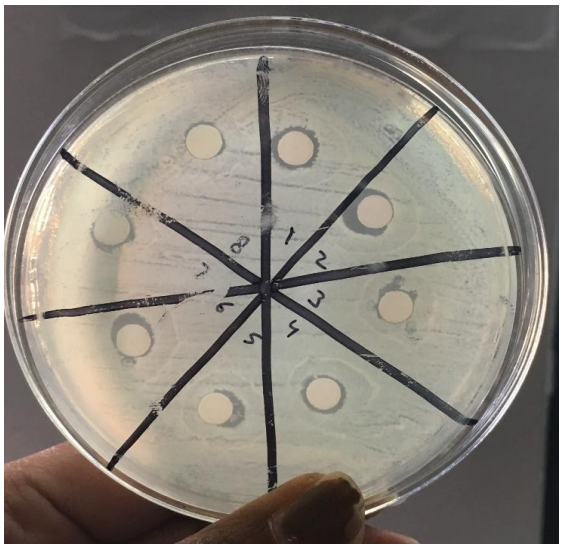
Fig 5: Graph showing carbohydrate content in red algae sample

4.2 Minimum Inhibitory Concentration Evaluation

On the basis of previous results of antibacterial and antifungal activity shown by four different extracts of red algae namely cow urine, benzene, acetone and ethanol against bacterial strains (bacteria#1 isolates, *Shigella flexneri)* and the fungal strains *(Aspergillus candidus, Aspergillus niger, Penicillium species, Penicillium corylophilum)* that were obtained, MIC is conducted further.

Cow Urine Concentrations(mg/ml)	Zone of Inhibition (mm)
200	9
100	11.5
50	9.5
25	7
12.5	10.5
6.25	9
3.125	10
1.5625	9

Fig 6: Plate and table showing Minimum Inhibitory Concentration of *Shigella flexneri*



Acetone Concentrations(mg/ml)	Zone of Inhibition (mm)
200	10
100	9
50	8
25	8
12.5	7
6.25	7
3.125	7
1.5625	6

Fig 7: Plate and table showing Minimum Inhibitory Concentration of bacteria#1isolates

Thus these results show MIC value of *Shigella flexneri* and bacteria#1 isolates to be 25mg/ml and 3.125mg/ml respectively.

Cow Urine Dilutions	Zone of Inhibition (mm)
200	10.5
100	9
50	8
25	6
12.5	6
6.25	6

Fig 8: Plate and table showing Minimum Inhibitory Concentration of *Aspergillus candidus*

Thus this result shows MIC value of *Aspergillus candidus* to be 50mg/ml.

Benzene Concentrations (mg/ml)	Zone of inhibition (mm)		
	Aspergillus niger	*Penicillium species*	*Penicillium corylophilum*
200	8.5	9	9
100	8	8	6.5
50	7.5	8	6
25	7.5	7	6
12.5	7	7	6
6.25	6.5	7	6

Fig 9: Table showing Minimum Inhibitory Concentration of all three fungal strains

Thus these results show MIC value of *Aspergillus niger*, *Penicillium species* and *Penicillium corylophilum* to be 6.25mg/ml and 6.25mg/ml and 100mg/ml respectively.

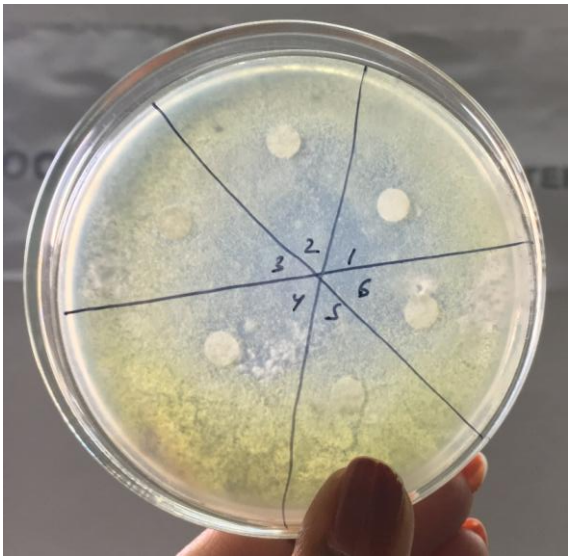


Fig 10: Plate showing MIC of *Penicillium corylophilum*

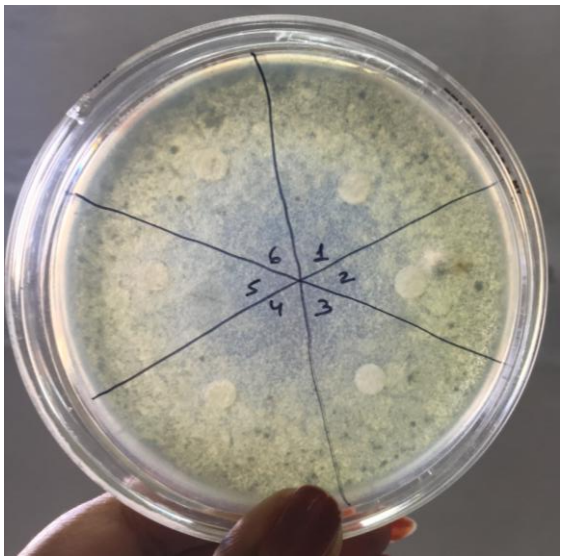


Fig 11: Plate showing MIC of *Penicillium species*

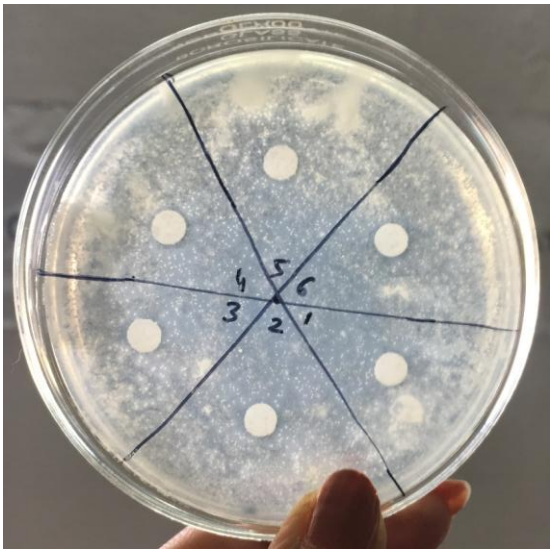


Fig 12: Plate showing MIC of *Aspergillus niger*

The Minimum Inhibitory Concentration of cow urine extract showed maximum inhibition against *Shigella flexneri* with a diameter of inhibition zone ranging from 7–11.5 mm and *Aspergillus candidus* with a diameter of inhibition zone ranging from 6–10.5 mm whereas the acetone dilutions have shown maximum inhibition against bacteria#1 isolates with diameter of inhibition zone ranging from 6–10mm. Similarly benzene extract showed maximum inhibition against *Aspergillus niger, Penicillium species, Penicillium corylophilum* with a diameter of inhibition zone ranging from 6–9 mm.

4.3 Thin Layer Chromatography

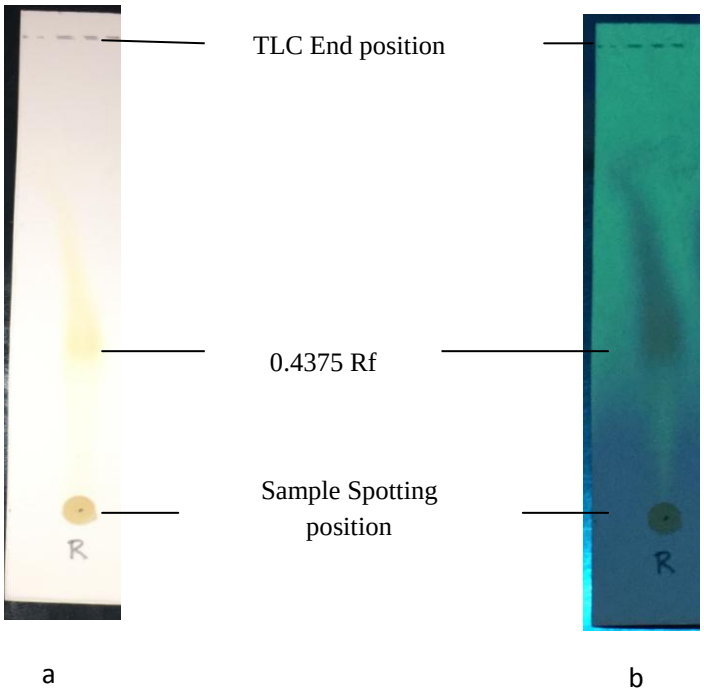


Fig 13: TLC of triterpenoids (a- under normal light and b- under UV) in methanolic extract of red algae

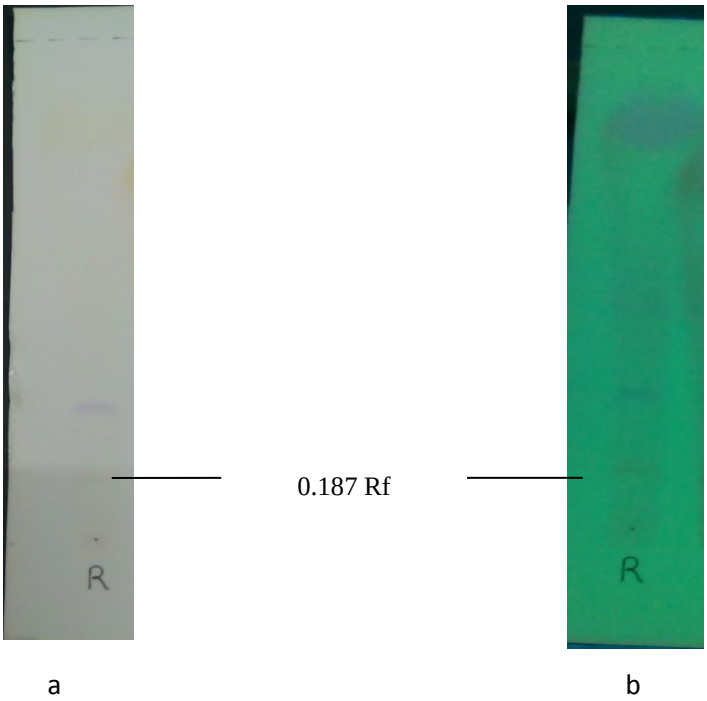


Fig 14: TLC of alkaloids (a- under normal light and b- under UV) in methanolic extract of red algae

4.4Evaluation of physical parameters

The following images and graph show the various physical parameters that are evaluated before proceeding for the formulation of any herbal product.

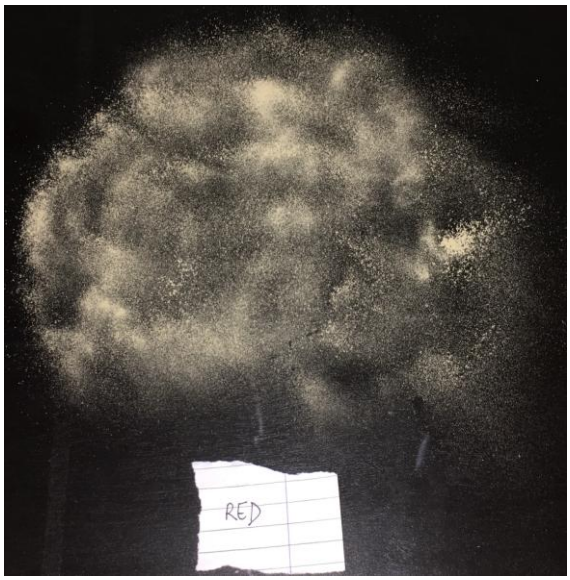


Fig 15: Foreign Matter Determination

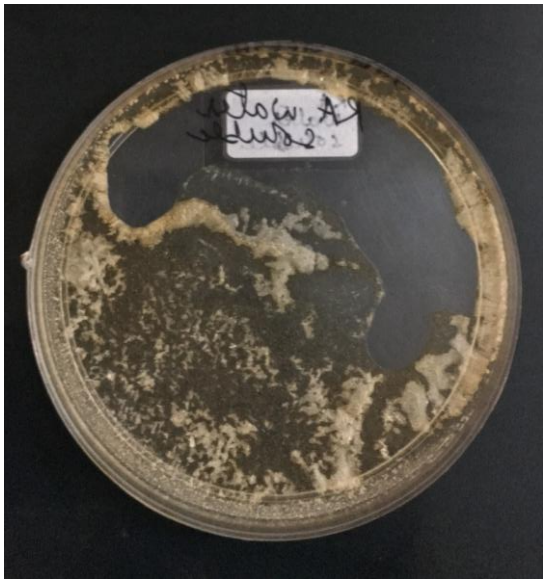


Fig 16: Water soluble extractive value

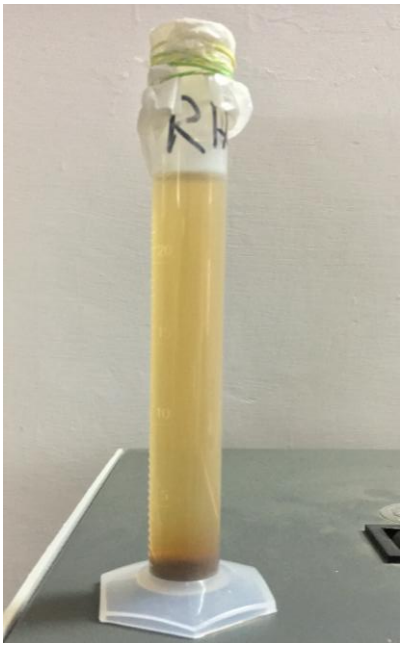


Fig 17: Swelling Index

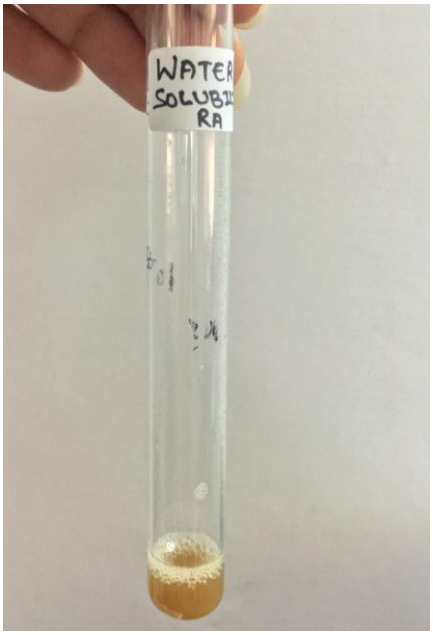


Fig 18: Solubility in water

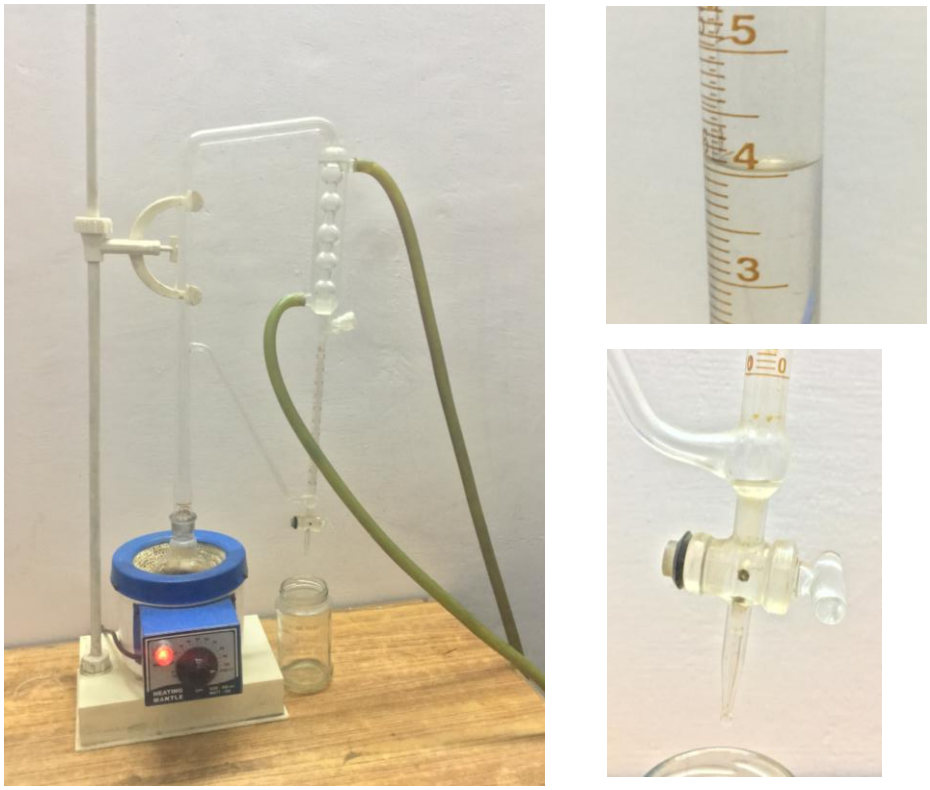


Fig 19: Volatile oil content calculation through Clevenger apparatus

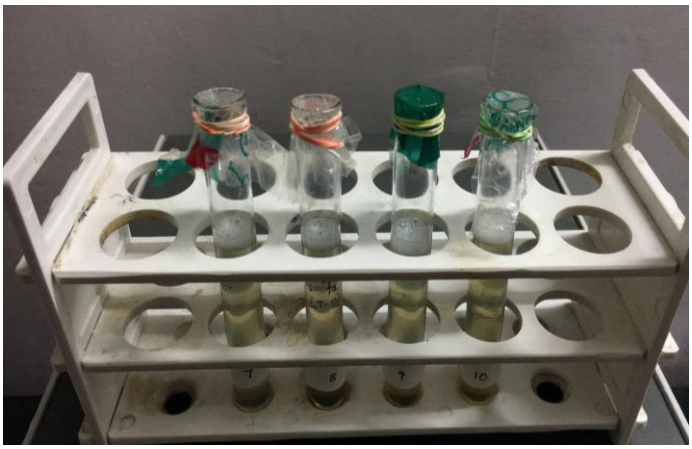


Fig 20: Foaming Index Calculation

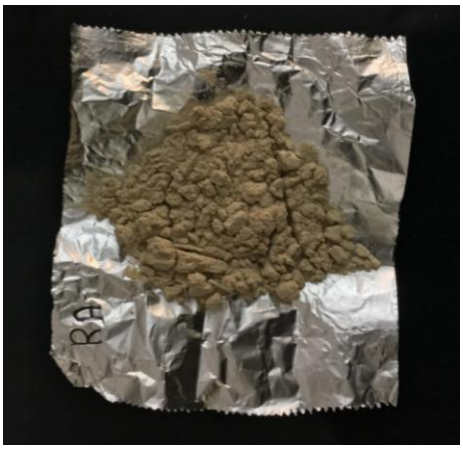


Fig 21: Moisture content

PARAMETERS	VALUE
Moisture Content	18.5%
Water Soluble Extractive	6.5%
Alcohol Soluble Extractive	0.5%
pH	8.1
Swelling Index	2 units of 25ml of measuring cylinder
Foaming Index	<1cm
Volatile Oil Content	Nil
Solubility in water	Highly soluble
Foreign Matter	Absent

Fig 22: Table showing results of evaluation of various physical parameters

4.4.2 Evaluation of biological parameters

The following images and graph show the various biological parameters that are evaluated (determining the microbial contamination in the sample and eliminating the specific microbes and making the sample pathogen free by filtration method) before proceeding for the formulation of any herbal product.

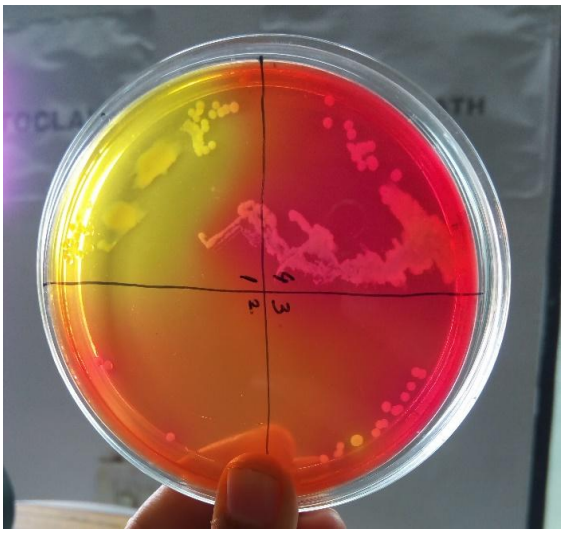


Fig 23: *Enterobacteria* on VRB Agar plate

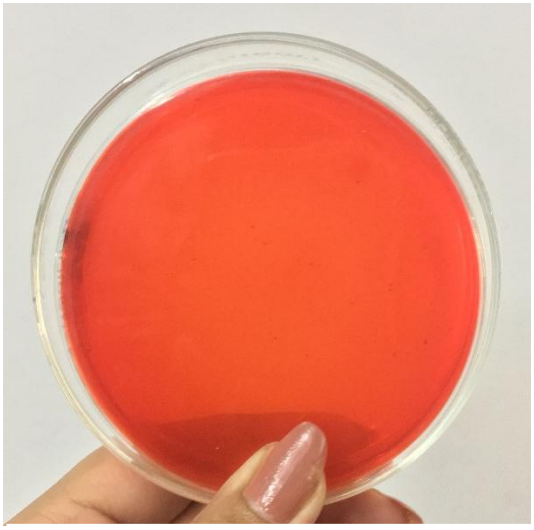


Fig 24: After filteration *Enterobacteria* is absent in the sample.

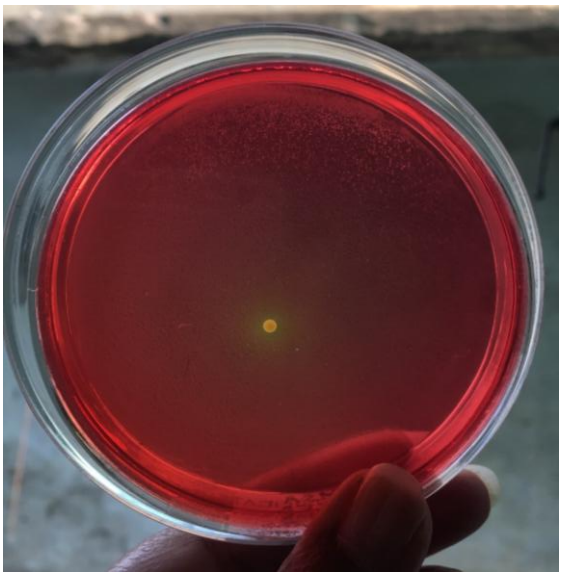


Fig 25: *Staphylococcus aureus* on Mannitol Salt Agar plate

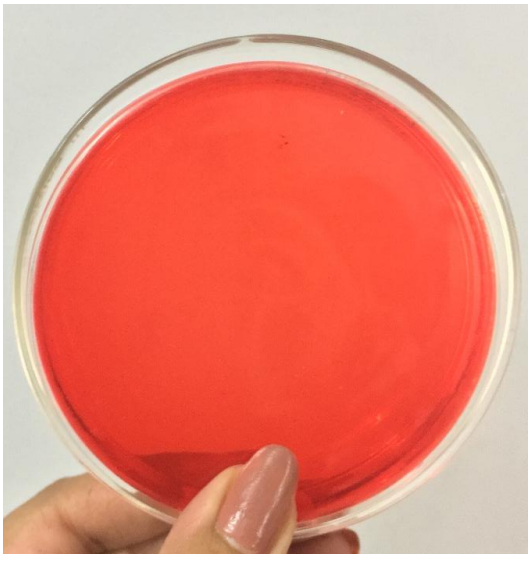


Fig 26: After filteration *Staphylococcus aureus* is absent in the sample.

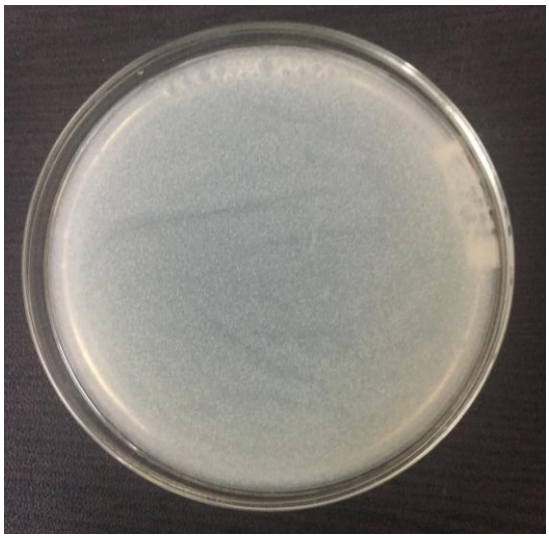


Fig 27: *Pseudomonas on Cetrimide Agar* plate

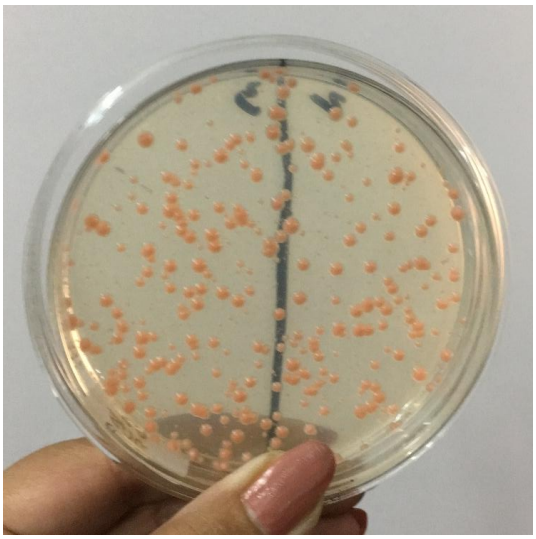


Fig 28: *Candida* on Sabourand Agar Plate

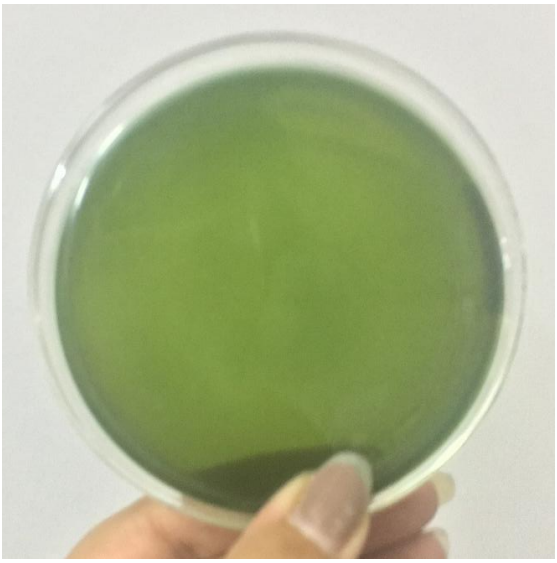


Fig 27: *Salmonella* on Brilliant Green agar plate

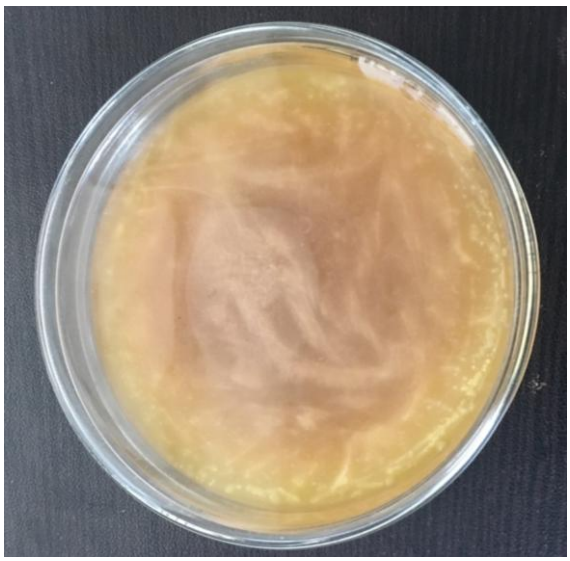


Fig 28: *Salmonella* on XLD agar plate

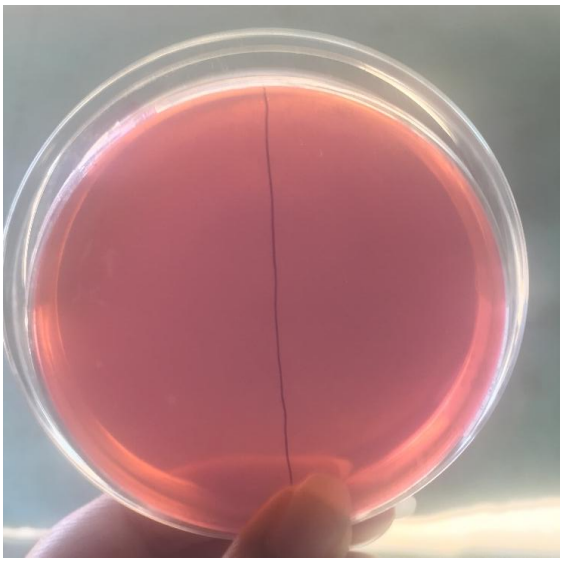


Fig 29*: E.coli* on EMB plate

Fig 30: *Clostridia* On Columbia Agar plate

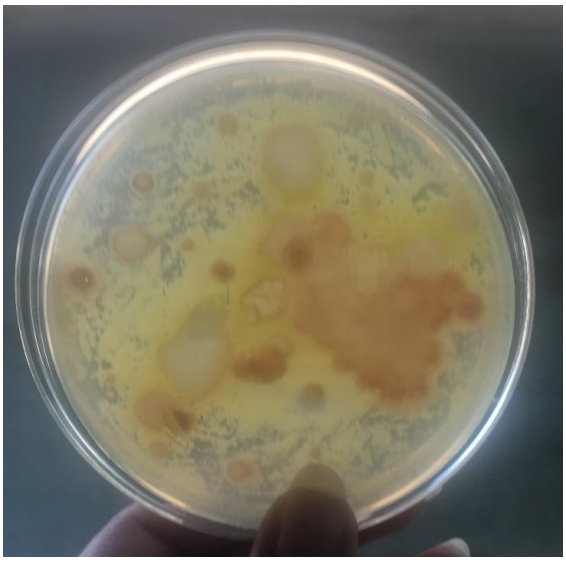


Fig 31: *Aspegillus brasiliensis* On Czapek Yeast Agar plate

Fig 32: Table showing results of Microbial contamination of various bacterial strains and a fungal strain (before and after treatment of red algae sample)

MICROBES	RESULTS	SYRINGE FILTERATION	RESULTS AFTER FILTERATION
Enterobacteria	Present slightly	Yes	Absent
E.coli	Absent	No	Absent
S.aureus	Present	Yes	Absent
Pseudomonas	Absent	No	Absent
A.brasiliensis	Absent	No	Absent
Clostridia	Present	Still in process	-
Candida	Present	Still in process	-

5. CONCLUSION

Red algae is rich in wide variety of phytochemicals like flavonoids, saponins, phytosterols, terpenoids, phenolic, reducing sugar and tannin that have been found to have antimicrobial activities. Upon Quantitative analysis of phytochemicals we found that flavonoids are present in huge amounts followed by alkaloids, phenols and carbohydrates. Minimum Inhibitory Concentration results reveal that cow urine dilutons have shown maximum inhibition against *Shigella Flexneri* as well as *Aspergillus niger*. Similarly, benzene extracts have shown maximum inhibitory activity against *A. niger, P. corylophilum* and *Penicillium species* with zone of inhibition varying from 6mm to 9 mm. TLC Analysis reported the presence of alkaloids and triterpiniods in the red algae sample. We have also screened the red algae sample for the evaluation of physical as well as biological parameters required to be checked before the formulation of a herbal product through it. The red algae extract screened for phytochemical constituents seemed to have the potential to act as a source of useful drugs in order to improve the health quality of the population as a result of the presence of various valuable compounds that are important for good health.

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