"Screening of phytochemical and antimicrobial activity of different solvent extracts of *Sargassum swartzii* and evaluation of herbal product formulation"

To be submitted as Major Project in partial fulfillment of the requirement for the degree of **M.Tech**

Submitted by

SHREYA JAIN

2K15/BME/11

Under the supervision of

DR. SAURABH C. SAXENA

(ASSISTANT PROFESSOR)

&

PROF. D. KUMAR

(HEAD OF DEPARTMENT)

DEPARTMENT OF BIOTECHNOLOGY

DELHI TECHNOLOGICAL UNIVERSITY

CERTIFICATE

This is to certify that the dissertation entitled: **"Screening of phytochemical and antimicrobial activity of different solvent extracts of *Sargassum swartzii* and evaluation of herbal product formulation"** by Shreya Jain (2K15/BME/11) in the partial fulfillment 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 honoring of any other degree.

Dr. Saurabh C. Saxena	Prof. D. Kumar
(Supervisor)	(Co Supervisor)
Assistant Professor	Head of Department
Department of Bio-Technology	Department of Bio-Technology
Delhi Technological University	Delhi Technological University

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This is to certify that **Ms. Shreya Jain,** D/o **Mr. Sudhir Kumar Jain,** student of M.Tech Biomedical Engineering, Final year, Enrollment No – 2k15/BME/11, from **"Delhi Technological University, Rohini, New Delhi"** has successfully completed her project of Six month, from 1st January 2017 to 30th June 2017, in our Biotechnology Division at Noida Campus with **A+ Grade** on the topic: **"Screening of phytochemical and antimicrobial activity of different solvent extracts of *Sargassum swartzii* and evaluation of herbal product formulation.",** this work has not be submitted anywhere for the award of degree.

We wish for her bright future.

Mr. Mayank Raj Bhardwaj **Certificate Number: RPTN-1707/54**

(Chief Executive Officer)

Rapture Biotech, India

RAPTURE BIOTECH D-201 Sector 10, NOIDA (U.P.), India Pin: 201301/ Tel: (0120) 4108608
Helpline: 9910181655, 9027326396 Email: info@rapturebio.com Website: www.rapturebio.com

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2K15/BME/11

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ABSTRACT

Phytochemical analysis and antimicrobial activity of different solvent extracts of *Sargassum swartzii* were investigated. *Sargassum swartzii*, a genus of brown algae possesses several therapeutic activities like anti-inflammatory, antioxidant etc.

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 brown algae were tested against various bacterial and fungal strains like *Aspergillus niger*, *Aspergillus candidus*, *Penicillium species* etc. using the disc diffusion assay.

Qualitative phytochemical analysis of the brown algae extract revealed the presence of flavonoids, saponins, tannin, phenolic, steroid, terpenoid, and phytosterols. The quantitative analysis of solvent extracts of brown algae has been screened. We have checked the antimicrobial activity of brown algae, extracted from different solvent against different pathogenic bacterial and fungal strains. Out of which cow urine extract and benzene extract was found to be very effective against majority of bacterial strains and fungal strains.

Minimum inhibitory concentration was also calculated for different solvent extract of brown algae. To confirm the occurrence of the bio active compounds in the crude extracts of *Sargassum swartzii* thin-layer chromatography (TLC) analysis was performed.

The findings of this study showed *Sargassum swartzii* extracts had significant therapeutic potential and could be introduced for the preparation of novel functional ingredients in cosmeceuticals. In order to identify the cosmetic potential of *Sargassum swartzii* physical parameters were evaluated. Therefore, further research studies are needed to exploit its maximum therapeutic potential in the field of cosmetic industry for unique and fruitful application.

Keywords: - *Antibacterial, antifungal, phytochemical, minimum inhibitory concentration, thin layer chromatography*

CHAPTER I

1. INTRODUCTION

1.1 Secondary Metabolites

Secondary metabolites are beneficial plant chemicals that show defensive nature against various microbial agent. Secondary metabolites are also known as phytochemicals. More than thousands of phytochemicals are present in nature. As in history, it reveals that by secreting these chemicals plant provide protection to itself. Now a day many research reveal that these phytochemicals provide protection to humans from infectious diseases.

Phytochemicals are natural compounds which are produced by plants as resistive chemicals. If they are not present in plants then it do not harm them. Such phytochemicals are alkaloids, phenolics, steroids, flavonoids, and tannins etc. They are produced biosynthetically resulting from primary metabolites. Phytochemicals are not needed to enhance the growth of plants, and they are not produce by metabolic pathways common to all plants. They are gathered in lesser qualities in plant cells as compared to primary metabolites. These metabolites are biologically synthesized in specialized plant cells at specific developmental stages in such a way that it causes difficulty to extract and purify them.

1.2 Minimum Inhibitory Concentration

MIC is used to determine the lowest concentration of sample with maximum inhibition effect against tested microorganisms. The length of inhibition zone determined whether the microorganism was considered to be liable, unaffected or intermediate to a specific antibiotic. Dilution techniques are used to determine the MIC of different antimicrobial sample. In dilution tests, 96 well microtiter plate wells containing serial dilutions of the antimicrobial sample is used to test the ability of microorganisms to produce visible growth in well.

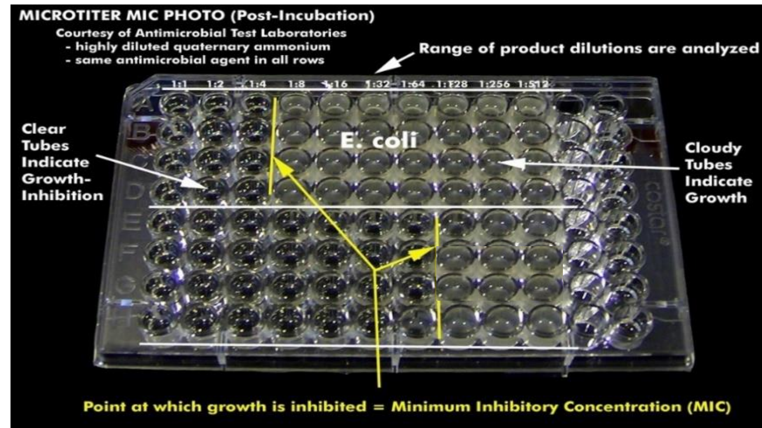


Figure 1 Minimum inhibitory concentration

In minimum inhibitory concentrations test, test sample was inoculates with a high number of microorganisms for 48hrs after that notice the wells properly to see if it changes from clear to hazy. If hazy appearance was visualised, that shows microorganisms was grown high in number, and the test sample was not resistive to them at that particular dilution. If wells were clear in appearance then that shows microorganisms was low in number or was inhibited by test sample.

1.3 Thin Layer Chromatography

Thin layer chromatography (TLC) is consider to be easier and better separation technique than other chromatography techniques. TLC principle is similar to paper chromatography. In TLC silica gel is used as a stationary phase and solvents system or mixture of solvent sytem with varies polarity is used as mobile phase. As compared with other chromatography technique, it has several advantage such as run faster, provide better separation, and choose absorbent according to sample. A TLC sheet is made up of glass, metal or plastic A TLC plate is covered with thin layer of a solid adsorbent. A slight amount of the sample to be examined is loaded at the bottom of the TLC plate. The TLC plate is then kept in a developing chamber having narrow pool of a solvent so that only the loaded portion in dipped in a liquid. As the solvent is rises up the loaded sample will move slowly in the TLC plate via capillary action. Carefully see and record the results of the chromatography in each solvent system. As you will notice all the components will get separated on the TLC sheet as polarity increases and vice versa.Best separation in TLC is always achieve by choosing the ideal solvent system.

1.4 Herbal Product Formulation

Evaluation of herb is based on its characteristics features. Macroscopically feature includes odour, taste etc and microscopically includes leaf content, trichome etc. For evaluation and standardization of herbal drug some physical and chemical test are performed.

Standardization of herb basically define the set of parameters starting from manufacturing process to product formulation It’s also include the study from plant origin to its clinical development. Identification, standardization and evaluation of herbal product formulation is based on physical, chemical and biological parameters which involves study on preformulation parameters and microbiological analysis of herb. This ensure formulated medicine contained correct ingredient in correct amount and will show therapeutic effect.

CHAPTER II

2. REVIEW OF LITERATURE

2.1 Brown Algae

Macro algae as well as micro algae are one of the major producers of biomass in the marine environment [1].They produce an inclusive variety of chemically active metabolites in their surroundings, potentially as a support to protect themselves against the other settling organisms. These active metabolites, are known as halogen compounds, alkaloids and terpenoids are produced by numerous species of marine macro and microalgae and have antibacterial, antiviral, and antifungal properties which are effective in the prevention of biofouling and have other likely uses, as in therapeutics [2].

Marine algae are classified into four namely Chlorophyceae (green algae), Phaeophyceae (brown algae), Rhodophyceae (red algae) and Cyanophyceae (blue-green algae) based on the type of pigments, morphological, anatomical and reproductive structures.

Algae placed in the phylum Phaeophyta (brown algae) are all multicellular. The modest species has branched, filamentous body composed of uniseriate cells, such as the species placed in the order Ectocarpales. Some of brown algae can form a marine forest attracting many marine organisms and drawing in abundant marine resources. Laminaria, one kind of kelps, tend to be dispersed in cold waters.

Brown algae usually comprise an accessory yellowish pigment, fucoxanthin, so they tend to appear in brown and also have chlorophyll a and c, β-carotene and lutein. Their food reserve include oils, laminarin and mannitol. Brown algae cell walls are rich in alginate, so they tend to be colloidal or thick characters and are usually used to make food additives. They are also used in the textile industry, the paint industry, or other industrial uses. Many brown algae are edible. As brown algae contain large quantities of vitamins and mineral salts, they are believed to be good dietary supplements. Past thousand years ago, the brown algae is used in medicine. It is written in several ancient Chinese medical books including “Compendium of Materia Medica”

and "Shennong's Classic of Materia Medica" that Undariaand Laminaria can help treat goiter, thyroid enlargement and oedema and that they induce urination.

Modern medical research also indicates that some brown algae can help lower blood pressure and prevent cancers. Scientists have essentially used alginate to make anticoagulants, haemostatic medicine, blood volume expanders, hemostatic gauze, hemostatic sponge, medical imprints and base. In short, brown algae have great economic value [3].

Sargassum, a genus of brown seaweed, commonly known as gulf-weed belonging to family Sargassaceae, order Fucales, subclass Cyclosporeae, and class Phaeophyceae, contains approximately 400 species [4, 5]. *Sargassum* species are reported to produce metabolites of structural classes such as terpenoids, polysaccharides, polyphenols, sargaquinoic acids, sargachromenol, plastoquinones, steroids, glycerides etc., which possesses several therapeutic activities like analgesic, anti-inflammatory, antioxidant, neuroprotective, anti-microbial, anti-tumor, fibrinolytic, immune-modulatory, anti-coagulant, hepatoprotective, anti-viral activity etc., Hence, *Sargassum* species have great potential to be used in pharmaceutical and neutralceutical areas.

Figure 2 Sargassum species

2.2 Theraputic potential of *Sargassum* species

2.2.1 *In vitro* antioxidant activity

Oxidative stress is the result of an imbalance between pro-oxidant and antioxidant homeostasis that leads to the generation of toxic reactive oxygen species (ROS) [6]. ROS such as hydroxyl, super oxide, and peroxyl radicals are formed in human tissue cells, which attack macromolecules such as membrane lipids, proteins, and DNA, lead to many health disorders such as cancer, diabetes mellitus, age-related degenerative conditions, neurodegenerative and inflammatory diseases with severe tissue injuries [7-9].

Antioxidant activities of *Sargassum* species have been determined by various methods such as 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging, 2,2'-azinobis-3-ethylbenzo thizoline-6-sulfonate (ABTS) radical scavenging, NO scavenging, lipid peroxide inhibition, superoxide and hydroxyl radical scavenging assays. Kim *et al*. [10] reported the sulfated polysaccharides of *Sargassum fulvellum* is more potent NO scavenging and DPPH scavenging activity than commercial antioxidants such as α-tocophorol.

2.2.2 Anti-cancer activity

Cancer is a leading cause of death worldwide and a diverse group of diseases characterized by the uncontrolled proliferation of anaplastic cells, which tend to invade surrounding tissues and metastasize to other tissues and organs. Cancer results from a mutation in the chromosomal DNA of a normal cell, which can be triggered by both external factors (tobacco, alcohol, chemicals, infectious agents, and radiation) and internal factors (hormones, immune conditions, inherited mutations, and mutations occurring in metabolism) [11].

Zandi *et al*. [12] reported that the cold water extract of *Sargassum oligocystum* showed the reasonable anti-cancer activity against tumor cells replication. Polysaccharides from *Sargassum fusiforme* showed significant anti-tumor activity both *in vitro* and *in vivo*, and improved the immune function in tumor-bearing mice [13].

2.2.3 Analgesic, and Anti-inflammatory activities

The inflammatory process involves a series of events that can be elicited by numerous internal or external stimuli. Therapy of inflammatory diseases is usually directed at the inflammatory processes. Anti-inflammatory refers to the property of a constituent or treatment that decreases inflammation [14].

Dar *et al*. [15] reported that butanolic extract of *Sargassum wightii* collected during winter season was most effective (86.7%) in reducing carrageenan-induced edema in rats at a dose of 100 mg/kg as compared to reference drugs aspirin (79.4%) and ibuprofen (57.3%). The dichloromethane extract of *Sargassum fulvellum* inhibited an inflammatory symptom of mouse ear edema by 79.1%. The ethanol extract of *Sargassum thunbergii* also inhibited edema by 72.1%, when evaluated against yeast-induced pyrexia, tail-flick test, and phorbol myristate acetate-induced inflammation (edema, erythema, and blood flow) in mice [16].

2.2.4 Other biological activities

Other pharmacological activity includes fibrinolytic, anti-diabetic, anti-bacterial, anti-plasmodial, Skin-whitening, gastric-protective activity etc.

Two bioactive products identified as 1-O-palmitoyl-2-O-oleoyl-3-O-(α-D-glucopyranosyl)-lycerol (POGG) and 1-O-myristoyl-2-O-oleoyl-3-O-(α-D-glucopyranosyl)–glycerol (MOGG) obtained from *Sargassum fulvellum* showed fibrinolytic activity in the reaction system of pro-u-PA and plasminogen [17].

Hot water extract of *Sargassum polycystum* in dose of 100 mg/kg maintains the acidity of gastric juice and improves the gastric mucosal injury in rats [18].Extracts of *Sargassum polycystum* and *Sargassum silquastrum* exerted *in vitro* inhibitory activity against tyrosinase and melanin production, which could be developed to a skin-whitening agent in cosmetics industry [19, 20].

CHAPTER III

3. MATERIALS AND METHODS

1. Sample collection (Soil,Brown Algae)

2. Preparation of media (Nutrient agar media and Potato dextrose agar)

3. Extraction and Evaporation of algae in different solvents

4. Isolation of bacteria from soil and fungus from soil

5. Identification of bacteria (Gram Staining and Biochemical Testing) and fungus

6. Qualitative and Quantitative analysis of phytochemicals

7. Antimicrobial activity test of algae through Disc diffusion method

8. Minimum inhibitory concentration analysis

9. Thin layer chromatography of brown algae sample

10. Herbal parameters evaluation in brown algae sample

3.1 Quantitative analysis of phytochemicals

According on the qualitative results, the quantitative analysis is performed for determination of total Alkaloids, Flavonoids, Tannins, Phenols, and Carbohydrates content.

3.1.1 Determination of Tannins Content:

Take 500 ml flask and add 500mg weighed sample to it. Add 50 ml distilled water in the flask containing the sample and shake it for1 h on a shaker. Pour the filtered into a 50 ml volumetric flask and make up the volume. In a clean test tube pipetted out 5 ml of the filtered and mixed with 2 ml of 0.1 M $FeCl_3$ in 0.1 N HCl and 0.008 M potassium ferrocyanide. The absorbance was measured at 605 nm within 10 min. Tannic acid was used as standard solutions.

3.1.2 Determination of Phenol Content:

Briefly, to the 50µl of the sample solution, 2.5 ml of Folin Ciocalteu reagent and 500µl of water added. Mix well and kept the mixture for 5 min incubation in dark at room temperature. Add 2 ml of 7.5% sodium carbonate to the mixture and finally kept the mixture for 1.5 hrs incubation in dark at room temperature. The absorbance was measured at 765 nm. Gallic acid dilutions were used as standard solutions. Gallic acid standard solution is prepared by dissolving 0.05 g of gallic acid in 50 ml of distilled water. 7.5% sodium carbonate solution is prepared by dissolving 1.125 g of sodium carbonate in 15 ml of distilled water. 0.2 N F_c reagent for 20 ml is prepared by adding 2 ml of 2N F_c reagent in 18 ml of water.

3.1.3 Determination of Alkaloid Content:

5 g of powdered sample was added to 20 ml of 10% acetic acid in ethanol, covered it properly and leave it for 4 hours. Reduce the volume of filtrate to one fourth using water bath Add Concentrated NH_4OH drop wise to the filtrate to get the precipitate. Completely settle the whole solution and wash the collected precipitate with dilute ammonium hydroxide and filtered it. The residue was dried and weighed.

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

3.1.4 Determination of Flavonoid Content:

Repeatedly extract 1 g sample with 100ml of 80% aqueous methanol at room temperature. Filter the mixture using Whatman No1 filter paper into a beaker. Evaporate the filtrate to dryness and weighed.

Flavonoid content = (weight of flavonoid/weight of sample)*100.

3.1.5 Determination of Carbohydrate:

For estimating the polysaccharide content, take 500µl of sample solution and add 2.5ml of water and then add 2 ml of DNS reagent mix well and leave for 10 minutes. Measure the absorbance at 540 nm against blank. Then compare it with standard solution of dextrose. To prepare blank, 3 ml of distilled water added to 2 ml of DNS reagent. DNS reagent is prepared by mixing reagent 1 and reagent 2. Reagent 1 is prepared by dissolving 5.625 g of sodium potassium tartarate in 9.375 ml of water. Reagent 2 is prepared by dissolving 0.1875g of DNS reagent in 3.75 ml of NaOH. 2M NaOH is prepared by dissolving 10 g of NaOH in 125 ml of water. DNS is prepared fresh by mixing reagent 1 and reagent 2 and make up the volume to 18.75 ml with water.

3.2 Preparation of Media

3.2.1 Preparation of NAM (Nutrient Agar Media)

NAM (Nutrient Agar Media) (200 ml):

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 was adjusted to 6.8 and the volume was made up to 200 ml with distilled water and agar was added and was kept for autoclaving. Pour the media into each plate and leave plates on the sterilized surface until the agar has solidified. Soil dilution was spread on plates and incubated at 37ºC for 24 hours. Then the colonies were purified by repeated streaking.

3.2.2 Preparation of PDA (Potato dextrose Agar)

PDA (Potato Dextrose Agar) (200 ml):

To 200 ml 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 was adjusted to 5.6 and the volume was made up to 200 ml with distilled water and agar was added and was kept for autoclaving. Pour the media into each plate and leave plates on the sterilized surface until the agar has solidified. Soil dilution was spread on plates and incubated at 30ºC for 7 days. Then the colonies were purified by repeated streaking.

3.3 Minimum inhibitory concentration

The MIC of the *Sargassum swartzii* extracts was evaluated using disc diffusion method. The MIC of the *Sargassum swartzii* extracts were analysed by diluting the several concentrations of cow urine and benzene respectively. 50µl of bacterial strain was swabbed on the Nutrient agar plates and 100µl of fungal strain was swabbed on the Potato dextrose agar plates. Then sterile Whatman number one filter papers (6 mm diameter disc) were dipped in different solvents extracts of *Sargassum swartzii* and placed on the spread-surface of the petri plates. After this, the plates were incubated in a vertical position at 37±2 °C for 24 hour (for bacteria) and at 30°C for 7 days (for fungi). Compare the result of positive test with the control. The sensitivity of the microbial species to the extract was analyzed by calculating the diameter of the inhibitory zones around the disc.

3.4 Thin layer chromatography analysis

TLC was performed silica gel plates having size 10 × 20 cm. The phenols, steroids and polysaccharide compounds present in *Sargassum swartzii* were qualitatively identified by TLC. To perform the TLC of phenols chloroform and methanol (9:1 ratio) was used as mobile and Fc reagent was act as spraying agents to detect the phenolic compound present in the *Sargassum swartzii*. If blue color spot is visualized in the TLC plate after spraying with Fc reagent then it indicate the presence of phenolic compounds in the *Sargassum swartzii* extracts. Similarly, to

perform the TLC of steroids benzene and methanol (9:1 ratio) was used as mobile .The butanol, acetic acid and water (6:3:1) was served as mobile phase for polysaccharide compound. 5% alcoholic sulphuric acid was act as the spraying agent to detect steroid and polysaccharide compounds present in the *Sargassum swartzii*. If bluish green colour and yellowish orange spot in the TLC plate then it indicat the presence of steroid and polysaccharide compounds in the *Sargassum swartzii* respectively.

3.5 Herbal Product Formulation

3.5.1 Determination of foreign matter

Weigh accurately 10 gm to 50 gm of *Sargassum swartzii* sample and spread it on a clean surface. Examine the sample with the unaided eye and remove the foreign matter completely. Weigh and determine the % of foreign matter from the weight of the sample taken.

3.5.2 Determination of moisture content

Weighed 2 gm of *Sargassum swartzii* sample and put it on an aluminum foil. Then place it in an oven and dried at 105°C for 3 hours and weigh the sample again. Process of drying and weighing was continued at 1 hour interval until the difference reaches to not more than 0.25% between two successive weighing. Once the constant weight is reached the reading was taken and the moisture content is determined.

3.5.3 Determination of extractive values

3.5.3.1 Alcohol- soluble extractive

Macerate 0.625 g of the air-dried *Sargassum swartzii* sample with 12.5 ml of methanol in aclosed flask for 24 hrs. Shaking frequently during 6 hrs and allow it to stand for 18 hrs. Rapidly filter the solution and evaporate 25% of alcohol soluble extractive. Weigh and calculate the alcohol soluble extractive value.

3.5.3.2 Water-soluble extractive

Macerate 0.625 g of the air-dried *Sargassum swartzii* sample with 3.125 ml of 25% chloroform and 9.375 ml of 75 % water in a closed flask for 24 hrs. Shaking frequently during 6 hrs and allow

it to stand for 18 hrs. Filter quickly and evaporate 25% of alcohol- soluble. Weigh and calculate the alcohol soluble extractive value.

3.5.4 Determination of pH

Dissolve 0.5 g of *Sargassum swartzii* sample and 50 ml of distilled water to it in a beaker. Incubate it for an hour. Filter the entire solution into another beaker and measure the pH through pH meter.

3.5.5 Solubility

Take 1gm of *Sargassum swartzii* sample and dissolve it in 10 ml of water. Vortex the sample properly and leave it undisturbed for 10 min. After 10 min, observe the sample solubility in water.

3.5.6 Foaming Index

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

3.5.7 Swelling Index

Take 1 g of *Sargassum swartzii* sample and add 25 ml of water to it in a measuring cylinder. Shake the mixture thoroughly every 10 minutes for an hour. Leave it for 3 hours at room temperature. Measure the volume occupied by the sample.

3.5.8 Volatile oil content

The distillation method (Clevenger apparatus) is used to determination of volatile oil content. 10 g weighed *Sargassum swartzii* sample is placed in a distillation flask with a mixture of 90 ml water and 1 ml glycerin and connected to receiver, which fitted with water and connected to the condenser. On distillation, the oil and water condense and the volatile oil which collects in the graduated receiver as a layer on top of the water is measured.

3.5.9 Microbial limit test for *Sargassum swartzii* sample

Microbial test are performed for the qualitative estimation of viable microorganisms present in *Sargassum swartzii* sample. If the test sample have any kind of contamination then it will be eliminated by syringe filtration method. Microbial test are as follows:

3.5.9.1 *Enterobacteria*

A. Media preparation:

- **Fluid Lactose Medium**

  Meat Extract - 0.03g

  Gelatin peptone - 0.05g

  Lactose monohydrate - 0.05g

  Water - 10ml

  pH - 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

B. Procedure

Take 1g or 1 ml of the test sample add 9 ml of Fluid lactose medium to form a solution. Transfer 1 ml to 9 ml to Enrichment Broth Mossel Medium and incubate at between 35ºC and 37ºC for 18 to 24 hours. Gently mix it and spread the sample solution on the surface of VRB agar with glucose. Incubate at between 35ºC and 37ºC for 18 to 24 hours.

C. Interpretation

If colonies are red or reddish in apperance, the sample may contain *Enterobacteria*.

3.5.9.2 *Escherichia coli*

A. Media preparation

- **Fluid Lactose Medium**

  Meat Extract - 0.03g

  Gelatin peptone - 0.05g

  Lactose monohydrate - 0.05g

  Water - 10ml

  pH - 6.7-7.1

- **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 - 6.9-7.3

B. Procedure

Take 1g or 1 ml of the test sample add 9 ml of Fluid lactose medium to form a solution. Take the Durham tube having 9 ml of EC broth and transfer 1 ml solution in the tube and incubate the tube at 44ºC for 24 hours in a water bath. If gas bubbles are present, the sample shows the absence for *Escherichia coli*. If gas bubbles are present, spread the sample solution on the surface of EMB agar. Incubate at between 30ºC and 35ºC for 18 to 24 hours.

C. Interpretation

Upon analysis, if colonies do not show both a characteristics metallic sheen and blue-black appearance under transmitted light, the sample shows the absence of *Escherichia coli*.

3.5.9.3 *Salmonella*

A. Media preparation

- **Fluid Lactose Medium**

Meat Extract - 0.03g

Gelatin peptone - 0.05g

Lactose monohydrate - 0.05g

Water - 10ml

pH - 6.7-7.1

- **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

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 - 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

B. Procedure

Take 1g or 1 ml of the test sample add 9 ml of Fluid lactose medium to form a solution. Incubate at between 30ºC and 35ºC for 24 to 72 hours. If growth is observed then pipet 1 ml sample solution into 9 ml of Fluid Rappaport medium and incubate for 12 to 24 hours. After the incubation, spread the sample solution on the surface of Brilliant green agar medium and XLD agar medium. Incubate at between 30ºC and 35ºC for 24 to 48 hours.

C. Interpretation

Upon analysis, if colonies do not show small, transparent and colorless, or opaque, pink or white on Brilliant green agar medium and red, with or without a black center on XLD agar medium, the sample shows the absence of *Salmonella*.

3.5.9.4 *Staphylococcus aureus*

A. Media preparation

- **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	- 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 - 7.2- 7.6

B. Procedure

Take 1g or 1 ml of the test sample add 9 ml of Fluid Soyabean-Casein Digest medium to form a solution. Incubate at between 30ºC and 35ºC for 24 to 48 hours, and pipet 1 ml into 9 ml of Fluid Soyabean-Casein Digest medium with 7.5% sodium chloride. If growth is observed then spread the sample solution on the surface of Mannitol- Salt agar medium and incubate at between 30ºC and 35ºC for 24 to 48 hours.

C. Interpretation

Upon analysis, if colonies do not show yellow colonies surrounded by yellow zone on Mannitol-Salt agar medium, the sample shows the absence of *Staphylococcus aureus*.

3.5.9.5 *Pseudomonas aeruginosa*

A. Media preparation

- **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 - 7.1-7.5

- **Cetrimide Agar Medium**

Gelatin Peptone - 0.5g

$MgCl_2.6H_2O$ - 0.075g

Potassium Sulphate - 0.25g

Cetrimide - 0.075g

Glycerine - 0.25g

Agar - 0.34g

Water - 25ml

pH before autoclaving - 7.2±0.2

B. Procedure

Take 1g or 1 ml of the test sample add 9 ml of Fluid Soyabean-Casein Digest medium to form a solution. Incubate at between 30ºC and 35ºC for 24 to 48 hours. If growth is observed then spread the sample solution on the surface of Cetrimide agar medium and incubate at between 30ºC and 35ºC for 24 to 48 hours.

C. Interpretation

Upon analysis, if colonies do not show gram negative rods with greenish fluorescence on Cetrimide agar medium, the sample shows the absence of *Pseudomonas aeruginosa*.

3.5.9.6 *Clostridia*

A. Media preparation

- **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 - 6.6-7.04

- **Columbia Agar Medium**

Columbia agar	- 1.1g
Water	- 25ml
pH after autoclaving	- 7.3±0.2

B. Procedure

Take 1g or 1 ml of the test sample add 9 ml of Reinforced Clostridium Medium to form a solution. Incubate at between 30ºC and 35ºC for 48 hours. If growth is observed then spread the sample solution on the surface of Columbia agar medium and incubate at between 30ºC and 35ºC for 48 to 72 hours.

C. Interpretation

Upon analysis, if colonies do not show white rod shaped on Columbia agar medium, the sample shows the absence of *Clostridia*.

3.5.9.7 *Candida glabrata*

A. Media preparation

- **Sabouraud Glucose Medium**

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

- **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

B. Procedure

Take 1g or 1 ml of the test sample add 9 ml of Sabouraud Glucose Medium to form a solution. Incubate at between 30ºC and 35ºC for 3-5 days If growth is observed then spread the sample solution on the surface of Sabouraud Glucose Agar Medium with Antibiotics and incubate at between 30ºC and 35ºC for 24 to 48 hours.

C. Interpretation

Upon analysis, if colonies do not show glossy pink round shaped on Sabouraud Glucose Agar Medium, the sample shows the absence of *Candida glabrata*.

3.5.9.8 *Aspergillus brasiliensis*

A. Media preparation

- **Czapek Dox Broth**

Sucrose	- 0.3g
Sodium nitrate	- 0.03g
Dipotassium phosphate	- 0.01g
$MgSO_4$	- 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

B. Procedure

Take 1g or 1 ml of the test sample add 9 ml of Czapek Dox Broth to form a solution. Incubate at between 25ºC and 30ºC for 48-72 hours. If growth is observed then spread the sample solution on the surface of Czapek Yeast Dextrose Agar Medium and incubate at between 25ºC and 30ºC for 48-72 hours.

C. Interpretation

Upon analysis, if colonies do not show white with black spores on Czapek Yeast Dextrose Agar Medium, the sample shows the absence of *Aspergillus brasiliensis*.

3.5.9.9 Microbial contamination treatment by syringe filtration method

Take 1 g of *Sargassum swartzii* sample and dissolve in5 ml of autoclave water. Mix the solution gently. Use 5 ml sterile syringe and autoclave cotton for filtration. In lamina air flow, open the syringe and put a layer of cotton inside it. After this, pour 3 ml solution in the syringe and press the syringe plunger gently to push sample through the cotton. One drop of filtrate sample passes through a needle is spread on specific media. Incubate the media and observe the result.

CHAPTER IV

4. RESULTS AND DISUCSSION

4.1 Quantitative analysis of phytochemical

The quantitative estimation of secondary metabolites showed that the various phytochemical constituents present in the *Sargassum swartzii* extract (Table-1).

S.NO.	PHYTOCHEMICAL	COMPOSITION
1.	Total Tannin content	0.494 mg/ml
2.	Total Phenol content	21.69 mg/ml
3.	Total Alkaloid content	6%
4.	Total Flavonoid content	57%
5.	Total Carbohydrate content	0.01185 mg/m/l

Table 1 Quantitative analysis of *Sargassum swartzii* extract

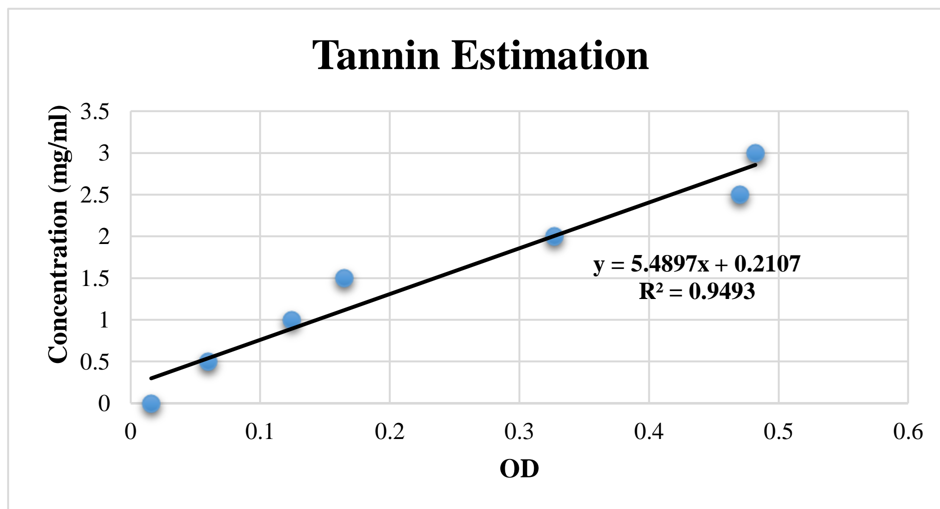


Figure 3 Total Tannin Content

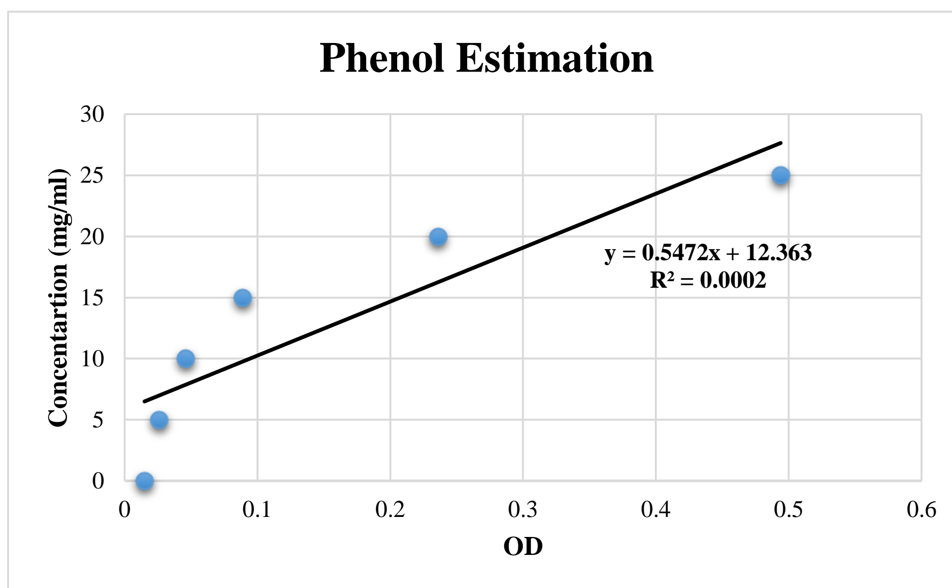


Figure 4 Total Phenol content

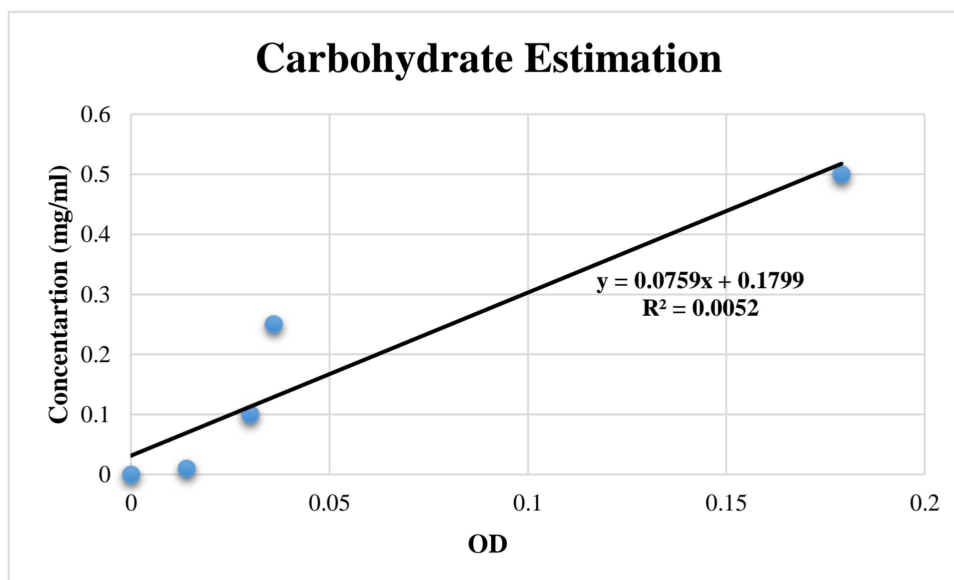
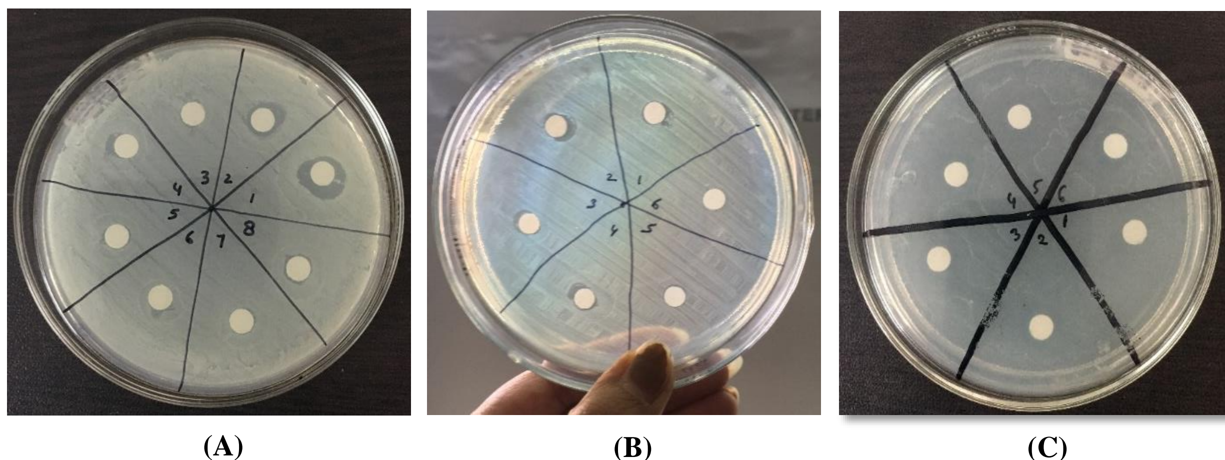


Figure 5 Total Carbohydrate content

The quantitative phytochemical screening of the methanolic extract revealed that of *Sargassum swartzii* contains 0.494 mg/ml tannin, 21.69 mg/ml phenol, 0.01185 mg/ml, 6 % alkaloids and 57 % flavonoids as shown in figure 7, figure 8 and figure 9.

4.2 Minimum Inhibitory concentration

The minimum inhibitory concentration of different solvent extracts of *Sargassum swartzii* were tested against bacterial strains and fungal strains as shown below:

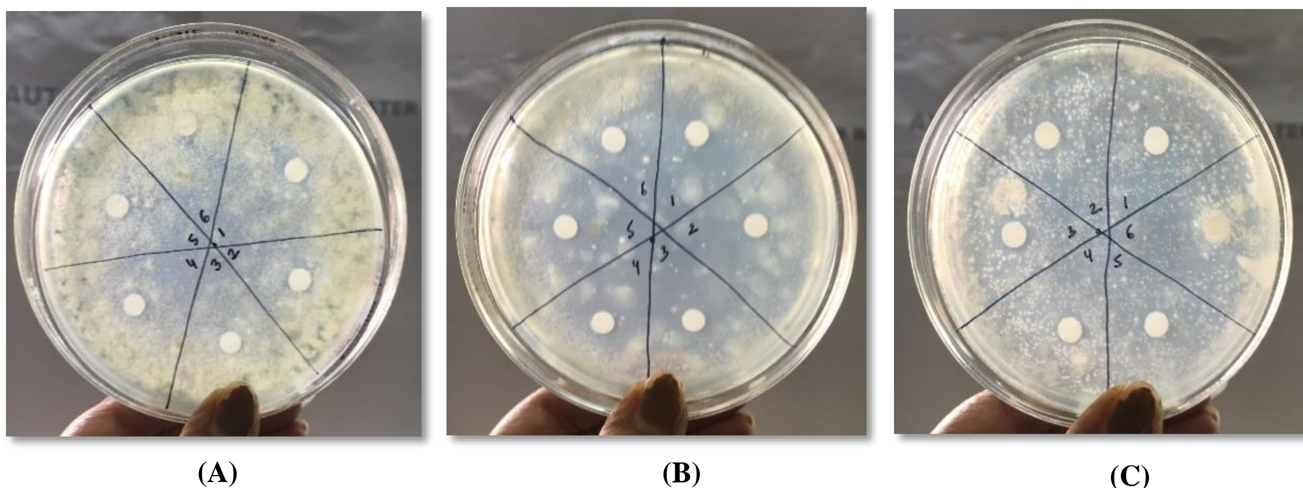


(A) (B) (C)

Figure 6 Minimum Inhibitory Concentration of (A) bacteria # 1 (B) *Shigella flexneri* and (C) *Enterobacter cloacae* respectively (Cow Urine dilutions)

Cow Urine Concentrations (mg/ml)	Zone of inhibition (mm)		
	Bacteria # 1	*Shigella flexneri*	*Enterobacter cloacae*
200	12	10	15
100	9.5	9.5	14
50	8	9	14
25	7	8.5	13
12.5	6	6	17.5
6.25	6	0	27.5

Table 2 Minimum Inhibitory Concentration of bacterial strains (Cow Urine dilutions)



(A) (B) (C)

Figure 7 Minimum Inhibitory Concentration of (A) *Penicillium species* (B) *Penicillium corylophillum* and (C) *Aspergillus niger* respectively (Benzene dilutions)

Benzene Concentrations (mg/ml)	Zone of inhibition (mm)		
	Penicillium species	*Penicillium corylophilum*	*Aspergillus niger*
200	10.5	17.5	12
100	10	16.5	11.5
50	9	15.5	11
25	8	15.5	10
12.5	7	15	7
6.25	0	10.5	0

Table 3 Minimum Inhibitory Concentration of fungal strains (Benzene dilutions)

The Minimum Inhibitory Concentration of cow urine extract showed maximum inhibition against bacteria #1 with zone of inhibition ranging from 6-12 mm in diameter, *Shigella flexneri* with zone of inhibition ranging from 6–10 mm in diameter and *Enterobacter cloacae* with zone of inhibition ranging from 13–15 mm in diameter as shown in figure 10 and Table 2 whereas the benzene extract showed maximum inhibition against *Aspergillus niger* with zone of

inhibition ranging from 7-12 mm in diameter, *Penicillium species* with zone of inhibition ranging from 7-10.5 mm in diameter, and *Penicillium corylophilum* with zone of inhibition ranging from 10.5-17.5 mm in diameter as shown in figure 11 and Table 3.

4.3 Thin Layer Chromatography

The thin layer chromatography end result shows the presence of different bioactive compounds in *Sargassum swartzii* as shown in figure. The results and observations were brief in Table 4.

S.No.	Bioactive Compounds	Solvent System	Rf Value
1.	Phlorotannin (Phenol)	Chloroform:Methanol (9:1)	0.76
2.	Steriod	Benzene:Methanol (9:1)	0.79
3.	Fucoidan (Polysaccaride)	Butanol:Acetic acid:Water (4:1:2)	0.57

Table 4 TLC fingerprinting of chloroform extract of *Sargassum swartzii*

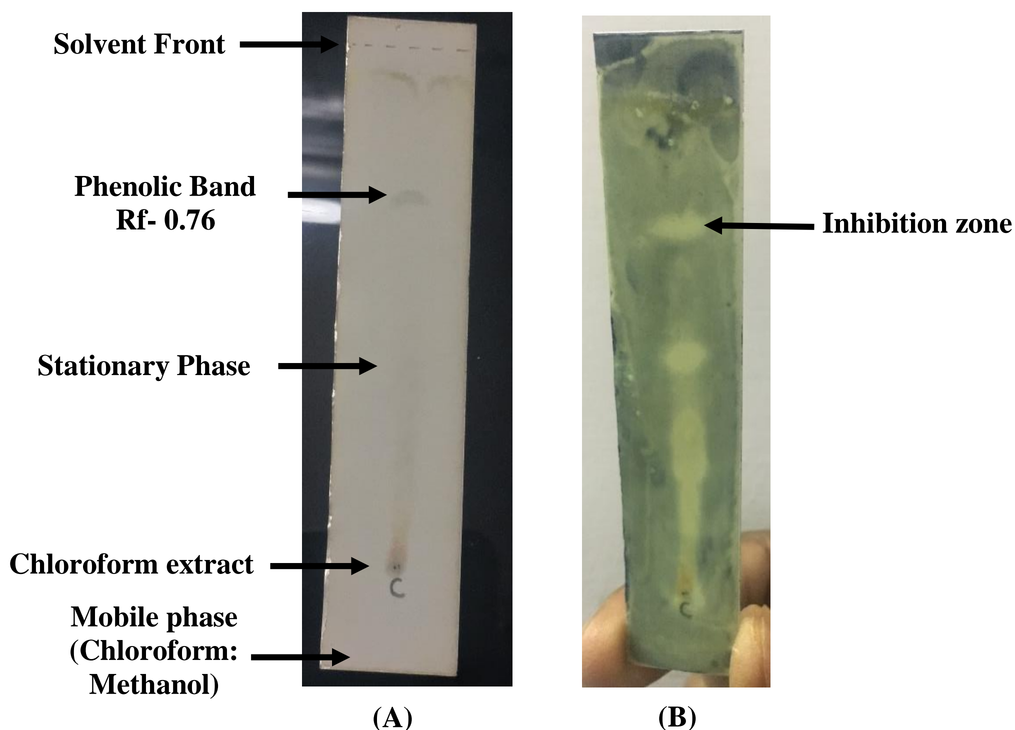


Figure 8 Phlorotannin (A) Before spraying (B) After spraying

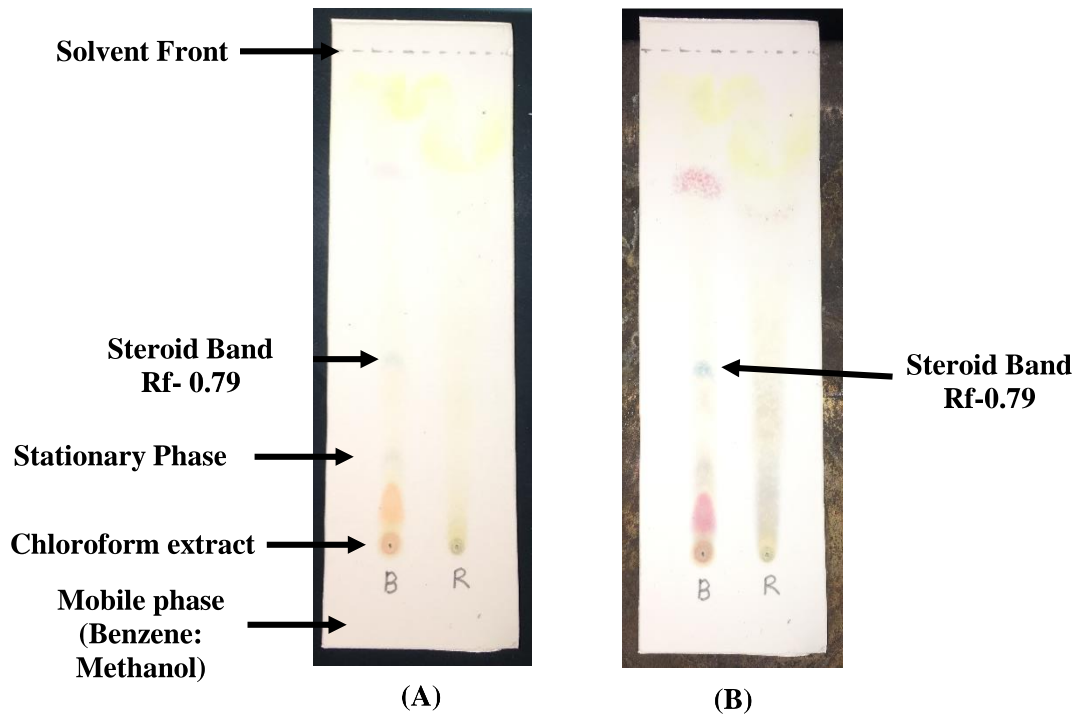


Figure 9 Steriod (A) Before spraying (B) After spraying

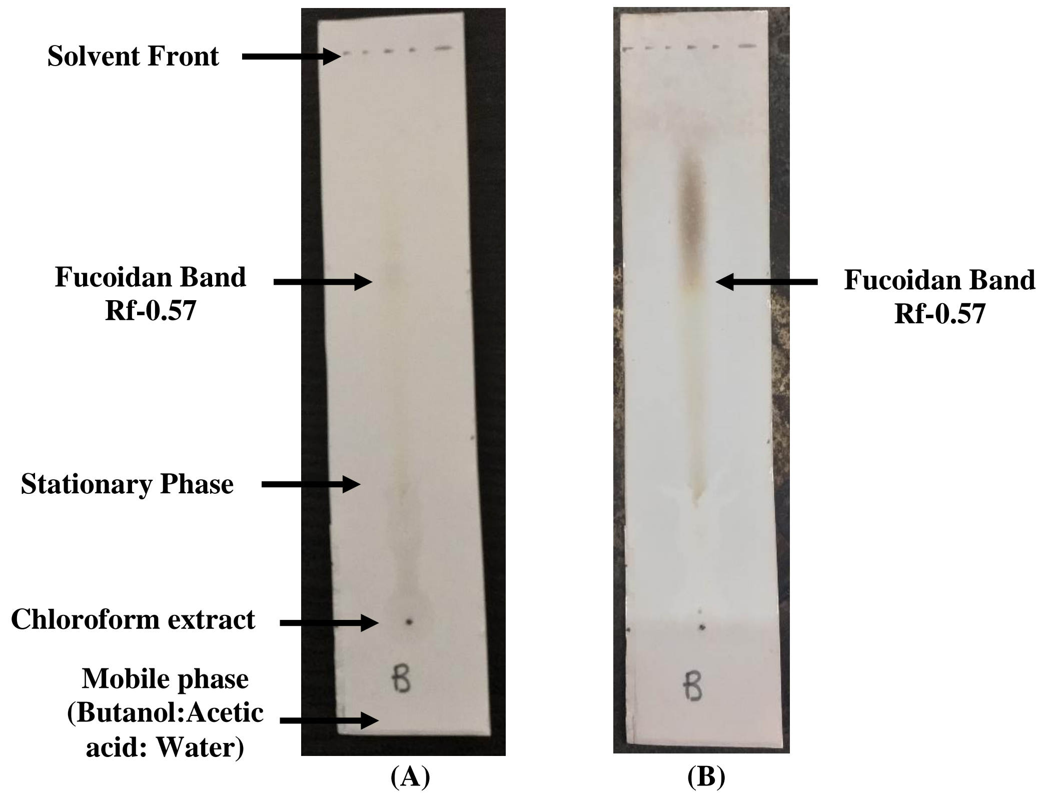


Figure 10 Fucoidan (A) Before spraying (B) After spraying

TLC profile of *Sargassum swartzii* chloroform extract showed one phenolic band with Rf 0.79 value (Figure 12a & 12b), one distinct steroid band was observed in the chloroform extract of *Sargassum swartzii* with the Rf value 0.76 (Figure 13a & 13b) and one polysaccharide band was observed in the chloroform extract of *Sargassum swartzii* with the Rf value 0.57 (Figure 14a & 14b) that confirmed the presence of fucoidan.

4.4 Herbal Product Formulation

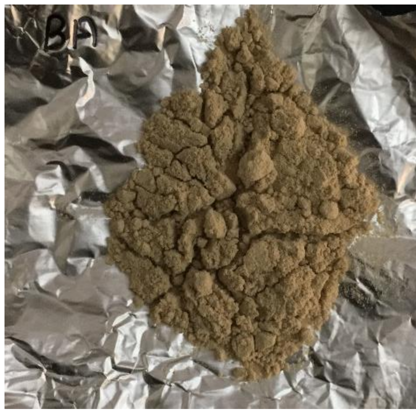
The outcomes of this study showed *Sargassum swartzii* extracts had important therapeutic potential and could be used for the preparation of new functional ingredients in the field of cosmetics. In order to identify the cosmetic potential of *Sargassum swartzii* various physical parameters were evaluated. The various physical parameters for identification, evaluation and standardization of herbal product development was shown in Table 5 & Table 6.

S.No.	Physical Parameters	Value
1.	Moisture Content	3%
2.	Water soluble extractive	8.3%
3.	Alcohol soluble extractive	0.5%
4.	pH	7.9
5.	Swelling Index	Absent
6.	Foaming Index	<1cm
7.	Volatile oil content	Absent
8.	Foreign matter	Absent
9.	Solubility	Highly soluble in water

Table 5 Various physical parameters for identification, evaluation and standardization of herbal product development

S.No.	Microbial Contamination	Before Filtration	After Filtration
1.	*Enterobacteria*	Present	Absent
2.	*E.coli*	Absent	_
3.	*Salmonella*	Present	Absent
4.	*Staphylococus aureus*	Present	Absent
5.	*Clostridia*	Present	Present
6.	*Pseudomonas aeruginosa*	Absent	_
7.	*Candida glabrata*	Present	Present
8.	*Aspergillus brasillensis*	Absent	_

Table 6 Microbial contamination in Sargassum swartzii

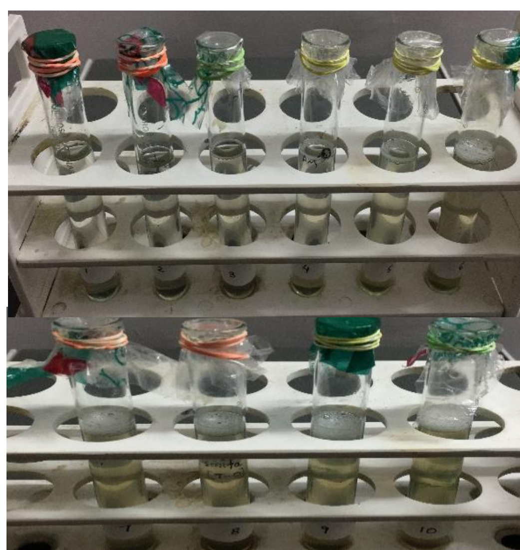


(A)

(B)

(C)

(D)



(E)

(F)

Figure 11 Physical Parameters (A) Moisture Content (B) Foreign Matter (C) Water Soluble Extractive (D) Swelling Index (E) Foaming Index (F) Water Solubility

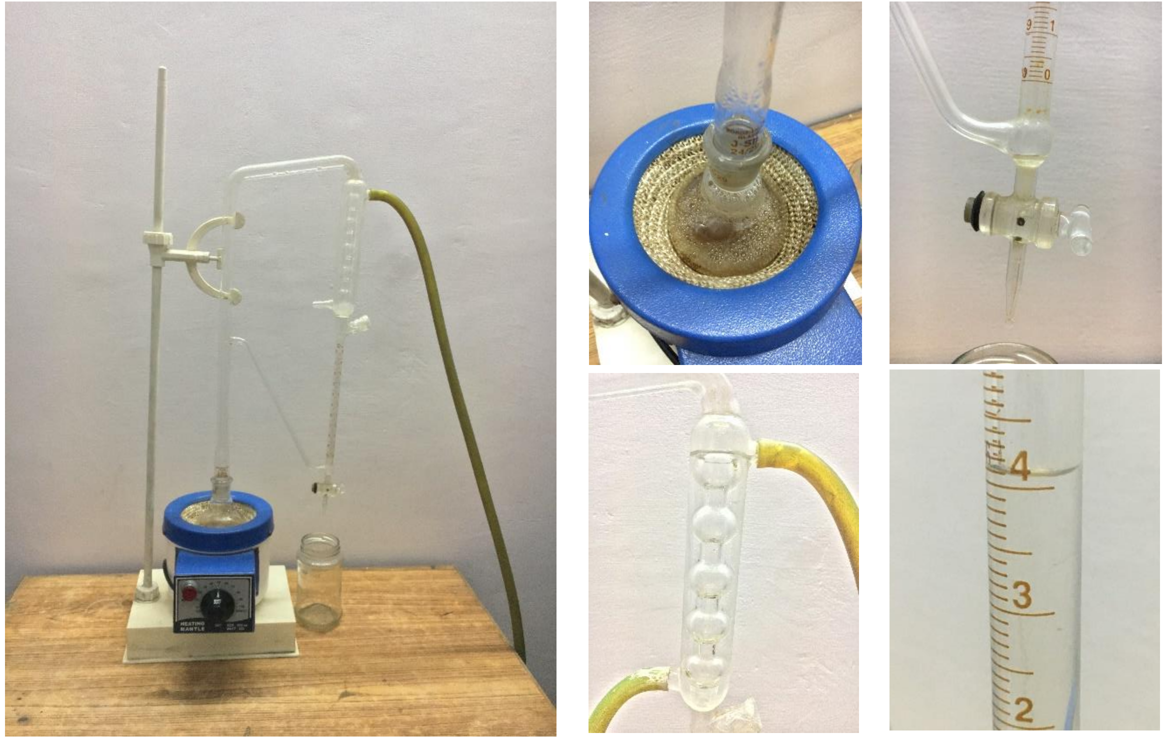
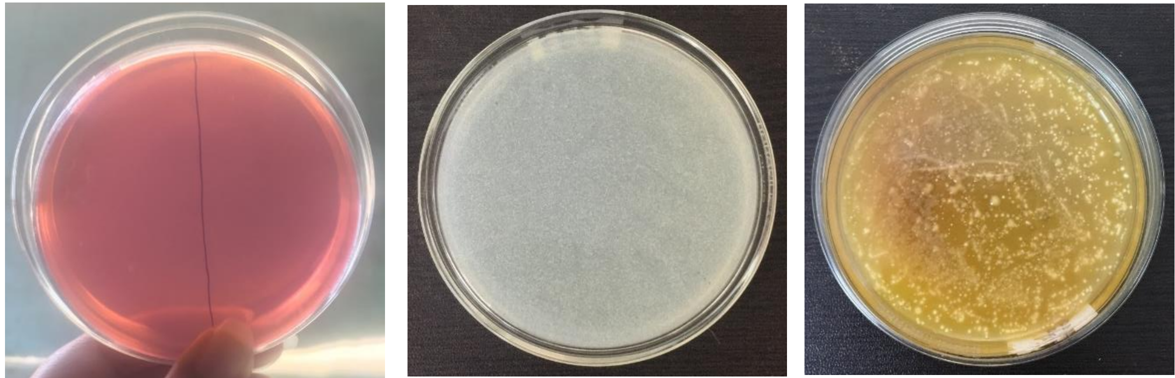
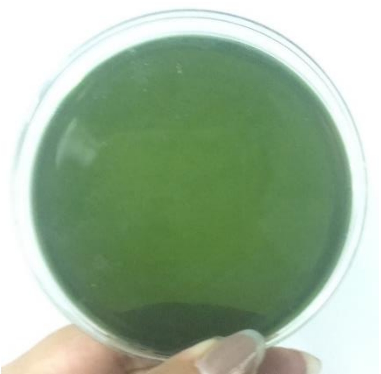


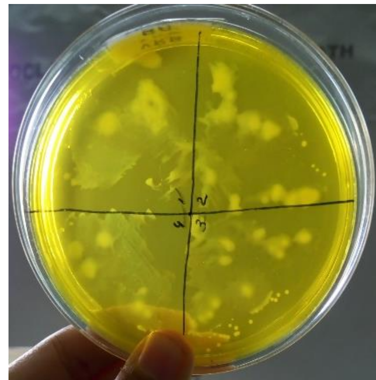
Figure 12 Volatile Oil Content



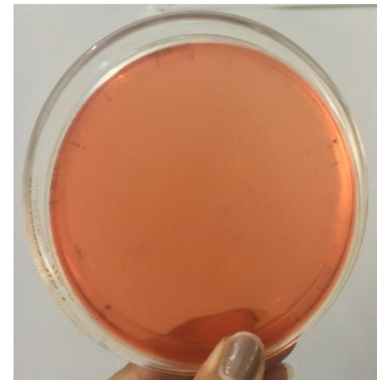
(A) **(B)** **(C)**



(D)



(E)



(F)

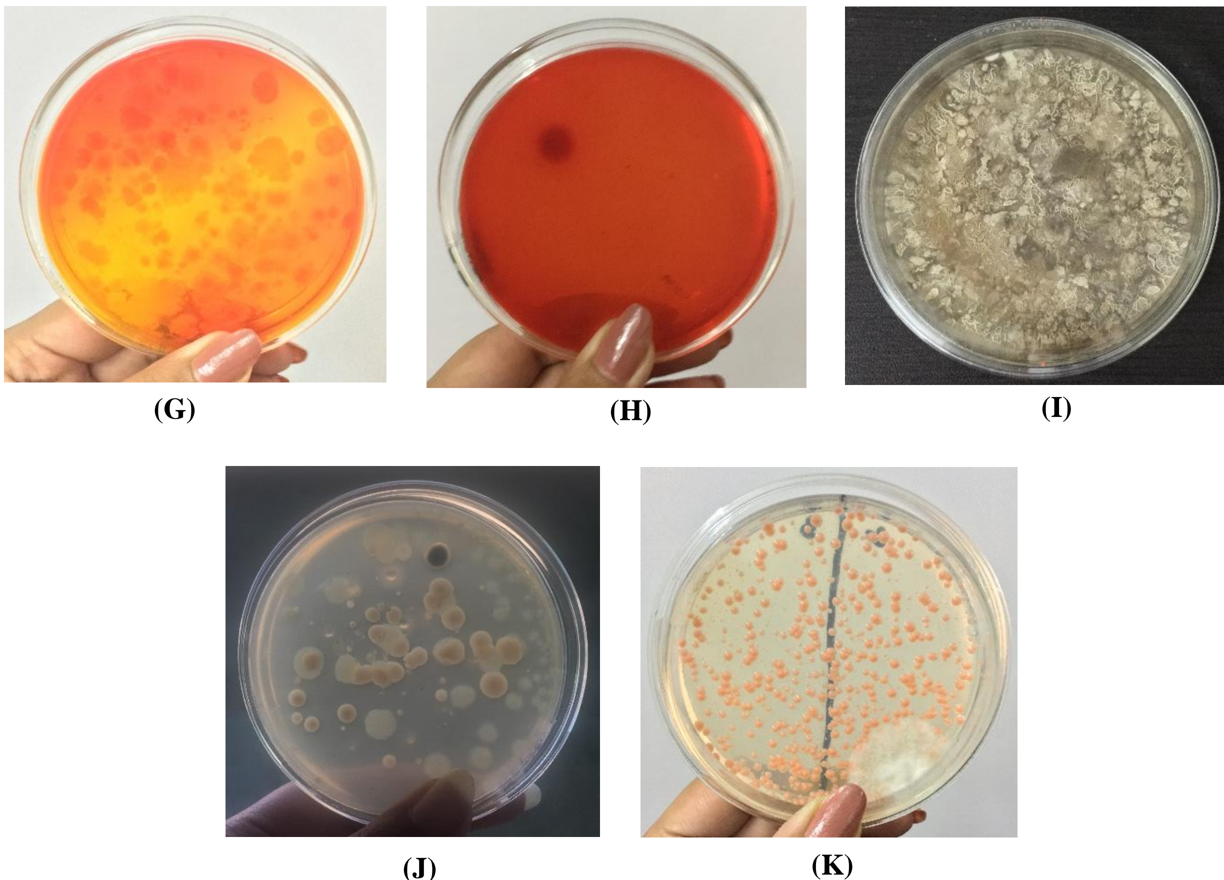


Figure 13 Microbial Contamination (A) *E.Coli* on EMB agar (B) *Psedomonas aeruginosa* on Soyabean Casein Digest agar (C) *Salomon*ella on Brilliant Green agar (D) *Salomonella* on XLD agar (E) *Enterobacteria* before filteration on VRB agar (F) *Enterobacteria* after filter (G) *Staphylococcus aureus* before filteration on Mannitol Salt agar (H) *Staphylococcus aureus* after filteration (I) *Clostridia* on Columbia agar medium (J) *Aspergillus brasiliensis* on Czapek Yeast Extract agar (K) *Candida glabrata* on Sabouraud Glucose agar

The herbal crude extract of *Sargassum swartzii* was prepared and subjected to evaluation of various parameters. Physiochemical analysis of *Sargassum swartzii* including foreign matter, solubility, pH, extractive value, loss on drying, swelling index, foaming index and volatile oil content were performed and results were reported in Table 5. Physiochemical analysis test results for *Sargassum swartzii* extract were within given specification. Hence, it complies with standards. Microbiological analysis of *Sargassum swartzii* including *E.Coli*, *Psedomonas aeruginosa, Salomonella spp.*, *Enterobacteria*, *S.aureus, Clostridia, Aspergillus brasiliensis* and *Candida glabrata* were performed. The absence of *E.Coli*, *Psedomonas aeruginosa, Salomonella spp.*, *Enterobacteria*, *S.aureus* and *Aspergillus brasiliensis* indicates that *Sargassum swartzii* extract complies microbiological test requirements of I.P. Hence, it was suitable for use in herbal product formulation. Presence of *Clostridia and Candida glabrata* in *Sargassum swartzii* extract were also reported. Results of microbiological analysis were reported in figure 17 & Table 6. The pH of the *Sargassum swartzii* extract was 7.9 which lies in the normal pH range of the skin. Solubility of *Sargassum swartzii* was higher in water as

compared to other solvents. Swelling index of *Sargassum swartzii* was absent and foaming index was found to be <1cm. Moisture content, Water and Alcohol soluble extractive value was found to be 3%, 8.3% and 0.5% respectively. Volatile content was also absent in *Sargassum swartzii* as shown in figure 16. Preformulation parameters of *Sargassum swartzii* was reported in figure 15. It was observed from preformulation parameter that *Sargassum swartzii* extract is fair to passable in flowing properties.

CHAPTER V

5. CONCLUSIONS

` In conclusion, as the methanolic extract of *Sargassum swartzii*, evaluated in this work has different varieties of phytochemicals that could be considered as responsible for their therapeutic effects. Alkaloids are the most significant compounds which are involved in the protective function in animals. Flavonoids have been shown anticancerous, antioxidant and anti-infective actions. Phenols potentially toxic to the growth and development of pathogens may play a role in inactivating carcinogens and inhibiting the expression of mutagens. Tannins inhibit the pathogenic fungi and can be toxic to filamentous fungi, yeasts, and bacteria. Steroids is responsible for anti-inflammatory activity [21].

Sargassum swartzii showed maximum inhibition against the bacteria and fungi. As cow urine extracts of *Sargassum swartzii* showed lowest MIC (25 mg/ml) against *Enterobacter cloacae* as compared to bacteria #1 and *Shigella flexneri.* Similarly, benzene extracts of *Sargassum swartzii* showed lowest MIC (6.25 mg/ml) against *Penicillium corylophilum* as compared to *Penicillium species* and *Aspergillus niger*. Its shows that cow urine extracts and benzene extracts of *Sargassum swartzii* showed significant inhibitory effect against tested microorganisms. The results of the MIC study would be interesting source for discovery of novel antimicrobial drugs from *Sargassum swartzii*.

The TLC results of the present investigation indicate that the chloroform extract of *Sargassum swartzii* contains several bioactive compounds. TLC gives an idea about the polarity of various compounds, on the basis of Rf value. Such that compound having high Rf valuehave low polarity and compound with less Rf value have high polarity. These potent bioactive compounds can be further used for development of different medicines in future and this study also help us to further research on characterization of the bioactive compound from the *Sargassum swartzii* using other various separation techniques.

Natural medications are more suitable as they are safer with rarer side effects than the manmade ones. Demand for Herbal formulations are growing in the world market. The present research

focuses on preformulation parameter that has been carried out with the aim of developing herbal product formulations containing *Sargassum swartzii* extract and studying their effectiveness in the field of cosmeceuticals. The research included evaluation of pH, moisture content, solubility, microbial limit test etc. with reference to the WHO Pharmacopoeia of India. The results of preformulation parameters and microbiological analysis of *Sargassum swartzii* extract showed significant therapeutic potential that could be used in herbal product formulation. Further studies on formulation and evaluation of herbal product containing *Sargassum swartzii* extracts are required to evaluate the maximum potential in the field of cosmetic industry.

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