EXTRACTION OF SECONDARY METABOLITES, PHYTOCHEMICAL SCREENING AND THE ANALYSIS OF ANTIBACTERIAL ACTIVITY IN *Spirulina platensis*

A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF SCIENCE IN BIOTECHNOLOGY

Submitted by

Jannatul Ferdaus

Student ID: 11136005

October, 2015

Department of Mathematics and Natural Sciences

Biotechnology Program

BRAC University

Dhaka, Bangladesh

Dedicated To

My Family & My Supervisor

DECLARATION

I hereby solemnly declare that the research work embodying the results reported in this thesis entitled **"Extraction of secondary metabolites, phytochemical screening and the analysis of antibacterial activity in *Spirulina platensis*"** submitted by the undersigned has been carried out under the supervision of Ms. Jebunnesa Chowdhury, Coordinator, Biotechnology Program, Department of Mathematics and Natural Sciences, BRAC University, Dhaka. It is further declared that the research work presented here is original and has not been submitted to any other institution for any degree of diploma. It is also to be declared that the research work presented here is based on actual and original work carried out by me. Information sources or reference to research works performed by other people or institution have been duly cited and referenced.

_____

Jannatul Ferdaus

ID: 11136005

Candidate

Certified

_____

Ms. Jebunnesa Chowdhury

Supervisor

Department Coordinator

Biotechnology Program

Department of Mathematics and Natural Sciences

BRAC University, Dhaka

ACKNOWLEDGEMENT

Getting through my undergraduate dissertation would never have been possible if I had to pursue it alone. First and foremost, I would like to thank **Almighty ALLAH** for guiding me in every step to complete my work properly.

I would like to express my sincere gratitude to **Prof. A. A. Ziauddin Ahmad**, Chairperson, Department of Mathematics and Natural Sciences; Prof**. Naiyyum Choudhury**, Chairman at Bangladesh Atomic Energy Regulatory Authority (Ex. Coordinator of the Microbiology and Biotechnology Programme, Department of Mathematics and Natural Sciences) for their exemplary supervision, cooperation and imperative suggestions for this study.

My deepest regards, gratitude and appreciation goes to my research supervisor **Ms. Jebunnesa Chowdhury,** Coordinator, Biotechnology Program, Department of Mathematics and Natural Sciences. Without her impeccable support, diligent guidance, encouragement and devoted involvement, this research would never have come to reality. I would like to thank you very much Madam, for inspiring me in every step of the way and for believing in me.

I am much obliged to my teacher **Ms. Romana Siddique**, Lecturer, Biotechnology Program, Department of Mathematics and Natural Sciences, who had given me valuable suggestions regarding experimental settings and for her enormous support and encouragement during my research.

Events of repeated failures and several uncountable obstacles would have inundated and subdued me if it was not for my senior Teaching Assistant **Mr. Shaan Mahmeed** in the laboratory. His advice and cordial assistance fortified me to endeavor my dissertation with patience and his unwavering support had led me to reach this pinnacle of being able to carry out this research. The completion of the work would not have been possible without his active support.

My indebtedness goes to my friend **Fatima Zohra** for her ardent support. I would also like to extend heartiest thanks to my friends **Mohammad Al Mahmud-Un-Nabi**, **Mashiat Nawar, Fatima Amin** , **Sihinta Shembil**, **Sadia Zaman Shormee, Ahmed Fahim** and **Taizina Momtareen** for accompanying me through the ups and downs during the whole research period with dedicated support.

I am also grateful to the lab assistants, **Forkan Mia** and **Arifuzzaman**, who had been always there to help me in the lab works.

Most importantly, I could never have persevered without my family who offered me their encouragement. I am forever grateful for having them in my life.

ABSTRACT

Spirulina platenis is a multicellular and filamentous blue-green microalgae which is most well-known and broadly accessible. It develops in water, can be reaped and prepared effortlessly and has fundamentally high large scale and micronutrient substance. In numerous nations of Africa, it is utilized as human nourishment as a critical wellspring of protein and is gathered from normal water, dried and eaten. It has increased impressive prevalence in the human wellbeing nourishment industry and in numerous nations of Asia it is utilized as protein supplement and as human wellbeing sustenance. *Spirulina* has been utilized as a reciprocal dietary element of food for poultry and progressively as a protein and vitamin supplement to aquafeeds. *Spirulina* seems to have significant potential for improvement, particularly as a little scale crop for wholesome upgrade, employment advancement and ecological alleviation. In the current research work , the crude extracts of *Spirulina platensis* were collected using three solvents namely- Methanol , Ethanol and Distilled Water and phytochemical assay was performed on it to identify the secondary metabolites present in them. Antimicrobial activity was performed on the three types of crude extracts (which were initially diluted with Dimethyl Sulfoxide -DMSO) against *Bacillus subtilis, Enterococcus faecalis , Staphylococcus aureus , Escherichia coli,* using a positive (antibiotic disk) and negative control (0.25% DMSO), The antimicrobial tests were carried in agar diffusion technique using Mueller Hinton Agar (M-H) media. The results obtained from this study can act as a stepping stone in investigating the antimicrobial properties of *Spirulina platensis* extracts. This study could be the beginning of discovering a new microbial agent against certain bacterial species.

TABLE OF CONTENTS

Appendices

CHAPTER 1
INTRODUCTION

Chapter 1 : Introduction

Overview of *Spirulina platensis*

Spirulina platensis which is a blue-green algae is an ancient algae which is predicted to be present since the beginning of life on earth. This alga has survived the test of time and climatic cataclysms. Basically, the algae is found to grow naturally in oceans and salty lakes in subtropical climates. The population who were among the first to cultivate this blue-green algae were the Aztecs in order to feed their very large population.

Studies published in the journal Cardiovascular Therapeutics shows that *Spirulina* was once classified as a plant as it was rich in plant pigments and it was able to photosynthesize. However, recent genetics, physiology and biochemical properties studies led to its shift to bacteria kingdom , initially in genus *Arthrospira* but later into genus *Spirulina. Spirulina platensis* is known to have high nutritional and potential therapeutic values.

Historical use of *Spirulina platensis*

Spirulina was rediscovered during a European scientific mission in Chad, as a traditional food of the locals called dihé. It was a blue-green, dried cake made of a micro-organism which grew in the natural alkaline lagoons found in this region. It was found that people had been consuming this for centuries not only in Chad, but also in Mexico and other areas. Even though people here had extremely poor diet, they did not suffer from malnutrition. The Kanembu populace living along the shores of Lake Chad gathered the wet green growth in earth pots, channel out the water through packs of fabric and spread out the green growth in the sandy shore of the lake for sun drying. The semi-dried green growth was then cut into little squares and taken to the towns, where the drying is finished on mats in the sun (Abdulqader, Barsanti and Tredici, 2000). At the point when dry, ladies took these green growth cakes available to be purchased in the nearby market. Dihé was disintegrated and blended with a sauce of tomatoes and peppers, and poured over millet, beans, fish or meat

and is eaten by the Kanembu in 70 percent of their dinners. Pregnant ladies eat dihé cakes straightforwardly on the grounds that they trust its dim shading will screen their unborn child from the eyes of alchemists (Ciferri, 1983).

Morphology and taxonomy of *Spirulina platensis*

Spirulina platensis is a considered to be a symbiotic , multicellular and filamentous blue-green microalgae. It can be either rod or disk shaped. The reason of bluish green color is due to the presence of photosynthetic pigment- phycocyanin which is actually blue in color. It also contains chlorophyll, carotenoids and some of them phycoythrin , which gives the bacteria red or pink color. Being photosynthetic, they are autotrophic and they reproduce by binary fission. The helical shape of this organism's filaments also known as trichomes is its characterstic and is maintained only in liquid environment or culture medium. Gas filled vacuoles in its cells accompanied by helical shape of filaments results in floating mats. The trichomes length have a range of 50 to 500 µm and a width ranging from 3 to 4 µm. *Spirulina* possess a cell wall that contain peptidoglycan which is a lysozyme-sensitive heteropolymer responsible for conferring shape and protection.

Domain	Bacteria
Kingdom	Eubacteria
Phylum	Cyanobacteria
Class	Cyanophyceae
Order	Oscillatoriales
Genus	*Spirulina*
Species	*Platensis*

Table1 :Scientific classification of *Spirulina platensis*

Fig1 : Microscopic view of *Spirulina platensis*

Natural habitat, source and growth of *Spirulina*

Spirulina platensis is mainly observed in soil, marshes, freshwater, brackish water , sea water and even thermal springs. Good production of *Spirulina platensis* includes presence of alkaline, saline water (>30 g/l) with pH (8.5-11.0) including high conductivity and presence of solar radiation in a specific area.If the cytoplasmic pH is relatively high i.e. around 4.2 to 8.5 , there is a possibility of this microorganism to utilize ammonia as a nitrogen source at high alkaline pH values (Sasson, 1997).It is considered to be an obligate photoautotroph as it cannot grow in dark on media containing organic carbon compounds. It actually assimilates nitrates and main assimilation product of *Spirulina* photosynthesis is glycogen, in light it reduces carbon dioxide. Under laboratory conditions it grows at an optimum temperature of 35°C and 37 °C and in the environment it can grows at a temperature upto 39°C for few hours and its photosynthetic ability remains intact. The other strains of *Spirulina* namely thermophilic or thermotolerantare cultivated at temperature range of 35°Cand 40 °C. Using this practice, microbial mesophilic contaminants can be removed . It is calculated that the lowest temperature at which *Spirulina platensis's* growth takes place is 15°C and that also during day time. It is found to be highly resistant to ultraviolet rays (Richmond, 1986). .However, at night *Spirulina platensis* can tolerate relatively low temperatures.

Cultivation of *Spirulina platensis*

If produced industrially: In green houses, large water tanks (raceway ponds), water, fertilizer, pump/ paddle wheel to move it.

A concentrated *Spirulina* culture is then used to seed the pond containing culture medium. This can be obtained from culture floating on an existing pond, or recently harvested. This is mixed into the culture medium and allowed to grow. It should be regularly agitated using an electric pump or by stirring manually. The temperature, pH and concentration of algae should be monitored. Once the concentration increases to about 0.5g/L (use a Secchi disk to measure) it must be harvested. This can be done by simply filtering it through a cloth to obtain a "biomass" of about 10% dry matter per litre. The biomass obtained is then pressed in a cloth to produce a kind of cake. The culture medium can then be reused, by adding any of the ingredients which were used up by the *Spirulina*.

Spirulina is most nutritious in its wet form. However this lasts at most for a few days if refrigerated, and only a few hours at room temperature. Hence if it needs to be transported or stored it must be dried. If dried and packaged well it can be stored for at least a year without losing nutritional value. However if dried it acquires an unpleasant smell and taste, and is inconvenient to use. It can then also be combined with various other food products or simply packaged on its own.

The production of *Spirulina* requires manufacturing of a tank. The size of this depends on the scale of production, and the number of tanks. 1 tank of $18m^2$ produces approximately 150g of *Spirulina* per day.

In Bangladesh, *Spirulina* is refined in diverse agro-mechanical squanders, for example, sugar plant waste gushing, poultry industry waste, manure production line waste, and urban waste and natural matte.

Medical importance of *Spirulina platensis*

Clinical trials have demonstrated that *Spirulina* can serve as a supplementary cure for some maladies. *Spirulina* containers have demonstrated powerful in bringing down blood lipid level, and in diminishing white blood corpuscles after radiotherapy and chemotherapy (Ruan, Long and Guo, 1988; Ruan, Guo and Shu, 1990), and in addition enhancing immunological capacity. *Spirulina* likewise is utilized for wellbeing sustenance, nourish and for the biochemical items since 1980s (Becker, 1988; Borowitzka, 1988; Richmond, 1988). *Spirulina* is rich in top notch protein, vitamins, minerals and numerous naturally dynamic substances (Becker, 1994). Its cell divider comprises of polysaccharide which has an absorbability of 86 percent, and could be effectively consumed by the human body. There are diverse classifications of *Spirulina* nourishment where pills and containers produced using dry *Spirulina* are critical.

Spirulina is thought to have ultra rich nutritional composition in such an amount that it allows preventing nutrient deficiencies and fighting malnutrition across the world. It is rich in 50-70% proteins , essential fatty acid gamma linolenic acid (GLA) , trace elements , minerals , magnesium , calcium , phosphor , potassium , selenium , sodium , chromium and essential amino acids. It also contains a large amount of chlorophyll that helps in elimination of toxins from blood and boosts the immune system. It also contains a very high concentration of bio-available iron which is beneficial to pregnant women, anemic patients and people suffering from constipation. *Spirulina* is enriched with calcium which makes it an excellent source for children, elderly people and pregnant women. Vitamins like beta-carotene , A , B1, B2 , B3 , B6 , B7 , B8 , B12 , D, E,K are also present. It also contains many elements that are required for the healthy functioning of the immune system and nervous system.

Spirulina , when consumed by athletes assist in avoiding deficiencies and muscular fatigue. It also helps to combat excessive blood cholesterol and diabetes for regulating the level of glycemia in the blood. *Spirulina* is helpful in reducing allergies and allergic reactions. *Spirulina's* phosphorus content makes it helpful as part of tooth remineralization regimen. *Spirulina* helps in stimulation of beneficial flora like *lactobacillus* and *bifidobacteria* in the

digestive tract which further promotes healthy digestion and proper bowel function. Its inflammatory properties have been helpful in reducing joint pains and inflammation.

Fig 2 :*Spirulinaplatensis* in two forms – powdered (left) and tablet form (right) , both forms can be taken as dietary supplement

Different secondary metabolites in *Spirulina platensis* and their importance

Alkaloids :Alkaloids assume a critical part in the nature of living beings which incorporate them. They assume a vital part in the resistance frameworks against pathogens and creatures. The utilizations of alkaloids are not constrained to natural control of herbivores but rather likewise have pharmacological, veterinary and medicinal significance. Alkaloids fitting in with beta-carboline gathering have antimicrobial, hostile to HIV and antiparasitic exercises.

Saponins : Normal saponins have been found to have noteworthy against malignancy properties. There are more than 11 recognized classes of saponins including dammaranes, tirucallanes, lupanes, hopanes, oleananes, taraxasteranes, ursanes, cycloartanes, lanostanes, cucurbitanes and steroids. Because of the colossal variability of their structures, saponins dependably show hostile to tumorigenic impacts through mixtures of antitumor pathways.

Steroids : A large portion of the steroids from these plants are changed over into hormonally dynamic substances that reproduce pregnancy and serve as antifertility or prophylactic mixes or as antiinflamatory medications, for example, cortisone and so forth that are utilized to treat various ailments, for example, joint inflammation and so forth.

Tannins: Numerous tannin particles have likewise been indicated to lessen the mutagenic action of various mutagens. The anticarcinogenic and antimutagenic possibilities of tannins may be identified with their antioxidative property, which is critical in securing cell oxidative harm, including lipid peroxidation. Tannins have additionally been accounted for to apply other physiological impacts, for example, to quicken blood coagulating, lessen pulse, diminish the serum lipid level, produce liver corruption, and balance immunoresponses.

Phenols:Phenols, has generally been considered as antinutritive mixes because of the unfriendly impact of one of their fundamental parts, tannins, on protein edibility. On the other hand, really there is an expanded enthusiasm for these mixes in light of the fact that they have been connected with the restraint of atherosclerosis and tumor. The bioactivity of phenolics may be identified with their cell reinforcement conduct, which is credited to their capacity to chelate metals, repress lipoxygenase and rummage free radicals.

Flavonoids:Current approved doctors are expanding their utilization of unadulterated flavonoids to treat numerous essential normal infections, because of their demonstrated capacity to restrain particular proteins, to reenact a few hormones and neurotransmitters, and to search free radicals. Flavonoids hinder or slaughter numerous bacterial strains, repress vital viral catalysts, for example, reverse transcriptase and protease, and devastate some pathogenic protozoans. Flavonoids are major useful segments of numerous home grown and creepy crawly arrangements for therapeutic utilization, e.g., propolis (honey bee's paste) and nectar, which have been utilized since old times.

Cardiac glycosides: The cardiac glycosides are an imperative class of normally happening medications whose activities incorporate both valuable and lethal consequences for the heart. Helpful utilization of natural cardiac glycosides keeps on being a wellspring of danger today.Plants containing cardiac steroids have been utilized as toxins and heart drugs at any rate following 1500 B.C. All through history these plants or their concentrates have been differently utilized as bolt toxins, emetics, diuretics, and heart tonics. Cardiac steroids are broadly utilized as a part of the present day treatment of congestive heart disappointment and for treatment of atrial fibrillation and shudder. Yet their danger remains a major issue.

Glycosides: There are various plants that store chemicals in the form of inactive glycosides which can be activated by enzyme hydrolysis, that causes the sugar part to be broken off, making the chemical available for use. These plant glycosides are used as medication purposes.However , in animals and humans, poisons are often bound to sugar molecules as part of their elimination from the body.

Purpose of phytochemical assay done for *Spirulina platensis*

Nature has given a complete storage facility of solutions for cure all illness of humanity. Utilization of plants as a wellspring of drug has been acquired from the onset of human progress and is an essential part of the social insurance framework. The restorative plants are valuable for mending and in addition for curing of human illnesses as a result of the vicinity of phytochemical constituents. Phytochemicals are normally happening in the restorative plants, leaves, vegetables and roots that have safeguard system and shield from different sicknesses. Phytochemicals are essential and optional mixes. Chlorophyll, proteins and normal sugars are incorporated in essential constituents and optional mixes have tannins, saponins, alkaloids phenolic mixes etc. In late years, substance examination and natural measures have started to assume a critical part in ethnobotanical studies (Jana et al., 2009). In a few cases, such examinations have prompted the revelation of novel bioactive phytochemicals.

Purpose of antimicrobial assay done for *Spirulina platensis*

Nature has been a wellspring of therapeutic operators for a large number of years and a noteworthy number of advanced medications have been confined from regular sources, of which numerous are in light of their uses in customary drug .Pharmaceutical medication disclosures, for the vast majority of the previous 40 years, have depended intensely on the procedure of observationally screening of huge number of unadulterated mixes to give new leads. Pathogen imperviousness to manufactured medications and anti-microbials that are as of now being used makes scan for plants with antimicrobial action more imperative, as they

can substitute for engineered anti-toxins and medications. *Spirulina platensis* or its concentrate show helpful properties, for example, the capacity to counteract diseases, lessening blood cholesterol level, decrease nephrotoxicity of pharmaceuticals and dangerous metals and give insurance against the hurtful impact of radiation. *Spiulina platensis* produces an assorted scope of bioactive particles, making them a rich wellspring of distinctive sorts of medicines. Some *Spirulina* species display antibacterial action . *Spirulina platensis* can possibly deliver countless substances, so they are considered as suitable life forms for misuse as biocontrol operators of plant pathogenic microscopic organisms and growths.

Aims and objectives of the study

The aim of this study was to perform phytochemical and antimicrobial assays on methanolic ,ethanolic and aqueous extracts collected from *Spirulina platensis*. In the phytochemical assays, the extracts were collected using methanol, ethanol and distilled water as solvents and then the secondary metabolites were determined by using different reagent tests .Antimicrobial testing was performed through agar diffusion method. Two types of controls were used- antibiotic disk as the positive control and 0.25% Dimethyl sulfoxide as the negative control. Prepared extracts were tested on several organisms namely *Bacillus subtillis, Enterococcus faecalis , Staphylococcus aureus , Escherichia coli*. This was followed by incubation period at 37°C for 24 hours. The zones formed in the quadrants of the plates are measured in mm. From the agar diffusion test, the minimum concentration of *Spirulina* extracts needed to visualize the zone of inhibition on the Mueller Hinton Agar was determined to be 60 µg/ml and maximum was 80µg/ml .

CHAPTER 2
MATERIALS & METHODOLOGY

Working laboratory

All the works were performed in the Microbiology Specialized Research Laboratory, Department of Mathematics and Natural Sciences, BRAC University, Mohakhali , Dhaka from January 2015 to September 2015. This Laboratory had got the Biosafety Label 2 (BSL-2) facility. All the microbiological works were done inside Biological Safety Cabinet.

Reference of collection sites

In this study, the *Spirulinaplatensis* powder was collected from Bangladesh Council of Scientific and Industrial Research (BCSIR), Dhaka. The reagents and chemicals used were used from Microbiology Specialized Research Laboratory, Department of Mathematics and Natural Sciences and some reagents were prepared in the laboratory only. The clinical strains of bacteria that were used during the study were B*acillus subtillis*, *Enterococcus faecalis* , *Staphylococcus aureus* , *Escherichia coli.* They were obtained from the preserved bacterial stock in the department laboratory.The antibiotics used were also obtained from the laboratory of department of Mathematics and Natural Sciences.

Phytochemical screening process

This process were carried out in three stages namely

i. Collection of required samples
ii. Carrying out extraction procedure from the sample
iii. Performing phytochemical screening on them in order to detect the availability of different secondary metabolites.

i.Collection of required samples

The *Spirulina platensis* sample was collected in the form of coarse powder from Bangladesh Council of Scientific and Industrial Research (BCSIR), Dhaka. The powder being coarse, were broken down into fine particles using pestle and mortar. Then the powder was stored in a plastic container which was labeled with required information.

ii. Carrying out extraction procedure from the sample

A total of three types of extracts were collected from the sample powders namely methanolic extract, ethanol extract and aqueous extract.

a) **Methanolic and Ethanolic Extracts**: For collection of both ethanolic and methanolic extracts 50g of *Spirulina* powder was packed in thimble and extracted in Soxhlet's apparatus using 250ml of Methanol and Ethanol respectively. The temperature was kept between 60-80ºC for both the cases. The samples in the thimble of the Soxhlet's apparatus was kept boiling for approximately 4 hours till the solution becomes clear and the dark colored extract was collected at the bottom of the apparatus. This was then collected in petri dishes and left to dry for 24hours. The dried extract having a sticky appearance was stored in 25 ml McCartney bottles at temperatures below 10 ºC in the refrigerator for further use. The whole process was repeated 3 times for the collection of a substantial amount of extracts for the study.

Fig 3 : *Spirulina* powder collected in Soxhlet's apparatus and extraction process being carried out (left) . Extract collected along with the solvent used (right)

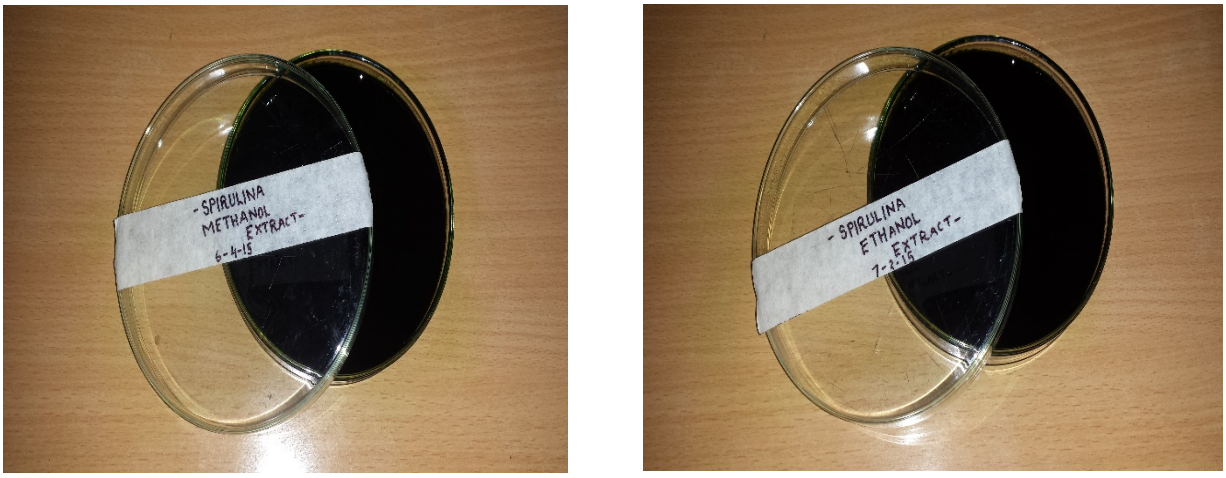


Fig 4 : Extracts collected in petri dishes and placed in fume hood for 24 hours for the solvent to evaporate. Methanol extract (left) and Ethanol extract (right) are appears similar.

Fig 5 : As the solvents evaporate out , the extracts are scraped out from the petri dishes and stored in McCarteny bottles and labelled properly so that two extracts can be distinguished.

b) **<u>Aqueous extract</u>**: Collection of aqueous extract was done in a different way than the other two. 40 gmof *Spirulina* powder was measured and mixed with 480 ml of distilled water. This was left for 2 days in sterile environment. The liquid extract was then filtered through WhatmanFilter paper. The filtrate was kept in waterbath at 80-90 ºC till the extract was dried out. The dried extracts were then stored at 10 ºC in McCartney bottles.

Fig6 : Aqueous extract collection procedure (left) . Filtering of extract after collection (right)

Stock solutions preparations

In order to carry out biochemical assays, the stocks were dissolved and a stock solution of 3.3µg/µl was made. This was done by mixing 3.3gm of crude extract with 100 ml of their respective solvents. All the three stock solution preparation was made in the same manner.

Fig 7: Stock solutions stored in reagent bottles

Phytochemical tests

Alkaloids test : This test follows the Dragendroff's test where to 2 ml of all the three extracts , 0.2 ml HCl is added., followed by addition of 1 ml Dragendorff reagent .If orange brown precipitate to be formed at the lower layer of all the three extracts , presence of alkaloids is confirmed.

Saponinstest: This test follows the froth test where to 3 ml of all the three extracts, 1 ml of distilled water is added and the test tubes are shaken vigorously for 30 seconds. If foam formation at the top layer of about 1cm is formed in the shape of a honey-comb structure , presence of saponins is confirmed**.**

Steroids Test : This test follows the Salkaowaski's test where to 2 ml of all the three extracts , 10 ml of chloroform is added, followed by addition of 10 ml of sulfuric acid to each test

tube. If formation of greenish brown ring with a glow at the bottom isformed, then presence of steroids is confirmed

Flavonoids Test: This test involves reaction with sodium hydroxide where to 1ml of all the three extracts, 2ml ofdilute sodium hydroxide is added, followed by addition of 1 ml of water . If yellowish precipitate isformed, presence of flavonoids is confirmed.

Tannins Test: This test follows the Gelatin test where to 3 ml of all the extracts, few drops of 1% lead acetate is added. If formation of a yellow precipitate at the bottom of the extracts takes places, presence of tannins is confirmed.

Phenol test: This test involves a process where to 2 ml of the extracts, a reagent of few drops (prepared from the mixture of 1% of 1 ml of potassium ferrocyanide and 1% of 1 ml ferric chloride) is added and the extracts are supposed to turn blue. If blue color is observed, presence of phenol is confirmed

Cardiac glycosides test: This test follows Keller-Killanis test where to 2ml of all the three extracts, 2ml of the glacial acetic acid, few drops of ferric chloride and 2 ml of concentrated sulfuric acid is added. If a brown ring at interface is formed in all the three extracts, presence of cardiac glycosides is confirmed.

Glycosides test: This test follows Killer-killanis test where to 2ml of algal extract 1ml of aqueous NaOH solution is added. The appearance of a yellow color indicates the presence of glycosides.

Antimicrobial assay

Disk diffusion test

In this method the standardized bacterial isolate is spread on an agar plate and then paper disc containing specific concentration of antibiotics are placed and incubated at 37°C overnight. If the isolate is susceptible to the antibiotic, it does not grow around the disk thus forming a zone of inhibition. Strains resistant to an antibiotic grow up to the margin of disk.

The diameter of zone of inhibition must be measured and result read from the Kirby Bauer chart as sensitive, intermediate or resistant.

In order to carry out disk diffusion test, these stages were followed namely

i.Preparation of stock solutions of methanolic, ethanolic and aqueous extracts using Dimethylsulfoxide (DMSO) at 80 and 60 concentrations.

ii.Subculturing test organisms on Nutrient Agar (NA) media

iii.Carrying lawn cultures on Mueller-Hinton Agar (M-H)to prepare lawns from cultures that were previously sub-cultured in Nutrient Agar media

iv.Carrying out antibiotic susceptibility test using positive control (antibiotic disk), negative control (0.25% DMSO) and the extracts.

Nutrient Agar Media

Nutrient Agar is a general purpose, nutrient medium used for the cultivation of microbes supporting growth of a wide range of non-fastidious organisms. Nutrient agar is popular because it can grow a variety of types of bacteria and fungi, and contains many nutrients needed for the bacterial growth and are also used for purity checking prior to biochemical or serological testing. Nutrient Agar is ideal for demonstration and teaching purposes where a more prolonged survival of cultures at ambient temperature is often required without risk of overgrowth that can occur with more nutritious substrate. This relatively simple formula has been retained and is still widely used in the microbiological examination of variety of materials and is also recommended by standard methods. It is one of the several non-selective media useful in routine cultivation of microorganisms. It can be used for the cultivation and enumeration of bacteria which are not particularly fastidious. Addition of different biological fluids such as horse or sheep blood, serum, egg yolk etc. makes it suitable for the cultivation of related fastidious organisms. Peptic digest of animal tissue, beef extract and yeast extract provide the necessary nitrogen compounds, carbon, vitamins and also some trace ingredients necessary for the growth of bacteria. Sodium chloride

maintains the osmotic equilibrium of the medium. The composition of Nutrient agar is as follows:

- 0.5% Peptone- It is an enzymatic digest of animal protein. Peptone is the principal source of organic nitrogen for the growing bacteria.
- 0.3% beef extract/yeast extracts - It is the water-soluble substances which aid in bacterial growth, such as vitamins, carbohydrates, organic nitrogen compounds and salts.
- 1.5% agar- It is the solidifying agent.
- 0.5% NaCl - The presence of sodium chloride in nutrient agar maintains a salt concentration in the medium that is similar to the cytoplasm of the microorganisms.
- Distilled water- Water is essential for the growth of and reproduction of micro-organisms and also provides the medium through which various nutrients can be transported.
- pH is adjusted to neutral (7.4) at 25 °C.

Preparation of Nutrient Agar media

- 28 g of nutrient agar powder was suspended in 1 litre of distilled water.
- This mixture was heated while stirring to fully dissolve all components.
- The dissolved mixture was autoclaved at 121 degrees Celsius for 15 minutes.
- Once the nutrient agar has been autoclaved, it was allowed to cool but not solidify.
- Nutrient agar was poured into each plate and plates were left on the sterile surface until the agar has solidified.
- The lid of each petri dish was replaced and the plates were stored in a refrigerator.

Mueller Hinton (M-H) Agar

Mueller Hinton Agar is used in antimicrobial susceptibility testing by the disk diffusion method. Mueller Hinton Agar is based on the formula recommended by Mueller and Hinton for the primary isolation of *Neisseria* species. This medium is low in sulfonamide, trimethoprim and tetracycline inhibitors, and provides satisfactory growth of most non-fastidious pathogens along with demonstrating batch-to-batch reproducibility. Mueller Hinton Agar is often abbreviated as M-H Agar, and complies with requirements of the World Health Organization. Mueller Hinton Agar used for food testing, and procedures commonly performed on aerobic and facultatively anaerobic bacteria.A variety of supplements can be added to Mueller Hinton Agar, including 5% defibrinated sheep or horse blood, 1% growth supplement and 2% sodium chloride.The composition of Mueller-Hinton Agar (1 L) is as follows:

Beef Extract -2 g

Acid Hydrolysate of Casein- 17.5 g

Starch-1.5 g

Agar -17 g

Final pH 7.3 ± 0.1 at 25°C

Preparation of Mueller Hinton Agar

1. 38 g of the medium was suspended in one liter of purified water.

2. The mixture was heated with frequent agitation and boil for one minute to completely dissolve the medium.

3.The media was autoclaved at 121°C for 15 minutes and then cooled to room temperature.

4. The cooled media was poured into sterile petri dishes on a level, horizontal surface to give uniform depth and then allowed to cool to room temperature.

5. The prepared Mueller Hinton Agar was checked to ensure the final pH is 7.3 at 25°C.

Test Organisms:

The bacteria strains used in the experiment were collected from ICDDR,Band preserved in biotechnology laboratory, BRAC University. The list of test organisms is as follows

- ✓ *Bacillus subtilis*
- ✓ *Enterococcus faecalis*
- ✓ *Staphylococcus aureus*
- ✓ *Escherichia coli*

Preparation of stock solution for antibacterial activity test

All three types of the dry extracts of *Spirulina platensis*that were collected were dissolved in 0.25% DMSO to make two different concentrations of extract solutions for the antibacterial activity test. 4gms of extract was dissolved in 50ml of 0.25% DMSO to prepare 80µg/µl. This was then diluted to prepare a solution that has a concentration of 60µg/µl. For each inoculation during the test the stock solution of extract was freshly prepared

Fig 8 : Each extract dissolved in 0.25% DMSO

Preparation of plating bacteria

The referred strains were taken from the stock culture. They were streaked onto Nutrient Agar (NA). The plates were incubated overnight at 37°C for growth in an incubator.

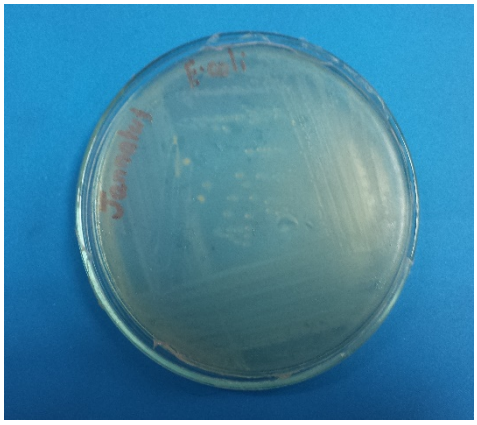


Fig 9 : Streaked plate of *E.coli*

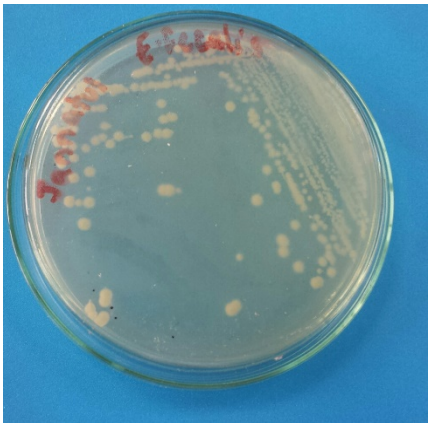


Fig 10 : Streaked plate of *E. fecalis*

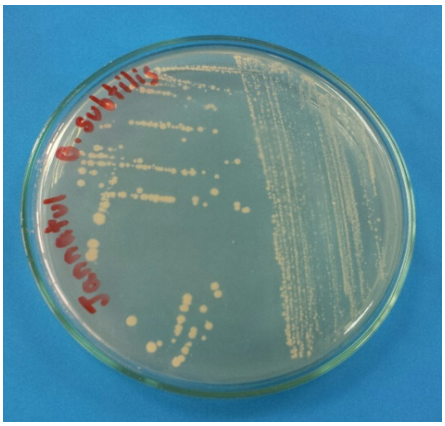


Fig 11 : Streaked plate of *B.subtilis*

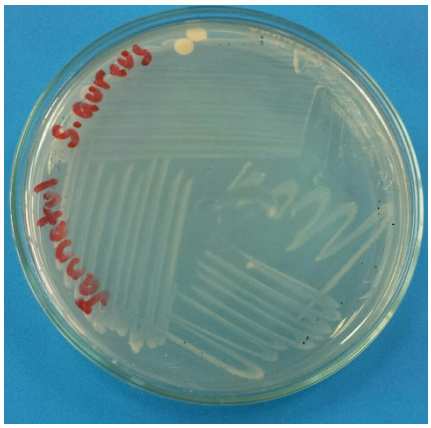


Fig 12: Streaked plate of *S.aureus*

Inoculation of test organisms

The test organisms were transferred using a loop in test tubes containing 5 ml of 0.9% saline to make cell suspension. They were then vortexed in a vortex machine for the organisms to mix properly in the saline solution. The concentration of the cells in each test tube was optimized using 0.5 McFarland solution that had an OD of 0.1 in 600nm wavelength when measured in a spectrophotometer. This was done so that there is equal number of cells in

every cell plate. Using a cotton swab the bacteria from the cell suspension was immediately inoculated in the freshly prepared MHA media. The lawn was done multiple times in each plate by rotating them 90º each time to make sure there was uniform distribution of organisms in the media. Using a sterile forcep, an antibiotic disk is picked from the cartridge and placed on the agar plate. This plate was incubated overnight at 37°C in an incubator. Next day the zone of inhibition (clear zone) was measured with a ruler and compared with the antibiotic disk zone diameter interpretation.

Placing extracts and controls in the plates

The inoculated plates were labeled and a cork borer was used to make a well in the media. 100 µl of stock extract that was previously prepared in concentrations of 80µg/ µl and 60 µg/ µl using freshly prepared 0.25% DMSO, were given in each well. A positive control and a negative control were used to compare the results. For positive control four different antibiotics were used against the organisms. Kanamycin was used against *Bacillus subtilis* and *Staphylococcus aureus*, Vancomycin against *Enterococcus fecalis*and Amoxicillin for *Eschirichia coli.* As negative control 0.25% freshly prepared DMSO was given in one of the wells. The plates were then labeled accordingly and incubated at 37 ºC for 24 hours.

Measuring Zones

Following 24 hours of incubation of the test plates the clear zones were measured using a ruler. This was done by measuring the entire diameter of the clear zone and the results were recorded.

Measuring the activity index

The inhibitory effects of the methanolic, ethanolic and aqueous extracts were calculated and compared by measuring the activity index. This was done by using the following formula:

Activity Index (AI)= Zone of inhibition of extract/ Zone of inhibition of antibiotic

Preservation of bacterial strains

In order to use the bacteria for a long period of time , it was necessary to preserve them in proper sterile conditions. So, from the preserved bacterial stock , selected stocks were prepared, sealed with Parafilm and stored for next use. It was ensured that no characteristic changes took place in the strains.

CHAPTER 3

RESULTS

Phytochemical Assay

Preliminary screening tests of all three extracts were done for testing various phytochemicals found in plants. The crude extracts were tested for the presence or absence of secondary metabolites such as alkaloids, steroidal compounds, phenolic compound, flavonoids, saponins, tannins and cardiac glycosides.

Amount of extracts collected

The extraction was done using 3 different solvents; methanol, ethanol and water. Different amount of extracts were collected during the experiment. The table shows the amount of extracts collected in grams after drying them in petri dishes

Extraction	Methanolic extract	Ethanolic extract	Aqueous extract
1	18.9 gm	25 gm	20.3 gm
2	20.46 gm	26.86 gm	19.54 gm
3	26.3 gm	25.56 gm	20.6 gm

Table 2 : Amount of extracts collected in grams after drying them in petri dishes

List of phytochemical tests and their results

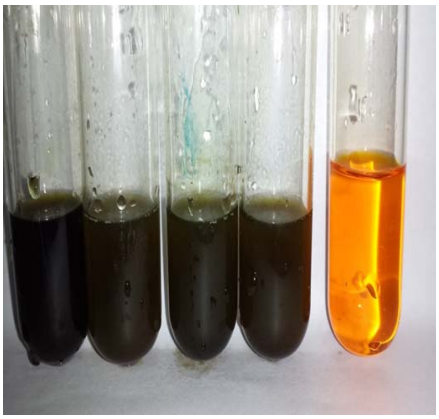
Alkaloids Test

Dragendroff's test :To 2 ml of all the three extracts , 1 ml of Dragendroff's reagent is added.

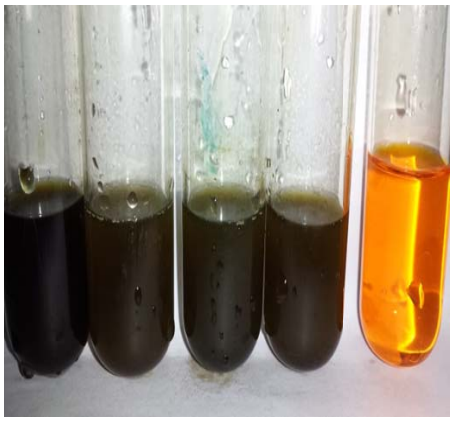
Expectation: Orange brown precipitate to be formed in all three extracts.

Result: Only in case of ethanolicextract, orange brown precipitate was observed.

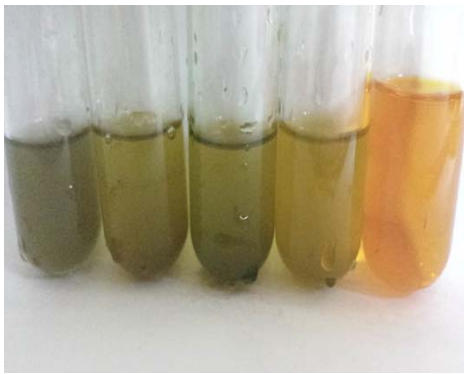
Inference:Ethanolic extract had a huge amount of alkaloids compared to methanolic and aqueous extracts.



Alkaloid test with Methanolic extract



Alkaloid test with Ethanolic extract



Alkaloid test with Aqueous extract

Fig 13 : Alkaloid tests with the three extracts

Saponins Test

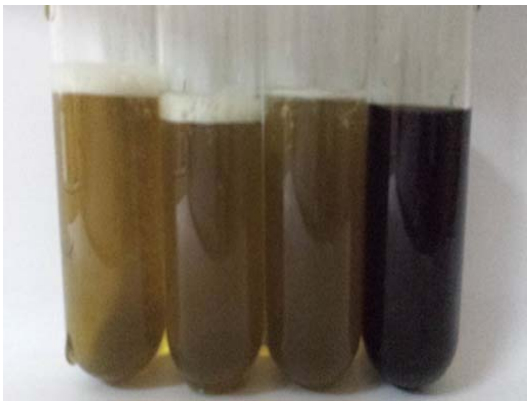
Froth test: To 1 ml of all the three extracts , 20 ml of distilled water was added and the test tubes was shaken vigorously for 15 minutes.

Expectation: Foam formation at the top layer of about 1cmis expected.

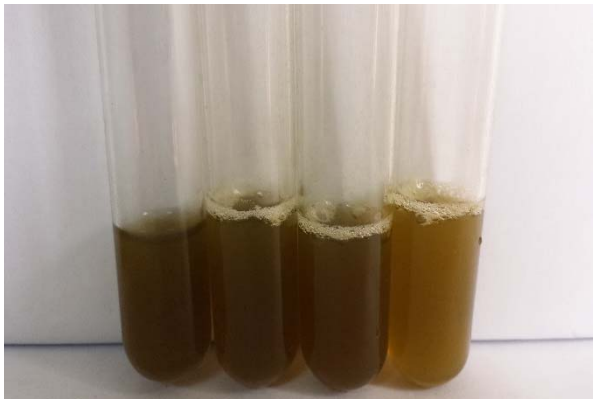
Result: Foam formation at the top layer of methanolic extract and ethanolic extract appeared more prominently.

Inference:Methanolic extract and ethanolic extract had huge amount of saponins compared to aqueous extract.

Saponins test with Methanolic extract



Saponins test with Ethanolic extract



Saponins test with Aqueous extract

Fig 14 :Saponin tests with the three extracts

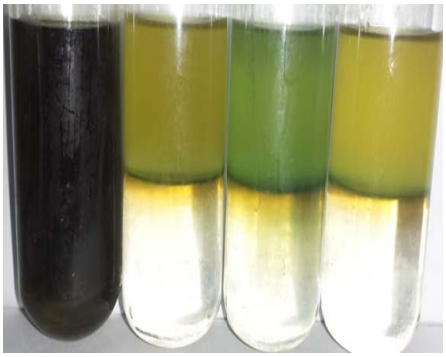
Steroids Test

Salkaowaski'stest:To 2 ml of all the threeextracts, 10 ml of chloroform was added, followed by addition of 10 ml of sulfuric acid to each test tube.

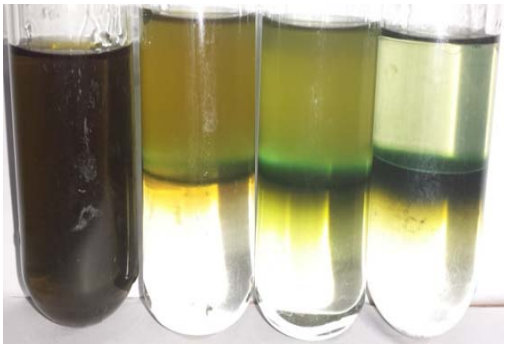
Expectation:Formation of greenish brown ring with a glow at the bottom is expected.

Result: Greenish brown ring with a glow the lower layer observed in all the test tubes.

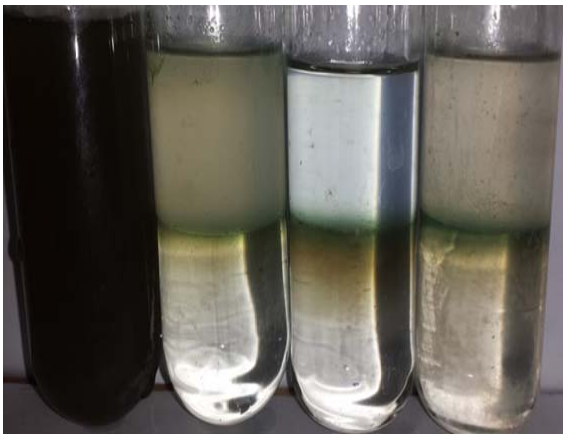
Inference: Steroids is present strongly in all the three extracts.



Steroids test with Methanolic extract



Steroids test with Ethanolic extract



Steroids test with Aqueous extract

Fig 15 : Steroid tests with the three extracts

Tannins Test

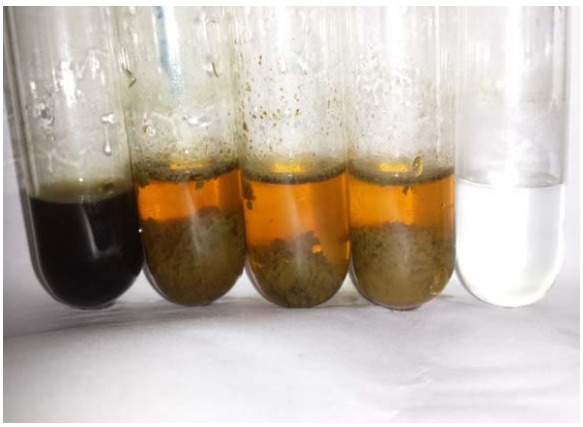
Gelatin test:To 3 ml of all the extracts , few drops of 1% lead acetate was added.

Expectation: Formation of a yellow precipitate at the bottom of the extract.

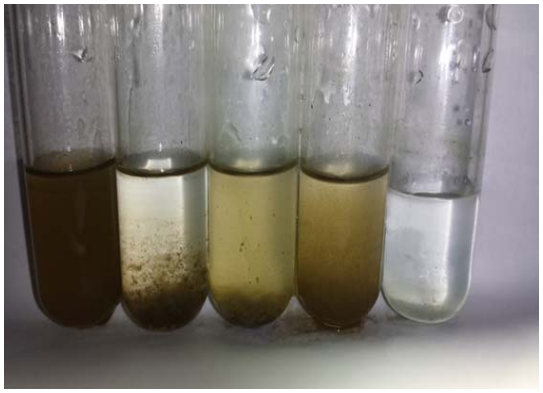
Result: In all the three extracts , the yellow precipitate was observed . In methanolicextract , the precipitate observed was a bit greenish.

Inference: Tannins is present in all the three extracts.

Tannins test with Methanolic extract



Tannins test with Ethanolic extract



Tannins test with Aqueous extract

Fig 16: Tannin tests with the three extracts

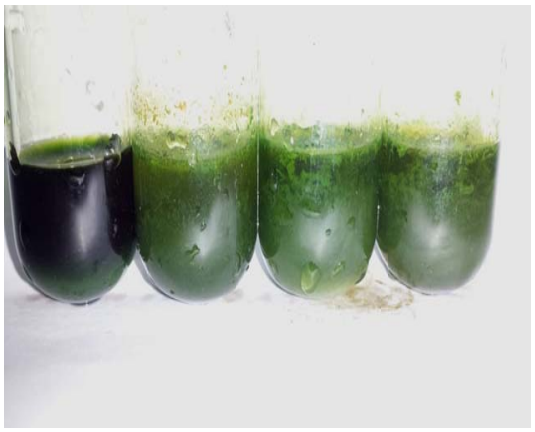
Flavonoids Test

Reaction with sodium hydroxide:To 1ml of all the three extracts ,2ml of the dilute sodium hydroxide was added , followed by addition of 1 ml of water.

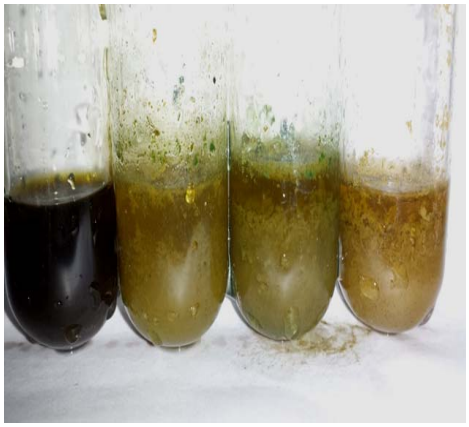
Expectation: Yellowish precipitate is expected.

Result: In case of Ethanolic extract, the yellow precipitate appeared more.

Inference:Ethanolic extract contains maximum amount of flavonoids compared to methanolic and aqueous extracts.



Flavonoids test withMethanolic extract



Flavonoids test with Ethanolic extract

Flavnoids test with Aqueous extract

Fig 17 :Flavonoid tests with the three extracts

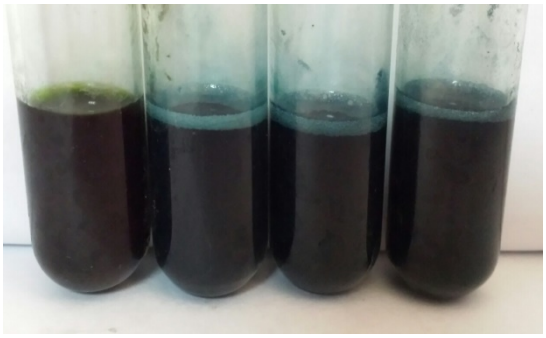
Phenol test

To 2 ml of the extract, a reagent of few drops (prepared from the mixture of 1% of 1 ml of potassium ferrocyanideand1% of 1 ml ferric chloride) was added.

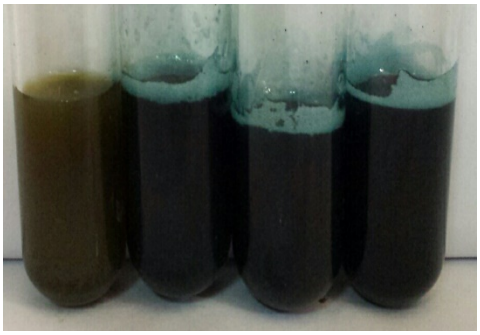
Expectation: The extracts are supposed to turn blue.

Result: The extracts appeared blue on reaction with the reagent.

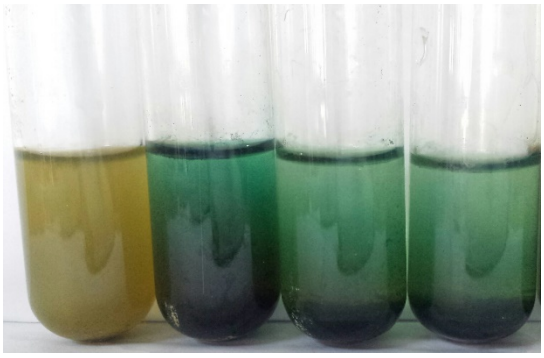
Inference:All the three extracts contain phenol.



Phenol test with Methanolic extract



Phenol test with Ethanolic extract



Phenol test with Aqueous extract

Fig 18 : Phenol tests with the three extracts

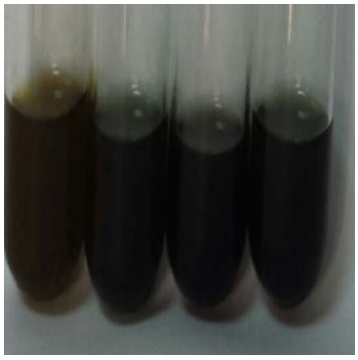
Cardiac glycosides test

Keller- Killani'sTest: To 2ml of all the three extracts , 2ml of the glacial acetic acid, few drops of ferric chloride and 2 ml of concentrated sulfuric acid was added.

Expectation: A brown ring at interface is expected in all the three extracts.

Result: Brown ring was observed in all the ethanolic extract and aqueous extract.

Inference:Cardiac glycosides is present in ethanolic extract and aqueous extract and not in methanolic extract.



Cardiac glycosides test with Methanolic extract

Cardiac glycosides test with Ethanolic extract

Cardiac glycosides test with Aqueous extract

Fig19 : Cardiac glycoside tests with the three extracts

Glycosides test

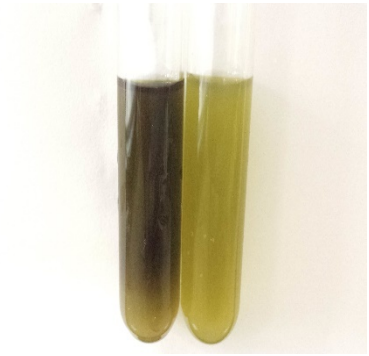
Keller Killanistest :To 2ml of all the three extracts , 1ml of aqueous NaOH solution was added.

Expectation: Yellow color appearance in all the three extracts

Result: Yellow color appeared in all the three extracts.

Inference : Glycosides is present in all three extracts.

Glycoside test with Methanolic extract



Glycoside test with Ethanolic extract

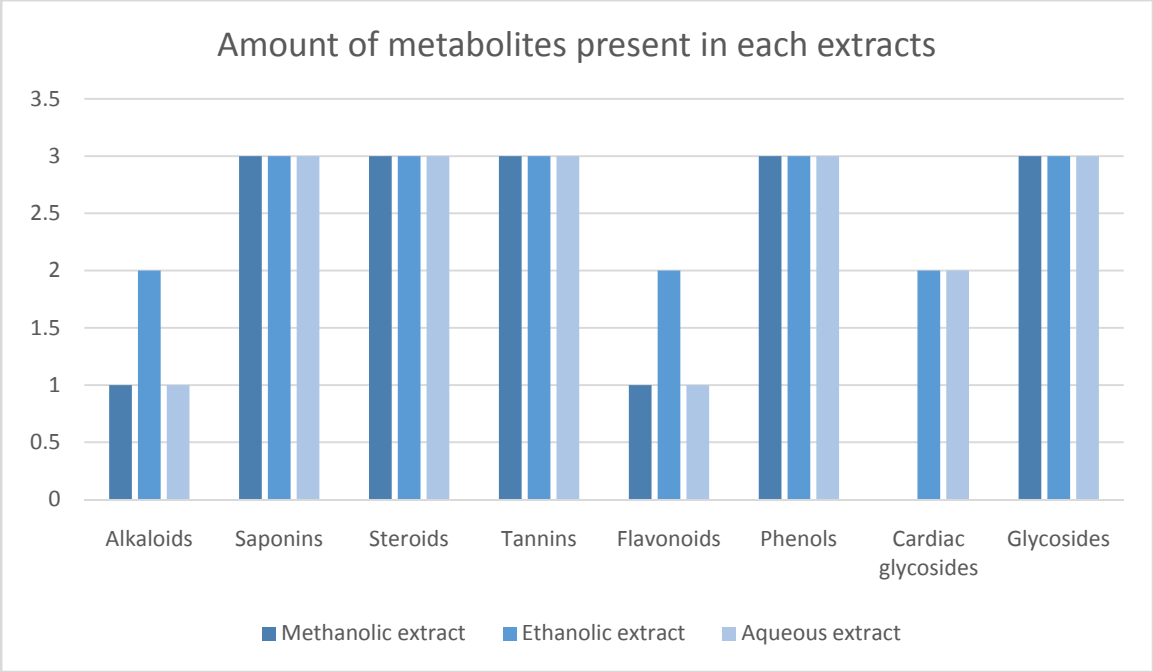
Glycoside test with Aqueous extract

Fig 20 : Glycoside tests with the three extracts

Constituents to be tested	Tests performed	Methanolic extract	Ethanolic extract	Aqueous extract
Alkaloids	Dragendroff's reagent test	+	++	+
Saponins	Foam test	+++	+++	+++
Steroids	Salkaowaski's test	+++	+++	+++
Tannins	Gelatin test	+++	+++	+++
Flavonoids	Sodium hydroxide test	+	++	+
Phenols	Ferric chloride and potassium ferrocyanide test	+++	+++	+++
Cardiac glycosides	Keller-Killanis test	-	++	++
Glycosides	Keller Killanis test	+++	+++	+++

Table 3 : The amount of constituents present in each extract performed during phytochemical assay

Key: +Positive(Slightly Present), ++=Moderately Present, +++ =Positive (Highly present)



Bar Graph1 : Represents the amount of metabolites in each extracts

Antimicrobial assay

The single colonies formed on Nutrient Agar were dipped in 0.9% saline solution, and at 600 nm , the optical density was measured which was against McFarland solution of standard value 0.5 having an optical density of 0.110.

Result

Enterococcus fecalis , *Bacillus subtilis* and *Staphylococcus aureus* gave clear zones in the three extracts and *Escherichia coli* gave no zones in any of the three extracts. Among the three bacterial isolates, that gave clear zones, *Staphylococcus aureus* had the maximum antimicrobial activity. The methanolic extract showed good amount of antimicrobial activity compared to ethanolic and aqueous extracts (Table 5&6).

Bacterial Isolates	**Optical Density of McFarland at 600 nm**	**Optical Density of bacterial suspensions at 600 nm**
Enterococcus faecalis	0.110	0.120
Bacillus subtilis		0.117
Staphylococcus aureus		0.114
Escherichia coli		0.118

Table 4 : Optical density of bacterial isolates

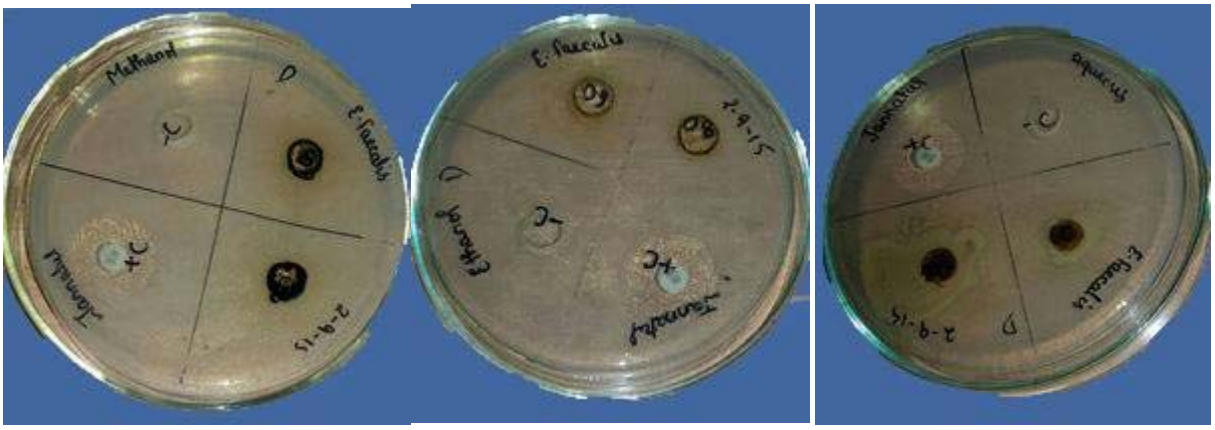


Fig21 : Antimicrobial assay of the three extracts against *E.faecalis*

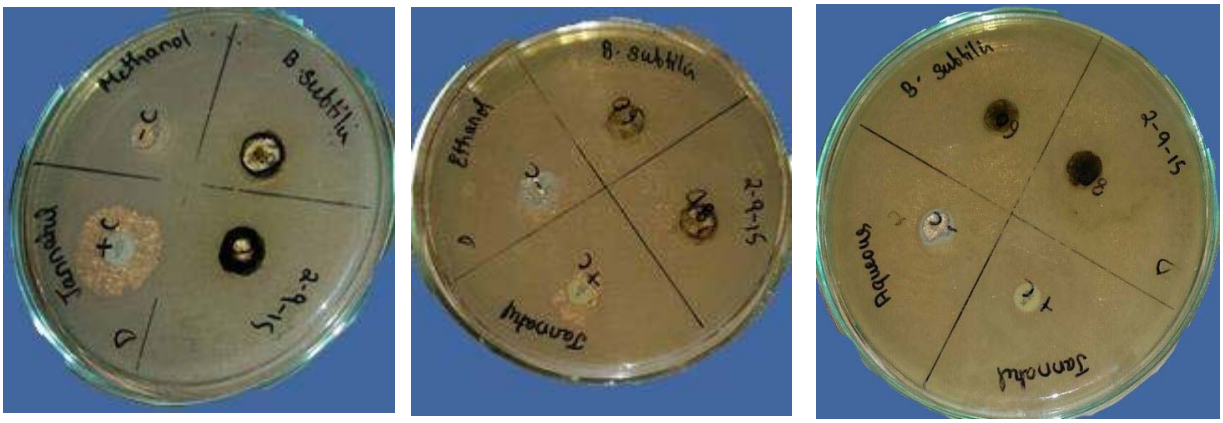


Fig22 : Antimicrobial assay of the three extracts against *B.subtilis*

Fig23 : Antimicrobial assay of the three extracts against *S.aureus*

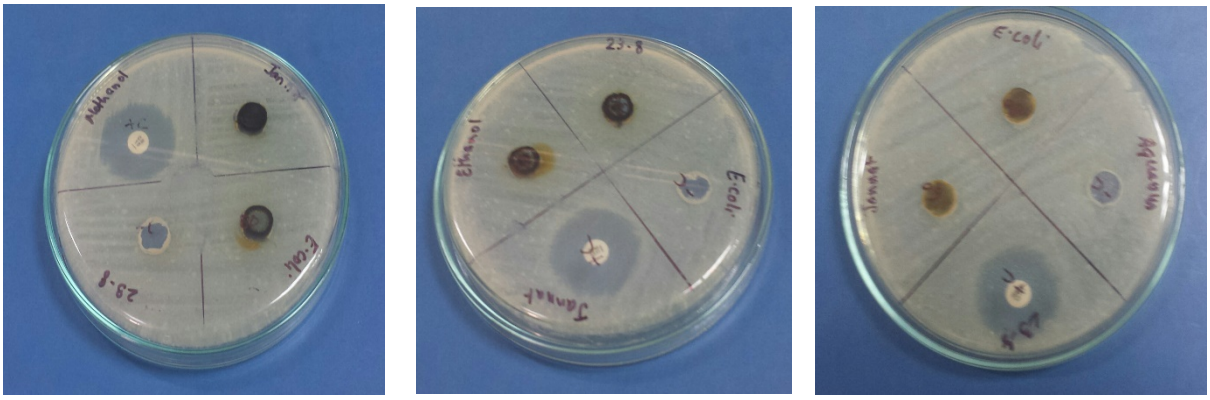


Fig 24 : Antimicrobial assay of the three extracts against *E.coli*

Bacterial Isolate	Zone (mm) for positive control	Zone (mm) for 80µg/µl	Zone (mm) for 60µg/µl
Enterococcus faecalis	21 mm	18 mm	0 mm
Bacillus subtilis	20 mm	15 mm	15 mm
Staphyloccusaureus	22 mm	19 mm	17 mm
Escherichia coli	20 mm	0 mm	0 mm

Table 5 : Measure (mm) for each zone formed for methanolic extract

Bacterial Isolate	Zone (mm) for positive control	Zone (mm) for 80µg/µl	Zone (mm) for 60µg/µl
Enterococcus faecalis	21 mm	0 mm	8 mm
Bacillus subtilis	20 mm	0 mm	0 mm
Staphylococcus aureus	20 mm	0 mm	0 mm
Escherichia coli	20 mm	0 mm	0 mm

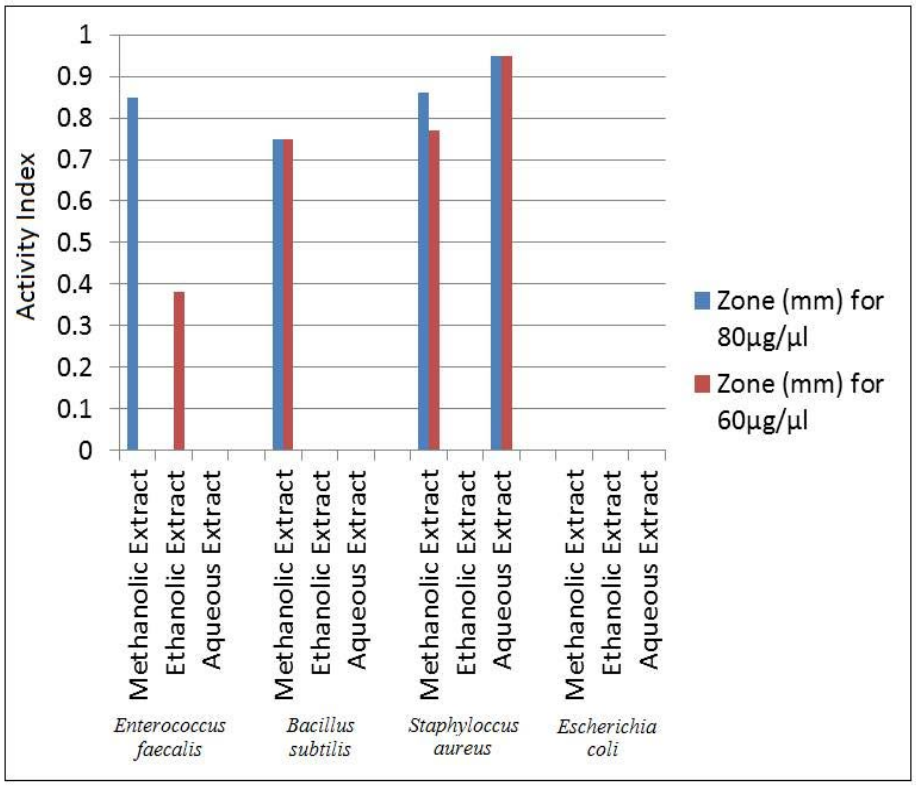
Table 6 : Measure (mm) for each zone formed for ethanolic extract

Bacterial Isolate	Zone (mm) for positive control	Zone (mm) for 80µg/µl	Zone (mm) for 60µg/µl
Enterococcus faecalis	21 mm	0 mm	0 mm
Bacillus subtilis	20 mm	0 mm	0 mm
Staphyloccusaureus	22 mm	21 mm	21 mm
Escherichia coli	20 mm	0 mm	0 mm

Table 7 : Measure (mm) for each zone formed for aqueous extract

Bacterial isolate	Methanolic extract		Ethanolicextract		Aqueous extract	
	80µg/µl	60µg/µl	80µg/µl	60µg/µl	80µg/l	60µg/µl
Enterococcus faecalis	0.85	0	0	0.38	0	0
Bacillus subtilis	0.75	0.75	0	0	0	0
Staphylococcus aureus	0.86	0.77	0	0	0.95	0.95
Escherichia coli	0	0	0	0	0	0

Table 8: Activity Index of the extracts collected from *Spirulina platensis*



Bar Graph 2: Graphical representation of the activity index of *Spirulina platensis*

CHAPTER 3
DISCUSSION

Chemical substances that produce a definite physiological action on the human body decides the medicinal values of plants of which the most important bioactive compounds of plants are alkaloids, flavonoids, tannins and phenolic compounds. Many plant leaves have antimicrobial principles such as tannins, essential oils and other aromatic compounds. In addition, many biological activities and antibacterial effects have been reported for plant tannins and flavonoids. The present work deals with the collection of three different types of extract (methanol, ethanol and water) and the biochemical assay to find the presence of various phytochemicals including alkaloids, steroids, phenols, flavonoids, tannins, saponins, cardiac glycosides and glycosides in *Spirulina platensis.*

The selected plant sample was collected and extraction was done using a Soxhlet apparatus. The highest amount of extract collected was from ethanol. The study also reported the antimicrobial activity of *Spirulina platensis* against common pathogenic organisms such as *Bacillus subtilis, Staphylococcus aureus, Enterococcus faecalis, and Escherichia coli*. The phytochemical assay results were obtained from the presence of phytoconstituents in all three extracts. For the alkaloid detection, Dragendroff's reagent test was carried out and it was found out moderate amount of it was present in ethanolic extract compared to methanolic extract. However, in another experiment carried out by Al-Rekabi (2007) on *Spirulina maxima*, it was found out that both alcoholic and aqueous extracts gave positive results for alkaloids. For the saponins detection, Foam test was carried out and it was found out that methanolic ,ethanolic and aqueous extract contains abundant amount of saponins. This test is similar to the test carried out by Islam et al (2012). For the steroids detection, Salkaowaski's test was carried out and it was found out that methanolic , ethanolic and aqueous extract contains abundant amount of steroids. According to Shalaby (2013), only methanolic extract gave positive results for steroids. For the tannins detection, Gelatin test was carried out and it was found out that methanolic ,ethanolic and aqueous extract contains abundant amount of tannins. However, there were negative results for tannins in the experiments carried out by Sudha et al (2011) but both alcoholic and aqueous extracts gave positive results for Tannins in the experiment carried out by Al-Rekabi (2007). For the flavonoids detection, Sodium hydroxide test was carried out and it was found out that, ethanolic extract contains moderate amount of flavonoids compared to methanolic and

aqueous extract. According to Shalaby (2013), methanolic extract had good amount of flavonoids. For the phenol detection , Ferric chloride and Potassium ferrocyanide test was carried out and it was found out that methanolic , ethanolic and aqueous extract contains abundant amount of phenol which is similar to the results of alcoholic and aqueous extracts of *Spirulina maxima* carried out in an experiment by Al-Rekabi (2007) For the cardiac glycosides detection, Keller Killanis test was carried out and it was found out that , ethanolic and aqueous extract contains moderate amount of cardiac glycosides. According to Shalaby (2013), this test is similar in results. For the glycosides detection, Keller Killanis test was carried out and it was found out that, all three extracts contain rich amount of glycosides. This study is similar to the test carried out by Arun et al (2012).

The results obtained also showed the antimicrobial properties of *Spirulina platensis*. Agar diffusion method was followed and the zones of inhibition were measured in millimeters. Kanamycin, Vancomycin and Amoxicillin were used as positive controls. The largest clear zone was seen in *Staphylococcus aureus*.

For *Bacillus subtilis* the clear zones were 15 mm at concentrations of 80µg/ µl and 60 µg/ µl for methanolic extract.In another experiment carried out by El-Sheekh (2014) *Bacillus subtilis* showed sensitive nature when carried out in 95% ethanol and other solvents.

Clear zones were found for *Staphylococcus aureus* at measure of 19 mm and 17 mm at 80µg/ µl and 60 µg/ µl for methanolic extracts and clear zones were observed at 21 mm in aqueous extract at 80µg/ µl and 60µg/ µl concentrations. These results are similar to the tests carried out by Arun et al (2012) where they found 35% sensitivity to methanolic extracts of *Spirulina platensis*. Moreover *Spirulina platensis* exhibited significant activity against *Staphylococcus aureus* in an experiment carried out by Shaieb et al (2014). In case of *Enterococcus faecalis* , clear zone was observed at 18 mm at 80µg/ µl for methanolic extract and 8 mm at 60µg/ µl for aqueous extract. However in an experiment carried out by Baz et al (2013) showed*Enterococcus faecalis* was affected only by the ethanolic extract of *Spirulinaplatensis.*In case of *Escherichia coli* , no zone of inhibitions were found in any of the three extracts at none of the concentrations. However for *Escherichia coli*, 11 mm zones were found in experiment carried out by Sudha et al (2011) and *Spirulina platensis* exhibited

significant activity against *Escherichia coli* in an experiment carried out by. Shaieb et al (2014). In another experiment carried out by Baz et al (2013) there was no inhibition zones with *Escherichia coli* for ethanolic extracts as well.

Overall, the results of antibacterial activity showed that *Spirulina platensis* is most effective against *Staphyloccus aureus* and *Enterococcus faecalis* in this experiment.

The different antimicrobial activities of *Spirulina platensis* could be attributed to various types of compounds belonging to a diverse range of chemical classes. The reason behind antimicrobial activity found in *Spirulina platensis* extracts could be due to presence of γ-linolenic acid , active fatty acid , synergetic effect of lauric and palmitoleric acid . However, the test microorganisms differ significantly in relation to their susceptibility to *Spirulina platensis* antimicrobial substances. Gram positive bacteria like *Enterococcus faecalis* and *Staphylococcus aureus* were more sensitive than the Gram negative bacteria. This may be ascribed to the way that cell wall in Gram positive bacteria consists of a single layer, whereas Gram negative bacterial cell wall is multilayered structure bounded by an outer cell membrane and or due to the permeability barrier provided by the cell wall or to the membrane accumulation mechanism. Then again, some bacterial species did not react to concentrates of *Spirulina platensis* .This might be due to the antibacterial activity masking by the presence of some inhibitory compounds in the extracts which were similar to the observations made by Sastry&Rao. The extraction procedure was carried out in a different way that is directly accumulating the extracts through Soxhlet apparatus. Had further steps been taken similar to Hossain et al (2013), may be positive results for all the expected bacterial isolates could be obtained.

CHAPTER 4

CONCLUSION

The extracts of *Spirulina* have been subjected to various pharmacological, clinical and toxicological investigations and results revealed interesting therapeutic applications throughout years. In addition there has been an immense interest in utilization of natural plant extracts as antimicrobial activity due to the increase in outbreak of food borne diseases and to minimize the health causing diseases over synthetic drugs (Gupta et al, 2009). The purpose of this study was to collect crude extracts and investigate the presence of phytoconstituents in *Spirulina platensis* that serves as an affect agent to treat many infectious diseases. The primary phytochemical analysis of ethanolic and methanolic extracts revealed the presence of alkaloids, flavonoids, saponins, tannins, steroids, phenols, cardiac glycosides and glycosides. Alkaloids, flavonoids and cardiac glycosides are present in lesser amount which could be responsible for the observed antimicrobial properties. Antibacterial tests show that the plant extracts may be used effectively as an antibiotic agent against microorganisms such as *Enterococcus faecalis* and *Staphylococcus aureus.* The isolation, identification and purification of phytoconstituents and determining their respective antibacterial potencies to evaluate and formulate chemotherapeutic agents could be the future frontier for this investigation. From the present study it can drawn to conclusion that the traditional use of the plant *Spirulina platensis* for infectious diseases is promising against many bacteria and disease causing pathogens. If further research is pursued in this field , then quantitative assays such as High Performance Liquid Chromatography (HPLC) can be performed as well as novel antibiotics can be developed. Moreover , antimicrobial assay can be applied to animal models to perform sensitivity tests .

REFERENCES

Cohen, Z., Reungjitchachawali, M., Siandung, W. and Tanticaroen, M.,(1993). Production and partial purification γ-linolenic acid and some pigments from *Spirulina platensis*. J. Appl. Phycol., 5:109-115.

Crasta, P.J., Raviraja, N.S. and Sridhar, K.R., (1997).Antimicrobial activity of some marine algae of southwest coast of India. Indian J. Mar. Sci., 26: 201-205.

Herrero, M., Simo, C., Ibanez, E. and Cifuentes, A., (2005). Capillary electrophorsis-mass spectrometry *Spirulina platensis* obtained by pressurized liquid extraction. Electrphoresis.,26:4215-4224

Kaushik, P. and Chauhan, A.,(2008). In vitroantibacterial activity of laboratory grown culture of *Spirulina platensis*. Ind. J. Microbiol.,48: 348-352

Khairy, H.H. and El-Kassas, H.Y. (2010).Active substances from some blue green algal species used as antimicrobial agents. Afr. J. Biotech., 9(9):2789-2800.

Medina-Jaritz, N.B., Perez-Solis, D.R., Ruiloba de Leon, F. and Olvera-Ramirez, R., (2011). Antimicrobial activity of aqueous and Methanolic extracts from *Arthrospira maxima*. Formatex.,1267-1271

Kumudha, A., Kumar, S.S., Thakur, M.S., Ravishankar, G.A. and Sarada, R., (2010). Purification, identification and characterization of methycobalamine from *Spirulina platensis*.J.Agri. Food. Chem.,58(18): 9925-9930.

Santoyo, S., Herrero, M., Javier, F., Cifuentes, A., Ibanez ,E. and Jaime, L., (2006). Functional characterization of pressurized liquid extracts of *Spirulina platensis*. Eur. Food Res. Technol., 224: 75-81

Sudha, S.S., Karthic, R., Rengaramunjan, J. and Athulya.,(2011) .Antimiicrobial activity of *Spirulina platensis* and Aphanothece sp. on selected clinical bacterial isolates and its antioxidant activity. South As. J. Biol. Sci.,1: 87-98

Tüney I, Cadirci BH, Ünal D, Sukatar A. Antimicrobial activities of the extracts of marine algae from the coast of Urla (Izmir, Turkey). Turk J Biol 2006; 30: 171-175

Xue C, Hu Y, Saito H, Zhang Z, Li Z, CaiY., et al. Mo- lecular species composition of glycolipids from *Spirulina platensis.* Food Chemistry 2002 ; 77: 9-13.

Aiba S, Ogawa T. Assessment of growth yield of a blue- green alga, *Spirulina platensis*, in axenic and continuous culture. J Gen Microbiol 1977; 102: 179.

.Katircioglu H, Beyatli Y, Aslim B, Yüksekdag Z, Atici T. Screening for antimicrobial agent production of some microalgae in freshwater. Internet J Microbiol 2006; 2: 2. www.ispub.com/ostia/index.php?xmlFilePath.../ ijmb/.../algae

Venkataraman, L.V. and Becker, E.W. (1985).Algal Cultivation. In: Venkataraman LV, Becker EW, (Ed.), Biotechnology and utilization of algae. The Indian experience 12-32.

Jain, S.C., 1976. Isolation of flavonoids from soapnut, SapindusemarginatusVahl.Indian J.

Pharm., 38 (6): 141.

Vonshak, A., Tomaselli, L., 2000. *Arthrospira (Spirulina)* systematics and ecophysiology. In:

Whitton B.A., Potts M., Editors. The ecology of cyanobacteria the Netherlands: Kluwer

academic publishers. regimes. Bioreso. Technol., 98: 1489–1493

Kamjunke, N., Boeing, W., Voight, H., 1997. Bacterial and primary production under

hypertrophic conditions. Aquat.Microb. Ecol., 13(1):29–35.

Borowitzka, M.A., 1995. Microalgae as sources of pharmaceuticals and other biologically

active compounds. J. Appl. Phycol., 7: 3–15.

Belay, A., Ota, Y., Miyakawa, K., Shimamatsu, H., 1993. Current knowledge on potential

health benefits of *Spirulina*. J. Appl. Phycol.., 5: 235-241

Ozdemir, G., Karabay, N.U., Dalay, C.M., Pazarbasi, B., 2004. Antibacterial activity of volatile

component and various extracts of *Spirulina platensis*. Phytother. Res. 18: 754–757.

Wendakoon, C., Calderon, P., & Gagnon, D. (2012). Evaluation of Selected Medicinal Plants Extracted in Different Ethanol Concentrations for Antibacterial Activity against Human Pathogens. Journal of Medicinally Active Plants, 1(2), 60-68

APPENDIX – I Reagents

1. **Dragendroff's Reagent** : Bismuth Nitrate solution; 8 grams Bismuth Nitrate in 12ml 30% Nitric Acid. Dissolve 27.2 gram Potassium Iodide in 50ml distilled water and put into the Bismuth Nitrate solution. Dilute with 100ml distilled water.

2. **1% Ferric Chloride**: 0.01 gram Ferric Chloride in 100ml distilled water.

3 .**1% Lead Acetate**: 0.01 gram Lead Acetate in 100ml distilled water.

4. **1% Potassium Ferrocyanide** : 0.01 gram Potassium Ferrocyanide in 100ml distilled water

APPENDIX – II Instruments

The important equipment used through the study are listed below

Equipment	Company
Autoclave	SAARC
Freeze (-20ºC)	Siemens
Incubator	SAARC
Micropipette (10-100µl)	Eppendorf, Germany
Micropipette (20-200 µl)	Eppendorf, Germany
Oven, Model :MH6548SR	LG, China
pH meter, Model: E-2010C	Shanghai Ruossua Technology Company China
Refrigerator (4ºC) Model: 0636	Samsung
Safety Cabinet Class II Microbiological	SAARC
Shaking Incubator, Model: WIS-20R	Daihan Scientific , Korea
Vortex Mixture	VWR International
Water Bath	Korea
Weighing Balance	ADAM EQUIPMENT™, United Kingdom