A DISSERTATION ON

Antioxidant and antimycobacterial efficacy of extracts from different cyanobacterial strains and their phytochemical analysis

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IN
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BY

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DECLARATION FORM

I, **Adila Salim**, a student of M.Tech Biotechnology IV Semester, Integral University have completed my six months dissertation work entitled **"Antioxidant and antimycobacterial efficacy of extracts from different cyanobacterial strains and their phytochemical analysis"** successfully from (Department of Bioengineering Integral University Lucknow) under the able guidance of **Dr. Alvina Farooqui**, **Professor and Head**, Department of Bioengineering.

I, hereby, affirm that the work has been done by me in all aspects. I have sincerely prepared this project report and the results reported in this study are genuine and authentic.

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I wish her good luck and bright future.

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DATE: **Adila Salim**

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ABBREVIATIONS

TB	Tuberculosis
M.tb	*Mycobacterium tuberculosis*
BCG	Bacillus Calmette–Guéri
IGRAs	Interferon-Gamma Release Assays
HIV	Human Immunodeficiency Virus
AIDS	Acquired Immune Deficiency Syndrome
INS	Isonicotinic acid hydrazide
HPV	Human Papillomavirus Infection
PUFAs	Polyunsaturated Fatty Acids
PBPs	Penicillin-binding proteins
PHA	Polyhydroxyalkanoates
CDW	Clinical Data Warehouse
WHO	World Health Organization
MIC	Minimal inhibitory concentration
MDR-TB	Multidrug-resistant-Tuberculosis
MDR/XDR-TB	Multidrug-resistant / extensively drug resistant TB
XDR-TB	Extensively drug resistant TB
EMB	Eosin methylene blue
DOTS	Directly observed treatment, short-course
pH	Potential of Hydrogen
UV-Vis	Ultraviolet–visible spectroscopy
EDTA	Ethylenediaminetetraacetic acid
BG11	Blue-green medium
RPM	Revolutions per minute
OD	Optical density
DMSO	Dimethyl sulfoxide
DPPH	2, 2-Diphenyl-1-picrylhydrazyl)
PSI	Pounds per square inch
REMA	Resazurin Microtitre Assay
FTIR	Fourier Transform Infrared Spectroscopy

1. INTRODUCTION

Algae are prokaryotic or eukaryotic autotrophic and heterotrophic organisms. They have a capability to convert nitrogen and phosphorus from the environment for their growth with the help of light, CO_2, and water. The resulting biomass can then be fractionated into various bioproducts (Omett *et al*., 2014). Algae can exist as mixotrophs or facultative heterotrophs as well as using sunlight for photosynthesis (Li-Beisson *et al*., 2019). Algae have multiplereproductive strategies and can be unicellular organisms or have complex multicellularity (Brodie *et al*., 2017). Algae can be categeorized into micro and macro algae. Microalgae (microphytes) are represented by green (*Chlorophyta*), blue-green (Cyanobacteria), yellow- green (*Ochrophyta Xanthophyta*), and golden (*Ochrophyta Chrysophyta*) algae, as well as diatoms (*Bacillaryophyta*). Red algae (*Rhodophyta*), green algae (*Chlorophyta*), and brown algae (*Ochrophyta*) are all examples of macroalgae, or simple algae (Lee *et al*., 2020; Chia et al., 2018; Sudhakar *et al*., 2019; Hong *et al*., 2019; Andreeva *et al*., 2021). Algae are rich sources of biologically active compounds with antiviral, antitumor, and anti-inflammatory properties, and they are also causes for plant growth stimulators or antioxidant agents (Pirian *et al*., 2018). Two types of algae are known i.e., microalgae and macroalgae. Microalgae are tiny, 150 µm diameter, unicellular, prokaryotic algae, sometimes referred to as blue-green algae or cyanobacteria. Some of them are capable of heterotrophic and photogenic growth. They have a carbon and radiant energy metabolism, which is similar to the oxygenated photosynthesis of terrestrial plants (Wolkers *et al*., 2011). Macroalgae are eukaryotic, macroscopic, multicellular macroalgae are commonly referred to as seaweed. Species of macroalgae live in seawater, or seawater that is the lightest. Eukaryotic, macroscopic, multicellular macroalgae are commonly referred to as seaweed. The seawater that is thelightest is the habitat of the macroalgae species (Milledge *et al*., 2014) like *Chlorophyceae* (green algae), *Phaeophyceae* (brown algae), and *Rhodophyceae* (red algae). The therapeutic potential of vaccines developed by microalgae (especially C. reinhardtii) are viable options for use as vaccine carriers because they are safe and contain a single chloroplast that expresses proteins at high levels.

Vaccines (Yan *et al*., 2016; Specht and Mayfield, 2014). Therefore, the microalgae have been reported to be a viable alternative to plant hosts. In a related study, Dermutas et al. inserted the gene of the HPV16T E7 protein into the genome of the chloroplast of C. reinhardtii expressing the E7GGG protein for therapeutic vaccines. Therefore, it has been reported that the microalgae represent a viable alternative to plant hosts (Demurtas *et al*., 2013). Nowadays, most monoclonal antibodies are produced in Chinese hamster ovary cell lines and are very expensive and carry the risk of contamination with human pathogens. Because of their advantages, microalgae are considered effective alternative host cells. These eukaryotic microorganisms are superior to bacterial cells in post-translational modification of human recombinant proteins. In addition, they are preferred over other eukaryotic hosts because of their high growth rate, convenience of handling, and ease of cultivation. In a study produced a monoclonal IgG antibody in engineered Phaeodactylum tricornutum against the Marburg virus nucleoprotein, which is a major cause of hemorrhagic fever in West Africa (Hempel *et al*., 2017; Fu *et al*., 2016). Tuberculosis (TB) is the most common human infectious disease, causing morbidity and numerous deaths world-wide. Tuberculosis (Accessed on March 03, 2023) *Mycobacterium tuberculosis* a bacterium, is the principal cause of this dangerous illness. Tuberculosis primarily affects the lungs, but can also affect the central nervous, lymphatic, andcirculatory systems (Kumar *et al*., 2007; Golden and Vikram, 2005). The process of diagnosing active contagious tuberculosis primarily involves radiological techniques, such as chest X-rays, but also includes microscopic examination and microbiologyical cultures of bodyfluids, such as multiple sputum cultures. The process of diagnosing active contagious tuberculosis primarily involves radiological techniques such as chest x-rays. This also includes microscopic examination and microbiological culture of body fluids such as blood vessels. B. several sputum cultures .For latent tuberculosis, but in which the bacteria present in the body remain dormant and do not cause symptoms, diagnosis is based on the Mantoux tuberculin skin test and/or interferon gamma release tests (IGRAs) of blood samples (Jacob *et al*., 2009). Prevention of TB mainly includes screening programs and vaccination such as Bacillus Calmette–Guérin (BCG) (Mainous and Pomeroy, 2012). According to WHO reports -

1.6 million Persons worldwide (including 187 000 HIV-positive individuals) died from TB in 2021. TB is the second most lethal infectiousdisease in the world, after COVID-19, and is the 13th greatest cause of death globally (behind HIV and AIDS).About 10.6 million tuberculosis (TB) infections were reported worldwide in 2021. 6,400,000 males, 3,400,000 women, and 1.2,000,000 kids. TB affects people of all ages and from all countries. TB, however, is treatable and preventable.The pathogen of *Mycobacterium tuberculosis*, a small aerobic, immotile bacillus, is the main cause of tuberculosis (Niederweis *et al*., 2010). Mycobacterial outer membranes: in search of proteins (Nicas *et al*., 2005). *Mycobacterium tuberculosis* and its very closely related seven species of mycobacteria (together form the classic *Mycobacterium tuberculosis* complex (Niederweis *et al*., 2010).The infectious dose of tuberculosis is very small and inhaling less than 10 bacteria can cause infection (Madison, 2001; Nical *et al*., 2005).The drug used for tuberculosis is Isoniazid (INH) is an antibiotic used for the initial treatment of active *Mycobacterium tuberculosis* (TB) infection. Infection Active TB infection may present with fever, chills, night sweats, coughs, hemoptysis, and/or weight loss. There are four CDC-recommended multidrug therapies that include INH for drug- sensitive strains .Strains The therapies consist of an initial phase of 2 months followed by a continuation phase of either 4 or 7 months (Blumberg *et al* .,2003).Isoniazid can also be used to treat latent tuberculosis infection, but rifampin-based therapies have recently replaced isoniazid as a recommendation. Isoniazid can also be used to treat latent tuberculosis infection, but rifampin-based therapies have recently replaced isoniazid as a recommendation. Rifampin-based therapies have shown similar efficacy with shorter treatment durations and better completion rates. Rifampin-based therapies have shown similar efficacy with shorter treatment durations and better completion rates (Huaman and Sterling, 2019). The mechanism of action of Isoniazid has been the main drug used to treat tuberculosis since 1952. It is a prodrug activated by catalase peroxidase KatG, which generates a variety of radicals and adducts that inhibit the production of the mycolic acids that make up the *Mycobacterium* cell wall. This activity makes INH a potent bactericidal agent. It also appears to work synergistically

With other species produced by KatG and other drugs used to treat tuberculosis (Chakraborty and Rhee, 2015). However, mutations in the genes katG, inhA, kasA and ahpC can lead to resistance to isoniazid therapy. This resistance develops more rapidly in *Mycobacterium tuberculosis* when treated with isoniazid monotherapy alone. The present study was designed to elucidate the antioxidant and antimycobacterial potential of extracts from different cyanobacterial strains. The results will enable the researchers and scientist to explore the therapeutic potential of cyanobacterial extract towards the development of drugs against tuberculosis.

OBJECTIVES

1. Screening of cyanobacterial strains.

2. To check phytochemical property and antioxidant activity of selected cyanobacterialstrains.

3. To elucidate the antimycobacterial activity of cyanobacterial strains.

2. REVIEW OF LITERATURE

2.1 Cyanobacteria

Cyanobacteria come from the color of the bacteria. In 1995 Edondson discovered cyanobacteria in the lake and Stainer first classified cyanobacteria in 1997 as Earth's oldest organism (fossil record - 3.5 billion years). Cyanobacteria are the only bacteria that can photosynthesize using light energy. Cyanobacteria are oxygenated photosynthetic prokaryotes that are found in soil and some of them can fix themselves in atmospheric nitrogen (Malik *et al*., 2001; Philipose, 1960; Vijaykumar *et al*., 2005; Muthukumar *et al*., 2007).

2.1.1 Classification of cyanobacteria

Kingdom: Monera

Division:Eubacteria

Class:cyanobacteria

Examples- Nostoc *sp., Spirulina sp., Azolla sp.* (Song *et al*., 2005)Cyanobacteria play an important role in maintaining and building up soil fertility, increasing temperature, biosorbent dosage and size, initial solute concentration, etc. (Vijayaragavan, 2008). Cyanobacteria, commonly known as blue, green, algae, are not actually eukaryotic algae. They are gram-negative prokaryotes, perform oxygenic photosynthesis and also fix atmospheric nitrogen.They are ubiquitous in ponds, lakes, streams, rivers and wetlands. They can easily live in harsh conditions such as hot springs, hypersaline water, cold temperatures, and arid deserts (Singh, 2014). Cyanobacteria can survive in a temperature range of 45-70 degrees Celsius (Castenholz, 1978) and a pH of less than 4-5 (Pfennig, 1969, 1974) with an optimal range of 7.5-10 (Fogg, 1956). Cyanobacteria induce soil aggregation and water permeability and are very useful in improving the quality of poorly structured soils in arid or subarid areas (Rogers and Burns, 1994). Cyanobacteria produce a variety of biologically active compounds with antibacterial, antifungal, antialgal, and antiviral potential (Teuscher *et al*., 1992; Dahms *et al*., 2006). Cyanobacteria produce a wide range of anti-algal compounds that inhibit the growth of pathogens by interfering with their metabolic and physiological activities (Dahms *et al*., 2006). Cyanobacteria have the ability to remediate a wide range of environmental toxins, including pesticides (Megharaj *et al*., 1994) crude oil (Sokhoh *et al*., 1992; Al-Hasan *et al*., 1998), and sewage naphthalene (Cerniglia *et al*., 1980a, b)

phenanthrene (Narro *et al*., 1992), phenol and catechol (Shashirekha *et al*., 1997), heavy metals (Singh *et al*., 2011b) and xenobiotics (Megharaj *et al*., 1987) either by accumulation or degradation.Some species of cyanobacteria survive in mixotrophic and heterotrophic conditions and as consortia with other microorganisms (Subashchandrabose *et al*., 2011) The production of cyanotoxins, such as microcystins, anatoxins, and saxitoxins, as well as other bioactive substances by cyanobacteria is also well documented (Kurmayer *et al*., 2016). Cyanobacteria can be employed as natural biofertilizers and contribute to higher production in a variety of agricultural and environmental circumstances (Song and Mårtensson *et al*., 2005). Cyanobacteria produce a variety of protein biomolecules such as phycobiliproteins, phycoerythrin, phycocyanin, and allopycocyanin, which are used as coloring pigments in foods, cosmetics, and as fluorescers in clinical or research centers (Parmar *et al*., 2016).Cyanobacteria can be used in a variety of applications including biopolymers and biodegradable plastics (Philip *et al*., 2020) medicinal and bioactive substances (Singh *et al*., 2005). Algae fall into three primary classes i.e. *Chlorophyceae*, *Phaeophyceae* and *Rhodophyceae* (Table 1).

Table 1: Classes of algae and its characteristics

Characteristics	***Chlorophyceae***	***Phaeophyceae***	***Rhodophyceae***
Habitat	Freshwater, brackish water and saltwater	Freshwater (rare), brackish and Saltwater	Freshwater, brackish and saltwater (rare)
Major pigment	Chlorophyll a, and Chlorophyllb	Chlorophylla, Chlorophyllc, Fucoxanthin	Chlorophyll a, Chlorophyll d, Phycoerythrin, Phycocyanin.

Stored food	Starch, pyrenoids, and oil droplets	Laminarin and Mannitol	Floridean starch
Cell wall	Cellulose	Phycocolloids, alginic acid	Cellulose, Phycocolloids
Flagella	2−82−8 equal	22 unequal	Absent
Vegetative Reproduction	Fragmentation and cell Division	Budding	Fragmentation
Asexual Reproduction	Zoospores and Aplanospores	Zoospores	Aplanospores
Sexual Reproduction	Isogamous, Anisogamous, and Oogamous	Isogamous, Anisogamous, and Oogamous	Oogamous. Male sex organ: Spermatogonium Malegamete: Spermatium Female sex organ: Carpogonium which produces egg. After fertilisation, carposporophyte is formed, which Produces carpospores.
Examples	*Chlamydomonas, Spirog yra, Chlorella, Ulothrix, Volvox*, and others.	*Dictyota, Laminaria, Sargassum, Fucus*, etc.	*Porphyra, Gracilaria, Gelidium, Polysiphonia*, and others.

2.2. Applications of cyanobacteria in various fields

2.2.1. Pharmaceutical

Studies on biomedical natural products have so far focused only on *cyanophyta* (blue- green algae) and *pyrrophyta* (dinoflagellates); most metabolites have been isolated from cyanobacteria (Moore, 1996; Beltron and Nielan, 2000). Cyanobacteria are considered to be a rich source of secondary metabolites with potential biotechnological applications in the pharmacological field. Recently, the production of bioactive compounds with commercial and medicinal applications has also increased interest in the study of these organisms. Natural products from marine cyanobacteria are used for drug discovery. Cyanobacterial metabolites exhibit interesting and exciting biological activities including antimicrobial immunosuppressive, anticancer, anti-HIV, antibacterial, anticoagulant, antifungal, anti-inflammatory, anti-Malaria, antiprotozoal, antituberculosis, antiviral, antitumor activities (Gademann and Portmann, 2008). The Anticancer Drugs from Cyanobacteria Are *Lyngbya.Bouilloni*, The bioactive compounds of *Lyngbya.bouilloni* are Apratoxins F And G And its function Is HCT-116 Colon Cancer Cells (Leusch H *Et Al*., 2001).The bioactive compounds of *Lyngbya.majuscula* Aurilide band its function is h-460 lung cancer (Nogle *et al*.,

2005) and the bioactive compound *Symloca* sp. is Belamide a and its function is hct-116 colon cancer (Macmillan *et al*., 1538). The effects of bioactive substances in the body may promote good health. They are being researched for the purpose of preventing diseases including heart disease, cancer, and others.

Table2: Cyanobacterial bioactive compounds and their activity.

S. No .	COMPOUNDS	CLASS	SOURCES	ACTIVITY	REFERENCE
1	Polysaccharides		Chlorella	Moisturizing andthickener Agent	Jain *et al*.,2005
2	Methanolic extracts of exopolysaccharides		Arthrospira platensis	Antioxidant	Raposo *et al*.,2015
3	Chrysolaminarin		Odontellaaurita	Antioxidant	Xia *et al*.,2014
4	Sulfated polysaccharides		Porphyridium and Rhodella reticulata	Antioxidant	Raposo *et al*.,2015
5	Beta- 1,3- Glucan		Chlorella Skeletonema Porphyridium Nostoc flegelliforme	Free-radical Collector Immune System	Spolaore *et al.,*2006; Koller *et al*., 2014.
6	Beta- carotenes		Dunaliella salina	Antioxidant	Hamed,2016
7	Asthaxanthin		Haematococuspluvialis	Antioxidant Sunscreen protection	Hamed,2016 Koller *et al*.,2014
8	Phycocyanobilin		Spirulina	Antioxidant	Hamed, 2016
9	Phycoerythrobilin		Porphyridium	Antioxidant Pigment for eye-liner and Lipsticks	Hamed,2016
10	Sulfolipid		Lyngbyalagerheimii , Phormidium tenue	Anti HIV-1 activity	Skulberg (2000)
11	Barbamide		Lyngbyamajuscula	Antumollusci icidal	Chang (2004)
12	Antillatoxin B		Majuscule	Neurotoxic Ichthyotoxic, activator of voltage-gated sodium Channel	Yokokawa *et al*., (2000)
13	Didemnin		Synechocystistrididemni	Anti-cancer Anti-viral Immunosupp Resive	Mitchell *et al*., (2000)

Cyanobacteria are a source of multiple secondary metabolites/bioactive compounds (i.e. PUFAs, PBPs, cartenoids and various enzymes, vitamins (Ghosh *et al*., 2016) and have great potential to be used as pharmacological agents in various diseases (Dunalp *et al*., 2007; Gademann and Portmann, 2008; Tan, 2007).

2.2.2. Applications of cyanobacteria in cosmetics

Cyanobacteria produce components that could be of interest for the cosmetic industry(personal care products) (Borowitzka, 1999).Cosmetics are products aimed at improving the structure, morphology and appearance of the skin, with the help of auxiliary substances and active ingredients adapted to different skin types (normal, oily, combination, sensitive, etc).Cosmeceuticals, although a term that is not officially recognized, are defined as cosmetic products containing biological ingredients that are said to have medicinal or drug- like benefits (Mourelle *et al*., 2015).

2.2.3. Biotechnological application of cyanobacteria

Other uses of cyanobacteria extracts include their use in scientific research experiments. For example, phycobiliproteins have fluorescent properties that can be used for flow cytometry and in immunoassay techniques (Amadu *et al*., 2021).These bioactive compounds include terpenoids such as terpenes, diterpenes, and sesquiterpenes.These organic compounds are widespread in cyanobacteria and used as natural ingredients in flavors and perfumes.Such applications are recently gaining ground in the therapeutic and pesticide industries (Singh *et al*., 2017).The rapid development of molecular tools for whole genome sequences encourages the use of omics technologies, i.e.; H. Transcriptomics, proteomics, and systems biology approaches to manipulate metabolicpathways to produce valuable products Among these, the most widely used fluorescent probe is phycoerythrin, used in biomedical research (Singh *et al*., 2020). Microorganisms have been found to contain a variety of storage substances, such as lipid, polyphosphate, sulfur polyamino acids, and glycogen. Polyhydroxyalkanoates (PHAs) are lipids that a wide range of microbes accumulate in the presence of ample carbon sources (Anderson and Dawes, 1990). PHA is a crystalline thermoplastic with properties comparable to polypropylene (Doi, 1990).PHA is also a biocompatible material and is being studied for its

application in the biomedical and biopharmaceutical field .The PHA granules can be stained with Nile blue a (Ostle and Holt, 1982). According to many studies (Vincenzini *et al*., 1990; Stal 1992; Arino *et al*., 1995; Carr 1996), the majority of known cyanobacteria that can manufacture PHAs often accumulate PHAs in amounts that are less than 6% by weight of their cell dry weight (CDW). Poly(3-hydroxybutyrate) [P(3HB)] is the most common type of PHA synthesized by most bacteria.The production of PHA is based on a readily available and free source of carbon and energy (sunlight).

2.3. Tuberculosis

Tuberculosis (TB) is a potentially fatal lung infection caused by the bacteria *Mycobacterium tuberculosis* World Health Organization (WHO). As a result, it necessitates immediate attention. However, tuberculosis is treatable and avoidable, thus ongoing research into natural bioactive substances, particularly those derived from endophytes, is helping to find new, better-tolerated treatments for the disease. At a minimum inhibitory concentration (MIC) of 0.78 g/ml, Gliocladium sp. Polyols 3 and 4 were able to be produced by MR41 and demonstrated inhibitory effects on *Mycobacterium tuberculosis*.(Uc-Cachón et al., 2019) Phomoenamide, abyssomicin, tenuazonic acid, and phomonitroester are other substances with possible antituberculosis properties.A variety of endophytic fungi, including but not limited to Fusarium oxysporum strain (KT166447) and Colletotrichum gleosporoides strain (KT166445), were found to be present on the Glycyrrhiza glabra L. plant grown in the Kashmir Himalayas. These fungi showed strong inhibitory potentials against *Mycobacterium tuberculosis* strain H37Rv, (Shah *et al*., 2016).

The development of drug resistance to *Mycobacterium tuberculosis* is *a* major problem with anti-tuberculosis drugs. Anti-TB drugs are a double-edged sword. While initially destroying the pathogenic bacteria, they later develop resistance and become ineffective against the disease. Global surveillance of TB drug resistance has become a major threat to TB control programs in many countries (Irene and Mark, 2014). Poorly managed TB care is the primary cause of the problem known as anti-TB drug resistance. Resistance to tuberculosis drugs is a phenomenon largely due to poorly managed tuberculosis care. Problems include improper drug prescribing practices by providers, poor drug quality or irregular drug supply, and patient non-compliance with therapy. Multidrug-resistant / extensively drug-resistant. TB MDR/XDR-TB is essentially a man-made probe *Mycobacterium* MDR-TB is caused by strains of *Mycobacterium tuberculosis* that are resistant to

both rifampicin and isoniazid, with or without resistance to other drugs Extensively drug-resistant tuberculosis (XDR-TB) is a rare form of MDR-TB that is resistant to both rifampicin (RIF) and isoniazid (INH) or any other. Although TB is a major cause of morbidity around the world, little is known about how it affects people's health and quality of life (James *et al*., 2015). The mainstay of TB treatment is the use of antibiotics, which are the most efficient substances against *Mycobacterium tuberculosis* that is actively growing. The unusual structural and chemical makeup of the *Mycobacterium's* cell wall makes it difficult for drugs to enter and complicates treatment. Drugs First-line medications like isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA), and ethambutol (EMB) are often administered for a 2-month initial phase before being continued for a total of 4 months with INH and RIF. This is the most widely used conventional chemotherapeutic regimen for the treatment of TB. Due to high rates ofresistance, streptomycin, a bactericidal antibiotic that affects polypeptide synthesis, is no longer regarded as a first-line treatment (Hobby and Lenert, 1957; Dover and Geoffrey, 2011). Second line Drugs when treating TB under unique circumstances like extensively drug-resistant tuberculosis (XDR-TB) or multidrug-resistant tuberculosis (MDR-TB), second-line medications are frequently employed. Second-line drugs (SLDs) come in a variety of categories and are used to treat tuberculosis. The second-line medications differ from the first-line medications in that they could be ineffective compared to the first-line medications (such as p-aminosalicylic acid), might have harmful side effects (such as cycloserine), or might not be available in many poor nations (such as fluoroquinolones) (Dover and Geoffrey, 2011; Kolyva and Karakousis, 2012). The poor administration of chemotherapy, which makes the treatment more complicated, lengthens it, and increases its adverse effects, is a factor in drug resistance, which is particularly significant in TB. Regardless of susceptibility or resistance to other medications, *Mycobacterium tuberculosis* strains that are resistant to both isoniazid and rifampicin are considered to have multidrug resistance. Directly observed treatment, short-course (DOTS) Plus MDR-TB is concerning because it has a high risk of mortality, but DOTS Plus allows for the management of drug resistance with additional first- or second-line medications (Kaona, 2004).

3. MATERIAL AND METHODS

3.1 Materials Used

3.1.1 Instrument used

Various instruments used during the research work are in listed in table 3.

Table 3: Instruments required for the fulfillment of the research.

Instruments	Model/ make	Uses
Electronic analytical balance	Shimandzu	Measure the mass Of chemicals
Hot Air Oven	Popular traders	For sample drying
Incubator	Globel scientific technology	To maintain optimum temperature for growth
Magnetic stirrer	REMI 5MLH	Mixing fluid sample
pH meter	EI-111	For maintain the pH Of media
Refrigerator	GL-P292KDSR	To preserve reagents and extracts of Samples
Microscope	QUANTA FEG 450	Imaging the algal cells
UV-Vis spectrophotometer	CARY 100	For taking OD for growth
Laminar air flow	Ikon instrument	For staining and plating
Heating mantle	KC/STI	For biomass drying
Autoclave	STI	For media autoclave

3.1.2. Chemicals used

All of the chemicals were of the analytical variety and were obtained from HiMedia.

Table 4: Chemicals used in the research

S.No	Chemical name	Chemical Formula	Company
1.	Sodium nitrate	$NaNO_3$	HIMEDIA
2.	Potassium hydrogen phosphate	K_2HPO_4	HIMEDIA
3.	magnesium sulfate heptahydrate	$MgSO_4.7H_2O$	HIMEDIA
4.	Citric acid	$CaCl_2.2H_2O$	HIMEDIA
5.	Ammonium ferric citrate	$(NH_4)_5[Fe (C_6H_4O_7)_2]$.	HIMEDIA
6.	EDTA	$C_{10}H_{16}N_2O_8$	HIMEDIA
7.	Sodium carbonate	Na_2CO_3	HIMEDIA
8.	Trace elements	H_3BO_3	HIMEDIA
9.	Manganese(II) chloride tetrahydrate	$MnCl_2.4H_2O$	HIMEDIA
10.	Zinc sulphate	$ZnSO4.7H_2O$	HIMEDIA
11.	Sodium molybdate	$Na_2MoO_4.2H_2O$	HIMEDIA
12.	Copper(II) sulphate	$CuSO_4.5H_2O$	HIMEDIA
13.	Cobalt(II) nitrate	$Co(NO_3)_2.6H_2O$	HIMEDIA
14.	Sulfuric acid	H_2SO_4	HIMEDIA
15.	Hydrochloric Acid	HCl	HIMEDIA

3.1.3. Glasswares used

Various glass wares used during the work are in listed in table 5.

Table 5: Glassware used in the research.

Glassware	Company
100-250 mL Erlenmeyer Flasks	Borosil, India
1.0-2.0 L round bottom flasks	Borosil, India
Test Tubes	Borosil, India
Petri Plates	Borosil, India
Measuring Cylinder	Borosil, India

3.2. Methods

3.2.1. Cyanobacterial strains selection

Cyanobacteria cultures of *Plectonema boryanum*, *Anabeana* sp. *was* obtained from the Department of Botany, University of Allahabad and Department of Biological Sciences, Allahabad Agricultural Institute Deemed University, Allahabad. The strains were maintained in the cyanobacterial culture chamber at Integral University in Lucknow Department of Bioengineering.

3.2.2. Culturing and maintenance of cyanobacterial strains

The maintenance of pure culture (test organism) was done in culture room at a temperature of 25 ± 5°C. BG11 medium with pH 7.0 was used for the optimum growth of the culture (Kuhl and Lorenzen, 1964; Rippka and Herdman, 1993), under the 75µmolm^{-2}s^{-1} photosynthetic photon flux density (PPFD) with 12:12 hours of photoperiod. The manual shaking of cultures was done for two to four times a day. The growth of the selected cultures was studied by plotting growth curve**.**

3.2.3. Growth curve

The cultures were centrifuge at 8000rpm for 10 minutes at 4ºC. Pellets were then collected in a petriplate and supernatant was discarded. Afterwards the collected biomass was dried at 60ºC and was stored in an airtight glass container. The cultures were maintained at desired growth parameters and optical density (OD) was recorded at 660nm each day and a graph was plotted (Miron *et al*., 2003).

3.2.4. Biomass collection

The cultures were treated to 12 hour light and dark cycles that were alternated. The cultures were filtered and centrifuged at 8000 rpm for 10 min at 4ºC. The biomass media were collected. After those pellets were collected in a petriplate and then supernatant was discarded. The harvested biomass was then dried and kept in an airtight container (M *et al*., 2008).

3.2.1. Extraction and yield %

The extraction was done via maceration method (Ngu *et al*., 2021). (1:50 ratio) 1g of dried algal biomass in 50 ml of solvents respectively (methanol and ethyl acetate) was taken in a flask and was kept on shaker for 48 hrs at 37ºC temperature. Afterwards, the cocktail was centrifuged at 8000 rpm for 20 minutes at 4ºC. After filtration Pellet was discarded and the supernatant was dried at room temperature.

The resulted extract was collected and stored at
-20ºC. Afterwards the extraction yield% was calculated with the below given formula-

[Extraction yield% = amount of crude extract/ amount of dried biomass*100]

3.2.2. Phytochemical analysis of cyanobacterial crude extracts

The many qualitative chemical assays can be used to determine an extract's profile for its bioactive components. Using the techniques described in the literature. The produced extracts made with methanol and ethyl acetate were examined for the presence of alkaloids, saponins, tannins, steroids, flavonoids, glycosides, proteins, amino acids, and reducingsugars (Srinivasan *et al*., 2017; Pant *et al*., 2017).

3.2.2.1. Test for Alkaloids: (Dragendorff's test)

Reagents	Amount
Dragendorff's reagent	1ml

Procedure: An orange-red precipitate was produced after adding 1 mL of Dragendorff's reagent to 2 mL of extract, proving the presence of alkaloids.

3.2.2.2. Test for flavonoids (Alkaline reagent test)

Reagents	amount
Sodium hydroxide	few drops
Dilute acid	few drops

Procedure: The presence of flavonoids is shown by the test solution developing a bright yellow when a few drops of sodium hydroxide solution are added, which then turns colorless after adding a few drops of diluted acid are added.

3.2.2.3. Test for terpenoids (Salkowoski test)

Reagents	Amount
Chloroform	3ml
Sulphuric Acid	2ml

Procedure: The presence of terpenoids is shown by the development of a reddish brown coloring at the interface following the addition of strong sulfuric acid and 0.4 ml of chloroform to 0.1 g of the extract.

3.2.2.4. Test for tannins

Reagents	Amount
DMSO	2ml
Ferric chloride	few drops

Procedure: Filtered, 0.1 g of the extract was heated in 2 ml of water/DMSO before being combined with a few drops of 0.1% ferric chloride. It was then examined for a brownish green or blue-black coloring.

3.2.2.5. Test for glycosides

Reagents	Amount
Glacial acetic acid	2ml
Concentrated sulphuric acid	1ml

Procedure: The development of a brown ring at the interface after the addition of 2 ml of glacial acetic acid containing one drop of ferric chloride solution, followed by the addition of 1 ml of concentrated sulfuric acid to 0.5 mg of extract diluted with 5 ml of water, is indicative of the presence of cardiac glycosides.

3.2.2.6. Test for phenols

Reagents	Amount
Distilled water	5ml
Ferric chloride	few drops

Procedure: When 50 mg of the extract were dissolved in 5 ml of distilled water and a few drops of a neutral 5% ferric chloride solution were added, the development of a dark green hue was considered a sign that phenolic chemicals were present.

3.2.2.7. Test for carbohydrates

Reagents	Amount
Diluted iodine	2-3 drops
Distilled water	5ml

Procedure: Add 2-3 drops of the iodine reagent to 1 ml of the prescribed solution in the test tube. Wait a while if a blue, reddish purple or reddish brown color appears to indicate the presence of carbohydrates.

3.2.3. Antioxidant Assay -2, 2-diphenyl-1-picrylhydrazyl (DPPH)

Diphenyl picrylhydrazyl (DPPH), a stable free radical, is one of the most often used Techniques (Blois, 1958) devised this approach with the goal of determining the antioxidant activity similarly by employing a stable free radical called DPPH ($C_{18}H_{12}N_5O_6$, MW = 394.33).

The test is based on the assessment of the antioxidants' ability to scavengeit.

The method of Brand-Williams, Cuvelier, and Berset (1995) that was slightly modified to measure the radical scavenging activity of extracts is detailed here. To make extract solutions, 0.025 g of dry extract was dissolved in 10 ml of methanol. Even after being treated for five minutes in an ultrasonic bath, acetone and ethyl acetate extracts did not completely dissolve in methanol; as a result, only the soluble portion was used for further analysis. Every day, prior to UV measurements, a solution of DPPH in methanol (610-5 M) was produced. In disposable microcuvettes with a 1 cm path length, three ml of this solution was combined with 77 (38 or 19 in further experiments) ml of extract solution (the final mass ratio of the extracts with DPPH was around 3:1, 1.5:1, and 0.75:1). The samples were held at room temperature in the dark for 15 minutes and the reduction in absorbance was then measured. Daily preparation and measurement of the absorption of a blank sample containing the same volume of methanol and DPPH solution. Three duplicates of the experiment were performed. The following formula was used to determine the amountof radical scavenging activity:

$$\textbf{\% Inhibition} = [(A_B - A_A)/A_B] \times 100$$

Where:

A_B—absorption of blank sample (t=0 min); A_A—absorption of tested extract solution (t=15 min).

3.2.4. Antimycobacterial effect of the crude cyanobacterial extracts

3.2.4.1. Collection of *Mycobacterium smegmatis* strain

The non-pathogenic (smegmatis MC$^{2)}$ *Mycobacterium tuberculosis* strain was purchased from MTCC (Microbial Type Culture Collection and Gene Bank Chandigarh). And the strain was then maintained in our lab for further experiments.

3.2.3.1. Media preparation (Middle brook media)

Add 2.08gm middle brook media with 400 ml of distilled water and add 0.2% of 0.8ml glycerol the media also. After that pH was maintained upto 6.8 ± 2. Afterwards the media was autoclaved at 121°C, 15 PSI for 20 minutes (Finegold *et al*., 1990).

3.2.3.2. Maintenance of the *Mycobacterium smegmatis* strain (pouring streaking)

For the maintenance of the culture the strain was revived in broth and on agar plated. For broth inoculation 6 test-tubes were taken in which 5 ml of autoclaved middle brook media in each test-tube. With the help of a sterile inoculating loop the culture was inoculated in each test-tube and kept it in the incubator at 37°C for 7 days (Lembi and Waaland, 1991). For streaking method: agar was mixed in the above left Middlebrook media and autoclaved. The autoclaved agar media was poured on the sterile petril plates and kept to get solidifies. These plates were then streaked with the help of sterile loop and kept at 37°C for 7 days.

3.2.3.3. Staining of *Mycobacterium smegmatis* and microscopy

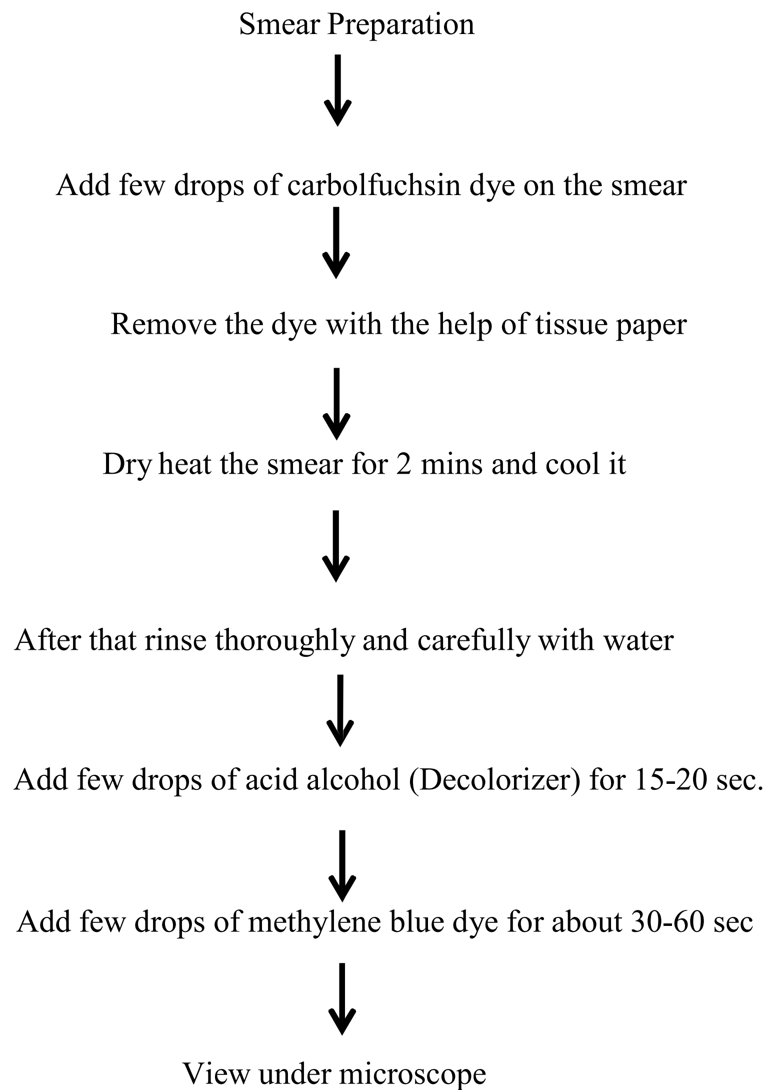
Acid Fast Staining.

This method of staining is to stain *Mycobacterium smegmatis* strains. Bacteria acquire pink colour after staining (Coherency and Berger, 2013; Fitzgerald *et al*., 2015; Murray, 2015). The procedure used is obtained from Dzodanu *et al*., 2019.

Table6: Dyes and chemicals used in acid fast staining method.

Primary dye	Carbolfuchsin	RED	RED
Decolorizer	Acid-alcohol	RED	COLORLESS
Counterstain	Methylene blue	RED	BLUE

Procedure: The procedure is given below-



3.2.3.4. Resazurin Microtitre Assay (REMA)

The REMA plate assay was performed according to (Taneja, N. K and Tyagi; J. S., 2007; Martin *et al*., 2016; Martin, 2003). A sterile flat-bottom 96-well plate 100 µl of broth in each well was used. 30µl culture was added. For every isolate, a sterile control and a growth control were also present. All perimeter wells received sterile water addition to prevent evaporation throughout the incubation. The plate was covered, bagged up, and incubated to 37 °C for 7 days. 30 µl of the resazurin solution were added to each well after the first 7 days of incubation, and the plate was then re-incubated overnight. Reduction in resazurin levels and thus a shift from blue to pink denotes bacterial growth. The least inhibitory concentration (MIC) was established as the lowest concentration of the medicine that prevented this change in color from blue to pink, which indicated the development of bacteria. When the growth control well became pink, it signified that the isolate was growing properly, and when it didn't, it meant that there were no contaminants present. The following medication concentration ranges were employed: 3.1-200 µg/ml.

4. RESULTS AND DISCUSSION

4.1. Growth curve of selected cyanobacterial strains-

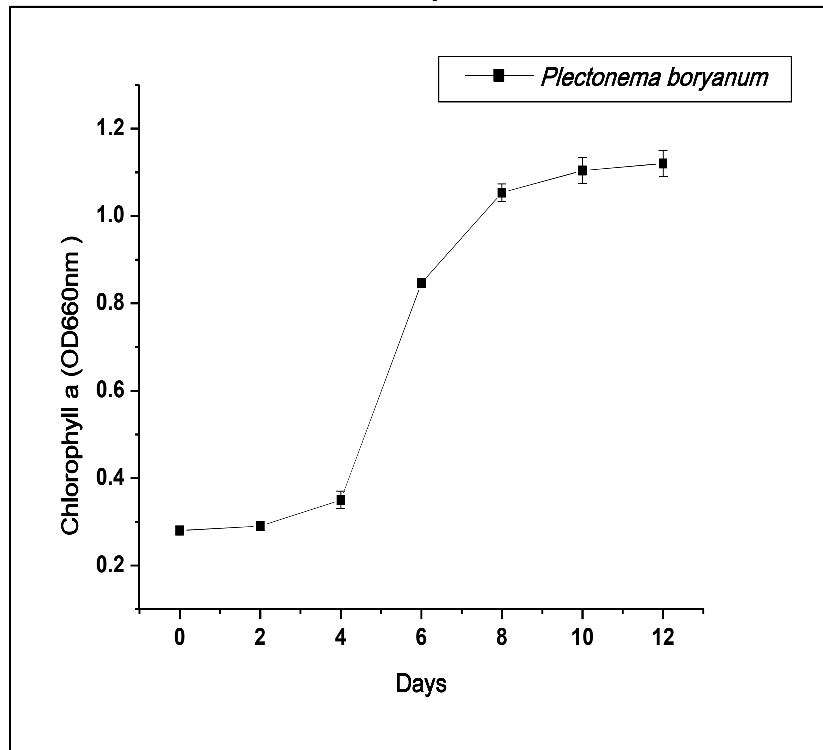


Figure 1: Growth curve of *Plectonema boryanum* at the absorbance of 660nm

The graph shown above is a growth curve of *Plectonema boryanum* at optical density (OD) 660nm which shows all growth phases. At 2nd day the log phase started and ends upon 8th day from where the stationary phase was started. The stationary phase from 8th to 12th day and after that decline phase was achieved.

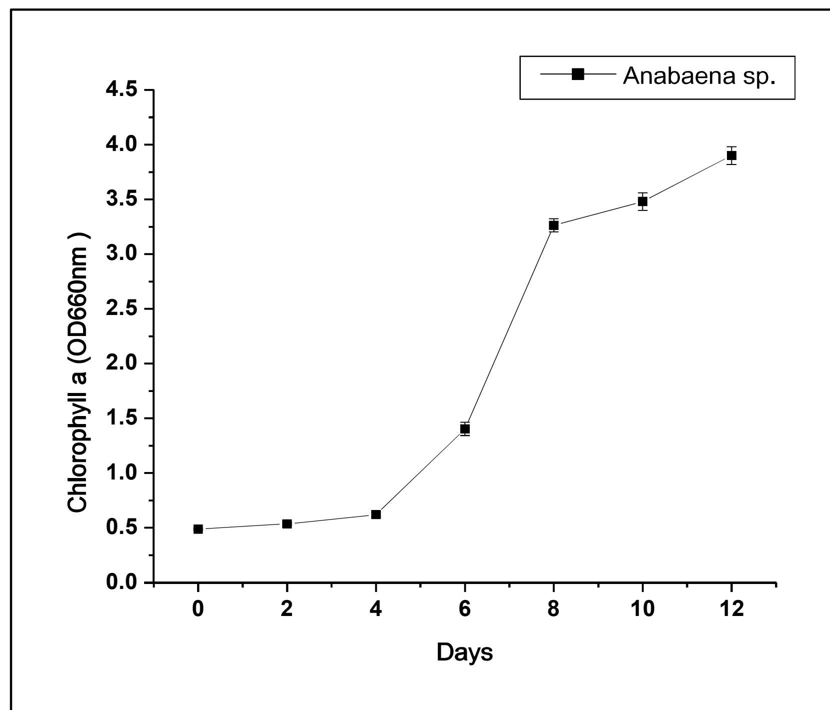


Figure 2: Growth curve of *Anabaena* sp. at the absorbance of 660nm

The graph shown above is a growth curve of *Anabaena* sp. at optical density (OD) 660nm which shows all growth phases. At 2nd day the log phase started and ends upon 8th day from where the stationary phase was started. The stationary phase is from 8th to 12th day and after that decline phase was achieved. According to Shamim *et al*., 2020 the stationary phase of the cyanobacterial strain 8 to 12 days and after that decline phase starts.

4.1.3. Biomass Collection

The results shown in the above table conclude the amount of biomass collected of both the strains (*Plectonema boryanum* and *Anabaena* sp.) suspended in 1 liter of their respective media i.e. BG-11 (Positive and Negative). The biomass collected of the *Plectonema boryanum* strain was 0.6854g/L^{-1} and from *Anabaena* sp. strain was 0.47g//L^{-1}.According to study by Yusoff, 2015; the cultivation of microalgae with nutrient source for the biodiesel production. IIRC, in the preliminary study, a correlation between the optical density of algal extracts and biomass weight was pre-determined. The microalgae biomass (g/L) collected dry weight (g/L) = 0.3793.

Table 7: Biomass collected from 1litre cyanobacterial culture

S.No.	Cyanobacterial Strain	Biomass Collected (g/l)
1.	*Plectonema boryanum*	0.6854g
2.	*Anabaena* sp.	0.47g

4.1.4 Extraction yield and yield %

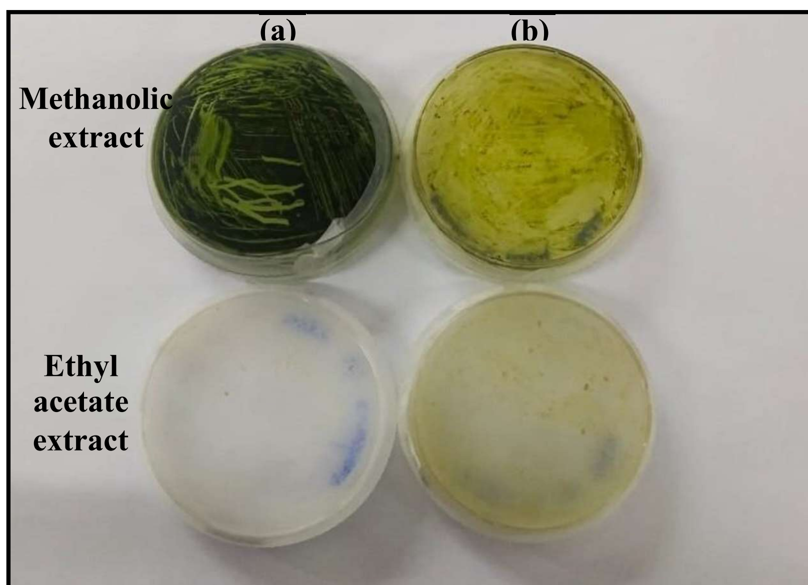


Figure 3: Methanolic and ethyl acetate extracts of *Plectonema boryanum* (a) and *Anabaena* sp. (b)

Table 8: Extraction yield of cyanobacterial strains in different solvents

S.No.	Cyanobacterial strains	Extraction method	Solvent used	Yield (%)
1	*Plectonema boryanum*	Maceration	Methanol	11.8
		Maceration	Ethyl acetate	18.1
2	*Anabaena* sp.	Maceration	Methanol	6.7
		Maceration	Ethyl acetate	5.8

Extract of *Plectonema boryanum* and *Anabaena* sp. was 0.118 g and 0.058 g. Extraction efficiencies for ethyl acetate extracts of *Plectonema boryanum and Anabaena* sp. were calculated to be 18.1 and 5.8% respectively. 11.8% and 6.7%. The extraction yield of the algal biomass is 3.4, 3.46, and 5.23 which is very much less in quantity. According to the (Ebrahimzadeh *et al*., 2018) the maximum extraction yield of microalgae ethyl acetate extract was 5.15 %. The results suggested that yield percentage of the methanolic and ethyl acetate extracts of *Plectonema boryanum* and *Anabaena* sp. The highest extraction yield was obtained in the ethyl acetate extract of *Plectonema boryanum.*

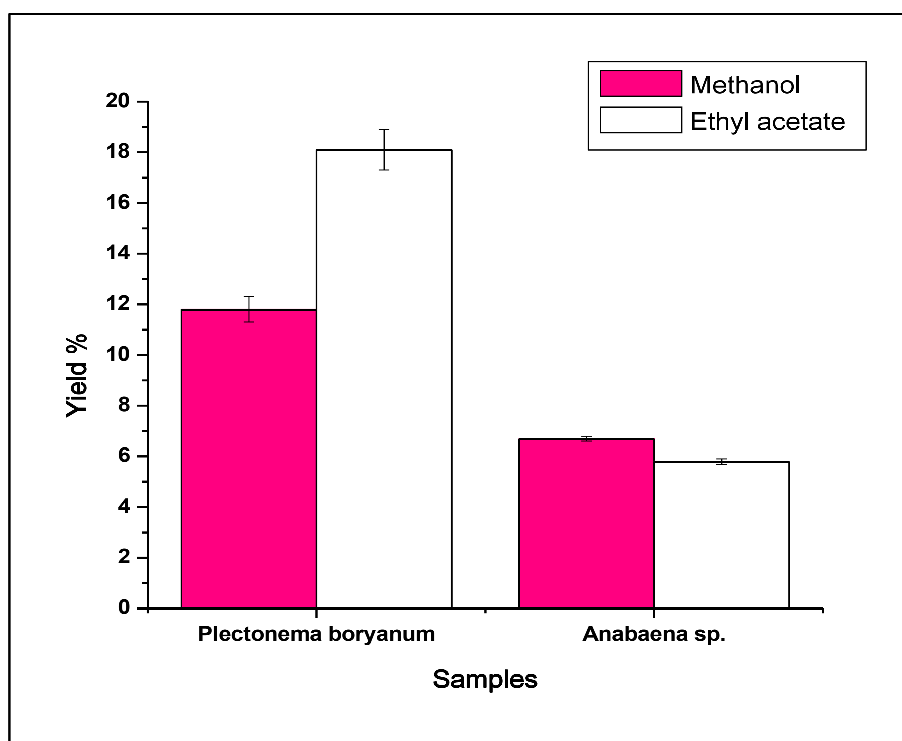


Figure 4: Percentage yield of dried crude extracts (methanolic and ethyl acetate) of *Plectonema boryanum* and *Anabaena* sp.

4.1.5. Phytochemical Test

Table 9 indicates the presence of different bioactive compounds in the cyanobacterial extracts where + represents the presence of respective bioactive compound and – represents the absence of that compound [+ denotes the presence of low intensity of colour, ++ denotes presence of moderate intensity of colour, +++ high intensity of colour and – denotes no colour change (negative results). The colour intensity denotes the concentration of the compounds.

Table 9: Phytochemical test of the cyanobacterial extracts

S.No.	**Bioactive Compounds**	***Plectonema boryanum***		***Anabaena* sp.**	
		Methanol	**Ethyl acetate**	**Methanol**	**Ethyl acetate**
1	**Tannins**	++	+	++	+
2	**Terpenoids**	+	+++	+	+++
3	**Alkaloids**	++	++	+	++
4	**Flavanoids**	+	+	++	+
5	**Glycoside**	+	+	+	+
6	**Phenolic compound**	++	+	++	+
7	**Carbohydrate**	-	+	-	+

Similarly in a study, the presence of common phytochemical constituents such as alkaloids, tannins, saponins, terpenoids, steroids, phenolic compounds and glycosides was tested qualitatively as per the methodology of Ahmad and Beg (2001). The bioactive compounds found in the extract of microalgae have alkaloids, terpenoids, steroids, phenolic compounds and glycosides. While in ethyl acetate extract there is absence of alkaloids.

4.1.6. Fourier transforms infrared analysis (FTIR)

Figure 5: FTIR analysis of methanol extract of *Plectonema boryanum*

Figure 6: FTIR analysis of ethyl acetate extract of *Plectonema boryanum*

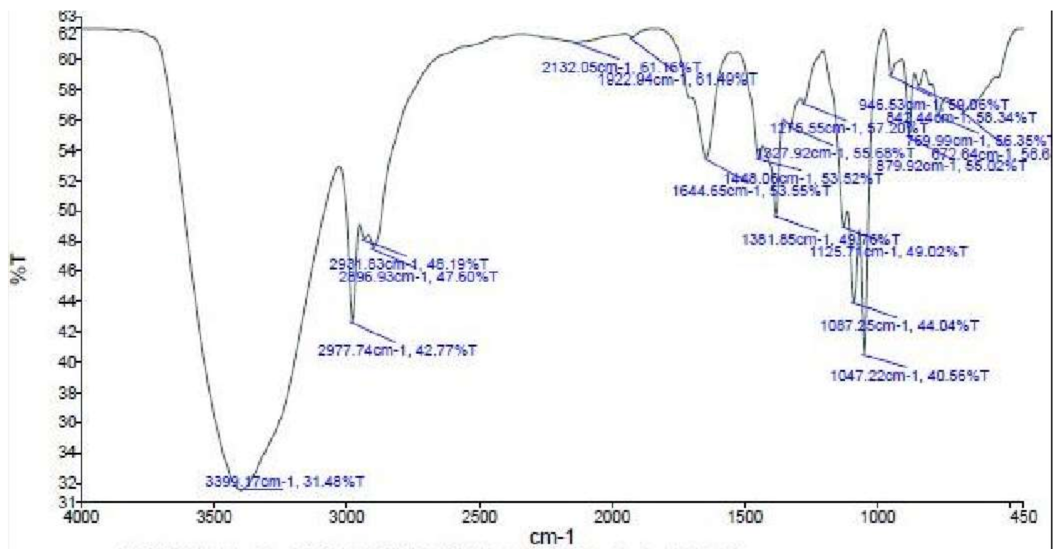


Figure 7: FTIR analysis of methanol extract of *Anabaena* sp.

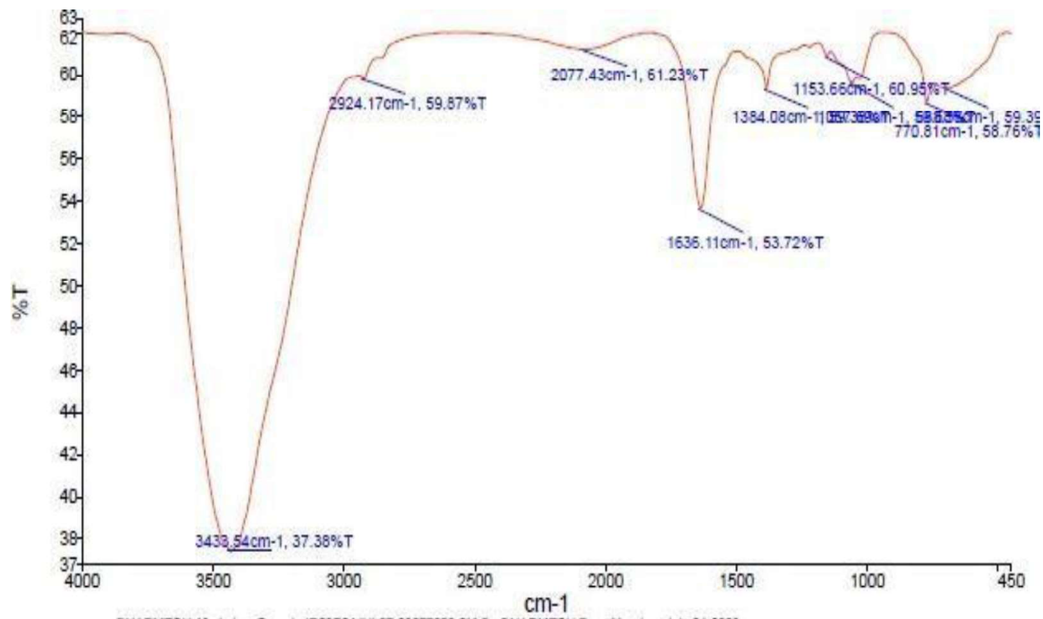


Figure 8: FTIR analysis of ethyl acetate extract of *Anabaena* sp.

S.No	Wavelength	Functional groups
1	3000-3600	-OH
2	2500-3000	$-O-CH_3$
3	1400-1700	N-H

Table 10: Interpretation of FTIR results

4.1.7 DPPH Assay

In the present work we observed pronounced antioxidant activity in a crude extract of cyanobacteria *Plectonema boryanum* and *Anabaena* sp.*).* Similar to this abd El-Baky and Athukorela et al 2008 reported for the antioxidant potential is well documented. Ethyl acetate extract of displayed *Plectonema boryanum* greater antioxidant potential (31.5% inhibition of (DPPH) than the positive control Ascorbic acid (28% inhibition) at 20 μgml^{-1} followed by methanol extract of *Anabaena* sp. (25%).

Table 11: Antioxidant activity and % inhibition of extracts of Cyanobacterial strains

S.NO.	Cyanobacterial strains	Solvents	%inhibition
1.	*Plectonema boryanum*	Methanol	28
		Ethyl acetate	31.5
2.	*Anabaena* sp.	Methanol	25
		Ethyl acetate	30.1

4.1.3 Antimycobacterial activity

4.1.3.1 *Mycobacterium smegmatis* colonies

Growth of *Mycobacterium smegmatis* in Middle-Brook media (Figure 11)

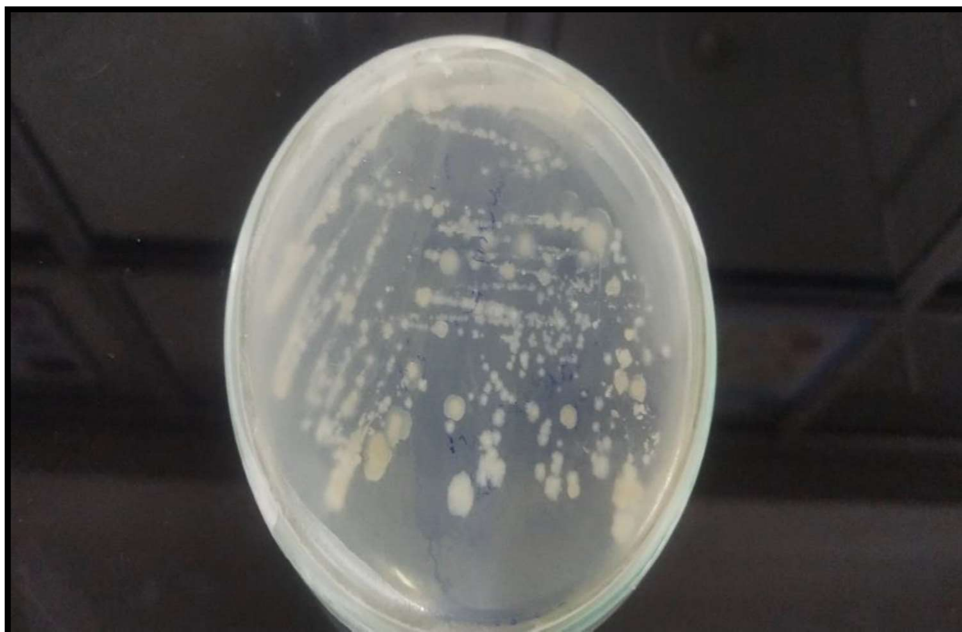


Figure 9: Plate showing colonies of *Mycobacterium smegmatis.*

4.1.3.1 Microscopic view of *Mycobacterium smegmatis* strain

The microscopic view of *Mycobacterium smegmatis* strain was taken at 10X (Figure 12).

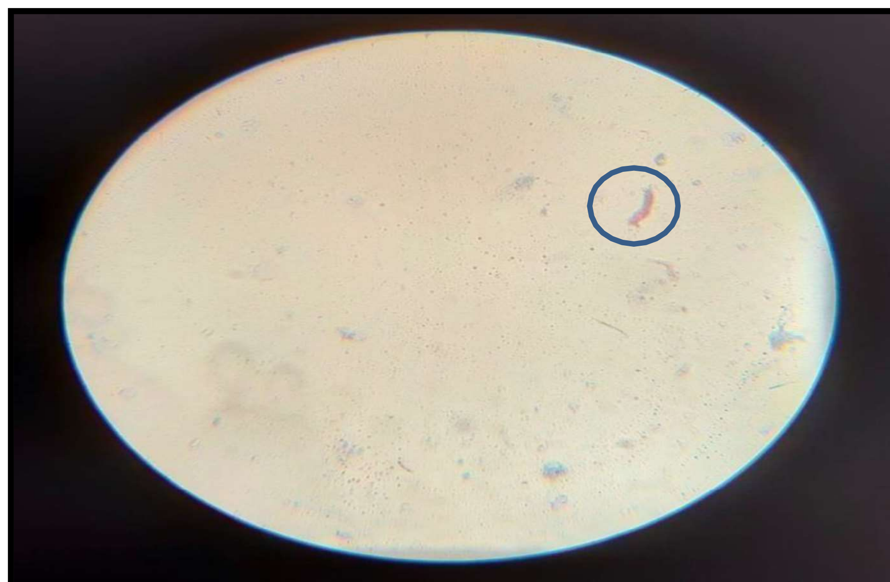


Figure 10: Microscopic view of *Mycobacterium smegmatis*

4.1.3.2 Resazurin Microtitre Assay (REMA)

REMA Assay for methanolic extract of *Plectonema boryanum*

Figure 11: 96 well plate showing inhibition of *Mycobacterium smegmatis*

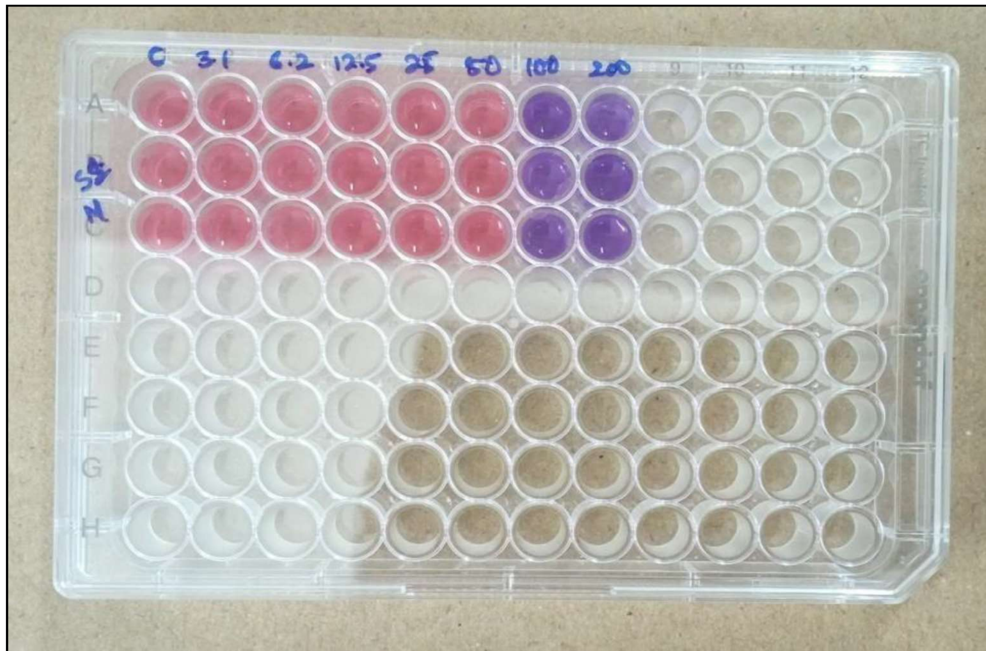


Figure 11:96 well plate showing inhibition of *Mycobacterium smegmatis* strain at different concentration of methanolic extract of *Plectonema boryanum.*

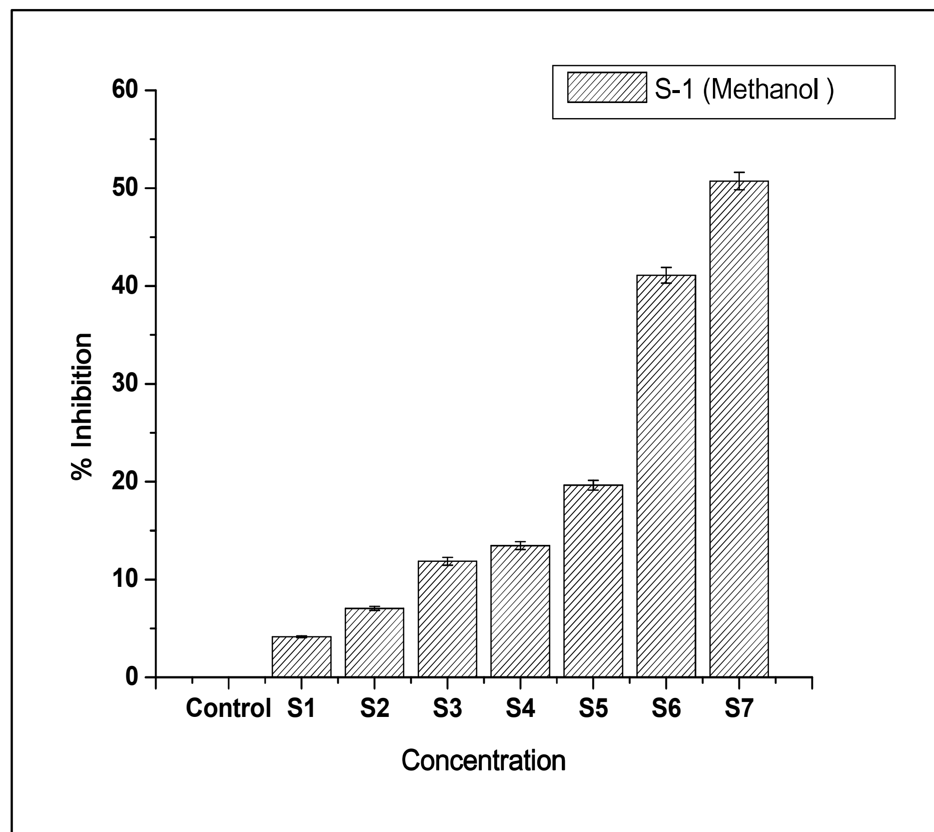


Figure 12: Inhibition percentage of methanolic extract of *Plectonema boryanum.*

The results suggested that the inhibition percentage of methanolic extract of *Plectonema boryanum* in which S1-S7 are the respective concentrations of methanolic extract (3.1µg/ml to200 µg/ml) (Fig 10) .The inhibition % increases on moving from less concentration to high concentration. The IC_{50} is between S6 to S7 (100-200µg/ml) concentration and the minimum inhibitory (MIC) concentration is at 100µg/ml.

- REMA Assay for ethyl acetate extract of *Plectonema boryanum*

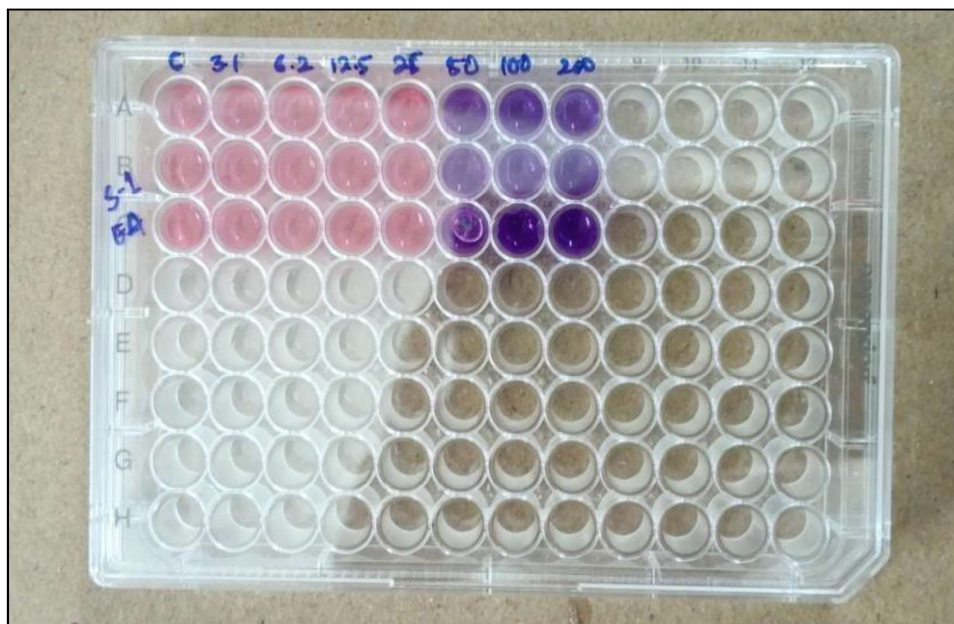


Figure 13: 96 well plate showing inhibition of *Mycobacterium smegmatis* strain at different concentration of ethyl acetate extract of *Plectonema boryanum*

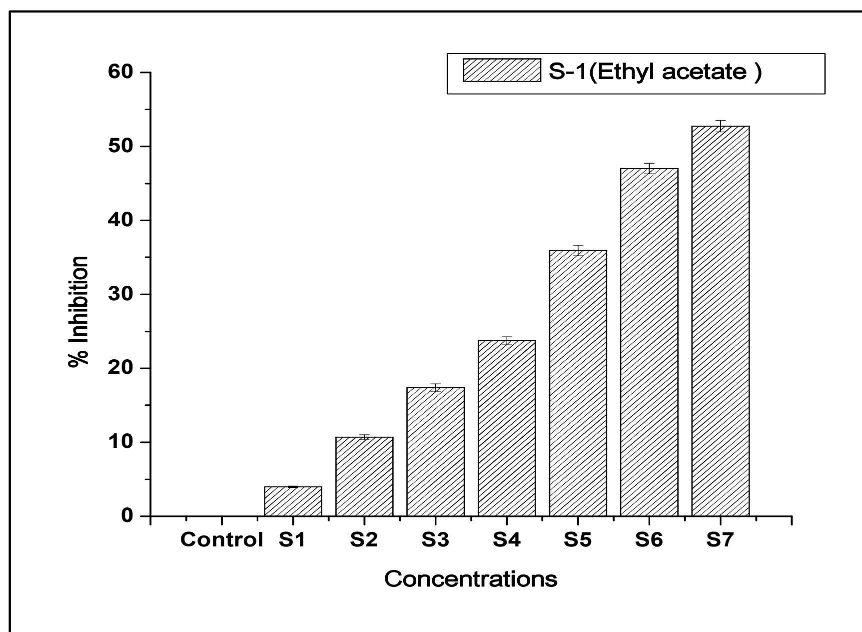


Figure14: Inhibition percentage of ethyl acetate extract of *Plectonema boryanum.*

The results suggested that the inhibition percentage of ethyl acetate extract of *Plectonema boryanum* in which S1-S7 are the respective concentrations of ethyl acetate extract (3.1µg/ml to200 µg/ml) (Figure 11). The inhibition % increases on moving from less concentration to high concentration. The IC_{50} is between S5 to S6 (100-200 µg/ml) concentration and the minimum inhibitory (MIC) concentration is at 50 µg/ml.

- REMA Assay for methanolic extract of *Anabaena* sp.

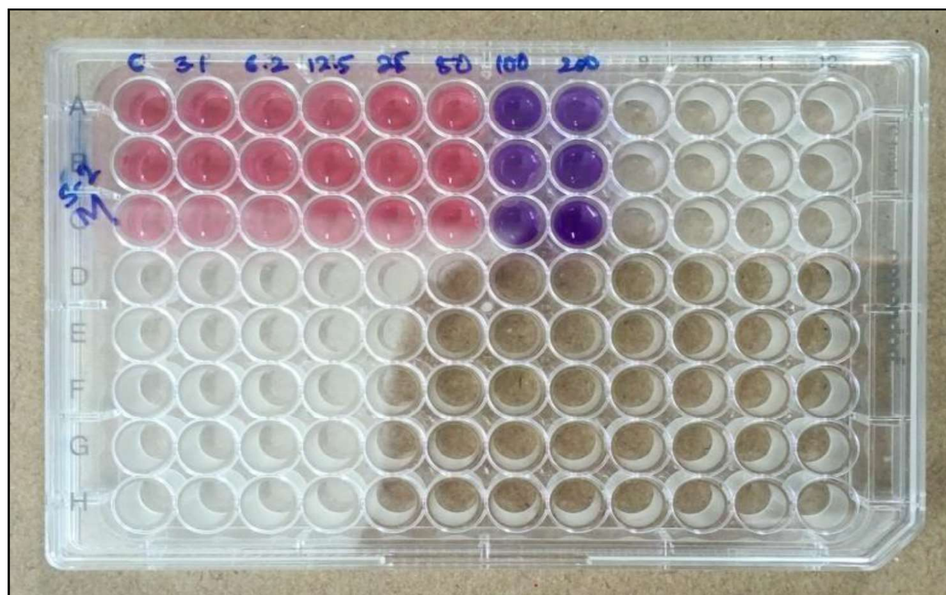


Figure15: 96 well plate showing inhibition of *Mycobacterium smegmatis* strain at differentconcentration of methanolic extract of *Anabaena* sp.

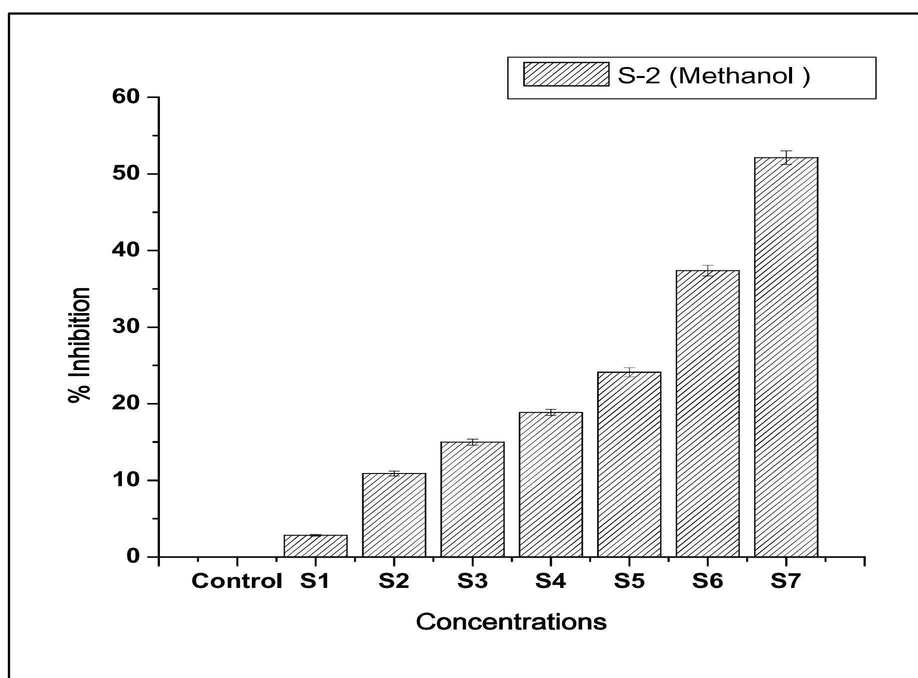


Figure 16: Inhibition percentage of methanolic extract of *Anabaena* sp.

The results suggested that the inhibition percentage of methanolic extract of *Anabaena* sp. in which S1- S7 are the respective concentrations of methanolic extract (3.1µg/ml to200 µg/ml) (Fig 12).The inhibition % increases on moving from less concentration to high concentration. The IC_{50} is between S6 to S7 (100-200µg/ml) concentration and the minimum inhibitory (MIC) concentration is at 100µg/ml.

- REMA Assay for ethyl acetate extract of *Anabaena* sp.

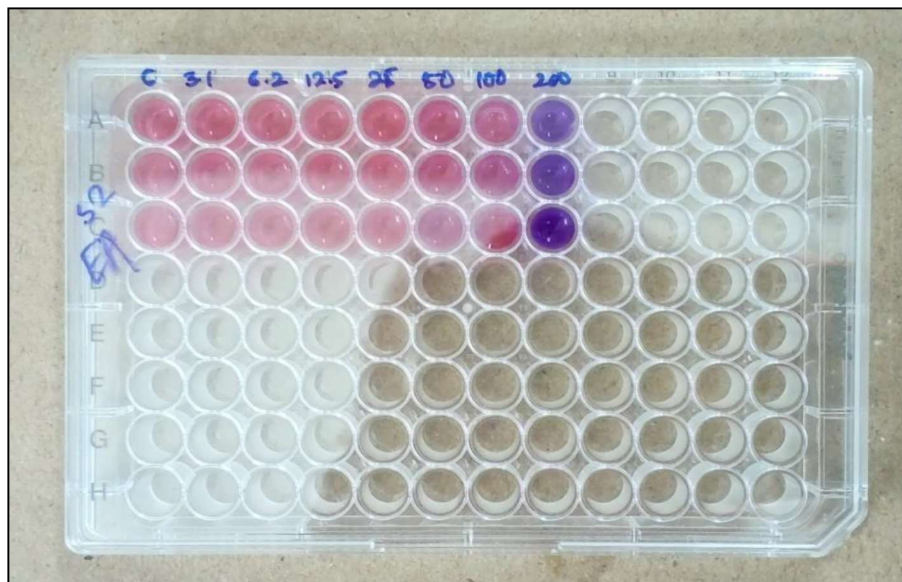


Figure 17: 96 well plate showing inhibition of *Mycobacterium smegmatis* strain different concentration of ethyl acetate extract of *Anabaena* sp.

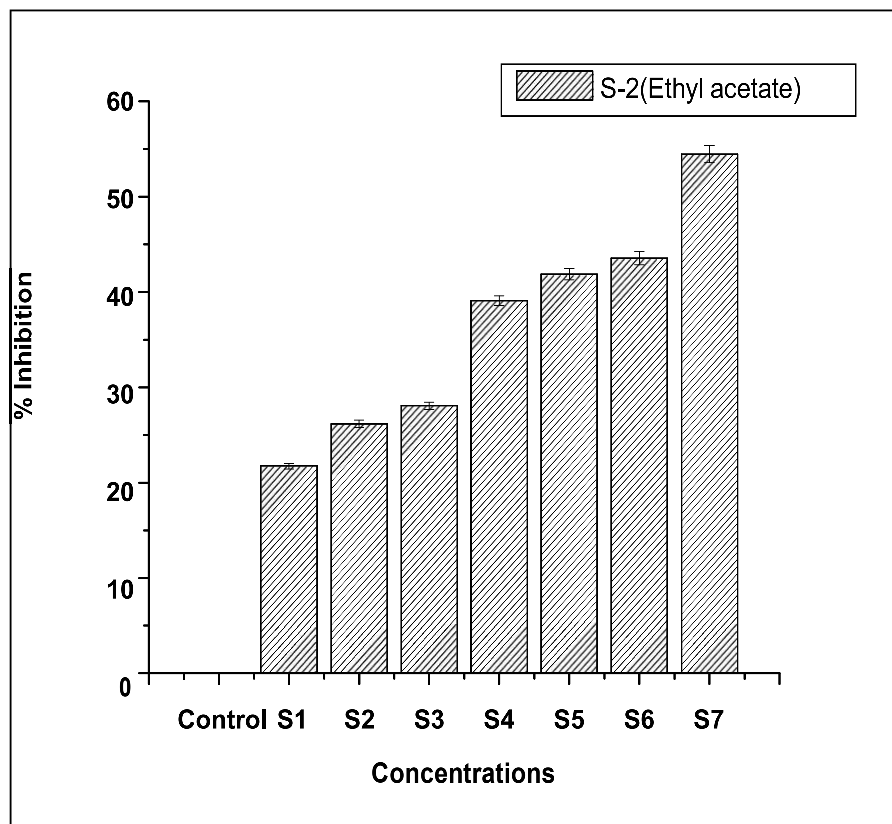


Figure 18: Inhibition percentage of ethyl acetate extract of *Anabaena* sp.

The results suggested that the inhibition percentage of ethyl acetate extract of *Anabaena* sp. in which S1-S7 are the respective concentrations of ethyl acetate extract (3.1µg/ml to200 µg/ml) (Fig 13). The inhibition % increases on moving from less concentration to high concentration. The IC_{50} is between S6 to S7 (100-200µg/ml) concentration and the minimum inhibitory (MIC) concentration is at 200µg/ml. The MIC of methanolic extract of *Plectonema boryanum* and *Anabaena* sp.is 100µg/ml And the MIC of Ethyl acetate extract of *Plectonema boryanum* and *Anabaena* sp.50 and 200µg/ml. According to Spavieri et al., 2010 The MIC of algal extract was 64µg/ml.

Table 12: Minimum Inhibitory Concentrations of methanolic and ethyl acetate extracts of *Plectonema boryanum* and *Anabaena* sp.

Cyanobacterial strains	**Solvents extract**	**Minimum Inhibitory Concentration (MIC) (µg/ml)**
Plectonema boryanum	Methanol	100
Plectonema boryanum	Ethyl acetate	50
Anabaena sp.	Methanol	100
Anabaena sp.	Ethyl acetate	200

5. CONCLUSION

The present study gives an insight about the antioxidant and antibacterial potential of *Plectonema boryanum* and *Anabaena* sp. extracts .The results concluded that among the two strains, the crude extract of *Plectonema boryanum* with ethyl acetate has a maximum antimicrobial and antioxidant activity of 50µg/ml and 31.5% respectively. The FTIR results detected the presence of high number of hydroxyl groups that reflects the presence of large number of alkaloids bioactive compounds. Owing to this high efficiency the cyanobacterial extracts can be further purified and used for the development of antimicrobial drug.

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