Phytochemical Investigations and Biological Activities of Cultured *Spirulina fusiformis* Isolated from Lake Chitu, Ethiopia.

Derartu Mitiku Jijo

A Thesis Submitted to the Department of Applied Chemistry
School of Applied Natural Science

Presented in Partial Fulfilment of the Requirement for the Degree of
Master's in Natural Product Chemistry

Office of Graduate Studies
Adama Science and Technology University

January 2024
Adama, Ethiopia

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DECLARATION

I hereby declare that this master's Thesis entitled "Phytochemical Investigations and Biological Activities of Cultured *Spirulina* Isolated from Lake Chitu, Ethiopia." is my original work. That is, it has not been submitted for the award of any academic degree, diploma, or certificate in any other university. All sources of materials that are used for this thesis have been duly acknowledged through citation.

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RECOMMENDATION

I/We, the advisor(s) of this thesis, hereby certify that I/we have advised and read the revised version of the thesis entitled "**Phytochemical Investigations and Biological Activities of Cultured *Spirulina* Isolated from Lake Chitu, Ethiopia**." Prepared under our guidance by Derartu Mitiku Jijo submitted in partial fulfillment of the requirements for the degree of Master of Science in Applied Chemistry specialization in Natural product chemistry. Therefore, I/we recommend the submission of the revised version of the thesis to the department following the applicable procedures.

Major Advisor	**Signature**	**Date**
Co-advisor	**Signature**	**Date**

APPROVAL OF ADVISORS

We, the advisors of the thesis entitled **"Phytochemical Investigations and Biological Activities of Cultured *Spirulina* Isolated from Lake Chitu, Ethiopia."** and developed by Derartu Mitiku, hereby certify that the recommendation and suggestions made by the board of examiners are appropriately incorporated into the final version of the thesis.

Major Advisor	**Signature**	**Date**
Co-advisor	**Signature**	**Date**

APPROVAL OF BOARD OF EXAMINERS

We, the undersigned, members of the Board of Examiners of the thesis by Derartu Mitiku have read and evaluated the thesis entitled **"Phytochemical Investigations and Biological Activities of Cultured *Spirulina* Isolated from Lake Chitu, Ethiopia."** and examined the candidate during the open defense. This is, therefore, to certify that the thesis is accepted for partial fulfillment of the requirement of the degree of Master of Science in Natural Product Chemistry.

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LIST OF ACRONYMS/ABBRIVATIONS

Symbol	Abbreviations
µ	Micro
^{0}C	Degree centigrade
L	Little
mL	Milliliters
Rpm	Revolution per minute
Mg	Milligram
µg	Micro gram
Ca-SP	Calcium *Spirulina*
CC	Column chromatography
DNA	Deoxyribonucleic acid
DPPH	1,1-diphenyl-2-picryl-hydrazyl
EFA	Essential fatty acid
EPA	Eicosapentaenoic acid
DHA	Docosahexaenoic acid
GC-MS	Gas chromatography mass spectrometry
GLA	Gamma linoleic acid
IC_{50}	The half maximal inhibitory concentration
IR	Infrared spectroscopy
LED	Light emitted diode
NADPH	Nicotinamide adenine dinucleotide phosphate
MHA	Mueller Hinton Agar
NMR	Nuclear Magnetic Resonance
PCs	Phenolic compound
PUFAs	Polyunsaturated fatty acids
PTLC	Preparative thin layer chromatography
RI	Retention index
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT	Retention time
SCP	Single cell protein
SOD	Superoxide dismutase
SPF	Sun protection factor
TLC	Thin layer chromatography
VOCs	Volatile organic compounds
UV-Vis	Ultraviolet Visible

ABSTRACT

*Now days a wide variety of secondary metabolites has been isolated from terrestrial plants however, unexplored natural sources of bioactive ingredients from marine are gaining much attention since they can lead to the discovery of new compounds or bioactivities. Microalgae have been proposed as an interesting, almost unlimited, natural source in the search for novel natural functional ingredients, and several works have shown the possibility to find bioactive compounds in these organisms. Among the micro algae Spirulina are blue-green alga that exists as a single-celled organism that converts sunlight into energy. This study investigated the indoor cultivation of Spirulina, followed by isolation and characterization of bioactive compounds with antibacterial, antioxidant, and sun protection potential. The indoor cultivation of Spirulina was done using appropriate media (zarrouk media) and growth condition such as pH., light, and aerator. While indoor cultivation minimized contamination risks, maintaining temperature and light within the ideal range presented a significant challenge. Total extraction was done by using Methanol and fractionated successively with Petroleum ether, Dichloromethane Ethyl acetate and Butanol by liquid extraction. TLC analysis guided further separation of the extracts, leading to Dichloromethane being chosen for subsequent column chromatography based on its promising profile which led to the isolation of two compounds namely stigmasterol and astaxanthin diester while Ethyl acetate extract furnishes hexadecenoic acid. The structures of these compounds were established using spectroscopic methods ^{1}H, ^{13}C NMR, and IR spectroscopy. Antibacterial activity was tested against two Gram-negative E. coli (ATCC 25922), P aeruginosa (ATCC 27825), and two Gram- Positive bacteria S. aureus (ATCC 25923) and S. pyogenes (ATCC 11778) at 25 mg/ml, 50mg/ml, 100mg/ml and 200mg/ml concentrations using agar disc diffusion method. The results indicate Methanol extract of Spirulina show the strongest antibacterial activity. Methanol extract showed a maximum zone of inhibition (18±.33) on S. aureus bacteria at concentration 200 mg/ml compared to the ciprofloxacin (16±.33) at a concentration of 10µg/ml followed by P. aeruginosa, E. coli and S. pyogenes at concentration 200mg/ml. The antioxidant activity of the Spirulina extracts (methanol and water extract) and isolates (stigmasterol and diester astaxanthin) was evaluated by DPPH free radical using concentrations 25, 50,100, 200µg/ml. Their IC_{50} inhibition was 3.248, 0.46, 12.19, and 3.14 respectively. The methanol extract of the microalgae Spirulina inhibited the DPPH radical by 66.66, 73.35, 76.4 and 79.5 at 25,50, 100 and 200 µg/ml respectively on the other hand, compound 36 displayed % inhibition of 79.8, 79.9, 80.2,83.6 at 25,50, 100 and 200 µg/ml respectively. In this study the sun protection potential of Spirulina extracts (water and methanol crude) and compound **37** were evaluated using the in vitro method. Among the tested samples, compound **37** exhibits the highest SPF value, which is 17.49. This study successfully isolated and characterized bioactive compounds from Spirulina and demonstrated their potential in diverse applications, including antibacterial, antioxidant, and sun protection. The findings highlight the exciting possibilities of exploring Spirulina for novel natural ingredients with significant health benefits.*

KEY WORDS: *Spirulina,* Secondary metabolites, Characterization, GC-MS, Biological activity

1. INTRODUCTION

1.1. Background of study

Aquatic bodies cover a large portion of Earth (70 percent of the total area) and provide diverse habitats for a wide range of organisms(Ebada & Proksch, 2011). The aquatic environment is a large reservoir of food and drugs, the exploration of new biomedically important molecules. Consequently, from marine flora and fauna resulted in the isolation of approximately 10,000 metabolites, which are a diverse group of aquatic photosynthetic organisms that account for 10% of the flora kingdom{Ullah, 2023 #1}. The term "algae" refers to a wide collection of organisms that produce their own food via the process of photosynthesis and may be found in a variety of habitats, including marine and freshwater environments. They are found in every part of the world and may be divided into two categories based on their biological structure: macroalgae (red, green, and brown seaweeds) and microalgae.

Microalgae are microscopic organisms found in both seawater and freshwater (Barbera *et al.*, 2016) and can be classified as eukaryotic microorganisms or prokaryotic cyanobacteria (blue-green algae), with more than 25,000 species already isolated and identified (Mata *et al.*, 2010). These microorganisms perform photosynthesis, which is an important natural mechanism for reducing atmospheric CO_2 concentrations. Microalgae are also characterized by a short generation time and multiplied exponentially under favourable environmental conditions (Buono *et al.*, 2016). They were a food source for ancient civilizations in some parts of the world such as Asia, Africa, and South America.

Spirulina is a microscopic blue-green algae that exists as a single-celled organism that converts sunlight into energy. It was one of the first life forms designed by nature more than 3.6 billion years ago. *Spirulina* contains billions of years of evolutionary wisdom in its DNA and is the offspring of the Earth's first photosynthetic life forms. Genus *Spirulina* has gained importance and international demand for its high phytonutrients value and pigments which have applications in healthy foods, animal feed, therapeutics, and diagnostics(Sivakumar *et al.*, 2011). It grows best in highly alkaline environments, with a pH of 10-12. *Spirulina* contains high-quality protein, standing for 50-70% of its dry weight(Marzieh Hosseini *et al.*, 2013), essential amino acids and fatty acids,

Ɣ-linolenic acid (GLA), vitamins, especially vitamin B12 and provitamin A (β-carotene) and dietary minerals(A. Belay *et al.*, 1993). Its cell wall is composed of polysaccharides, which are 86% digestible and are easily absorbed by the human body. Furthermore, phytochemical screening of *Spirulina* species revealed alkaloids, terpenoids, steroids, tannins, saponins, flavonoids, phenols, coumarins, quinones, glycosides, vitamin E, and various light-absorbing pigments (phycocyanin, chlorophylls, carotenoids, etc.)(K. Kumar *et al.*, 2005). Those phytochemicals have therapeutic effects for various health problems and various medical purposes, such as anti-inflammatory, antihypertensive, diuretic, antimicrobial, antioxidant, antidiabetic, antihyperlipidemic, antitumor, antipyretic, antiulcer, and hepatoprotective effects (Belay, 1997). Therefore, *Spirulina* extract may play a key role in disease prevention, control, and reversal of malnutrition, especially in developing countries, such as Ethiopia.

In Ethiopia, except for the limited work by (Ogato *et al.*, 2014) on the evaluation of growth and biomass production of *Spirulina fusiformis*, on the morphological variability of *Spirulina fusiformis* (Cyanophyta) in relation to environmental variables in Lake Chitu, by(Getachew *et al.*, 2019) on some nutritive values and toxicity tests, no previous research has been conducted on the detailed investigation of the chemical composition, isolation, and biological activities of *Spirulina fusiform* . Therefore, in this work the phytochemical and biological activity of crude extracts and isolated compounds of the microalgae *Spirulina* found in Ethiopia has been investigating.

1.2. Statement of the Problem

The issue of microbial resistance to medicines is getting worse, and its yet unclear how antimicrobial drug use will develop in the future (Aslam *et al.*, 2018). Most clinically prescribed antibiotics have problems, such as toxicity, ineffectiveness, prohibitive cost, and frequent use, which promote the evolution of resistant strains of bacteria. These highlight a great need for the discovery and development of non-toxic, affordable, and effective novel natural drugs.

Excessive levels of free radicals have been shown to cause various diseases. Oxidative molecules may react with proteins, lipids, deoxyribonucleic acid (DNA), and other cellular components through chain reactions, resulting in various diseases related to protein/lipid oxidation and DNA damage. Synthetic antioxidants have been used to combat oxidative stress. However, long-term use of synthetic antioxidants has been reported to be dangerous for humans, and natural antioxidants have received increasing attention (Lourenço *et al.*, 2019).

In addition, most skin cancers occur in areas of the body that are most frequently exposed to the sun, such as the face, neck, head, and back of the hand. The harmful effects of solar radiation are predominantly caused by the ultraviolet (UV) region of the electromagnetic spectrum. Ultraviolet radiation has been implicated as a causative factor in skin cancer(Cummings *et al.*, 1997) . As a result, various types of sunscreen and sunblock formulations are there in the market such as cream lotion etc., but most of them have chemical agents as lead molecule for showing sunscreen and sunblock activity(Romano, 2006). From long back, the use of chemicals in sunscreens as photo protective agent in the formulation is a common practice. Owing to their harmful effects, they are becoming less popular now a day. Several people with sensitive skin, such as those suffering from skin hypersensitivity don't want to use chemical sunscreens due to concern about skin exposure to unknown chemicals. Therefore, assessment of the sun protection potential and antioxidant activity of *Spirulina* extract can overcome these limitations.

Another major concern is that early research findings on natural product chemistry were largely restricted to terrestrial plants. Marine is a large reservoir of natural products and novel drugs, and several microalgae-derived products have not been explored. Recently, researchers have focused on the marine flora and fauna(Imhoff *et al.*, 2011). Several researchers have reported that marine-derived secondary metabolites, such as alkaloids, flavonoids, terpenoids, glycosides, tannins, and saponins, have shown several biological activities, and very few researchers have focused on microalgae. In addition, there has been no previous research on the phytochemistry, isolation, or biological activities of *Spirulina* specifically in Ethiopia. Therefore, the present study aimed to investigate the phytochemical profile and screen the potential antioxidant, antibacterial, and sun protection effects of the marine microalgae *Spirulina*.

1.3. Objectives of the Study

1.3.1. General Objective

The main objective of this study was to investigate phytochemicals and to evaluate the biological activities of indoor cultured microalga *Spirulina* isolated from Lake Chitu.

1.3.2. Specific Objectives

- To isolate and culture *Spirulina* in the laboratory.

- To extract *Spirulina* with methanol and partition the extract with solvents such petroleum ether, dichloromethane, ethyl acetate and butanol using liquid-liquid fractionation method.
- To extract essential oils by hydro-distillation and analyze using GC-MS.
- To carry out preliminary phytochemical screening of the extracts.
- To isolate compounds from crude microalga *Spirulina* using CC, TLC, and PTLC.
- To characterize the structure of the isolated bioactive compounds using combined spectroscopic technique (GC-MS, 1H and 13C NMR and FT-IR).
- To evaluate *in vitro* antibacterial activity of the crude extracts and isolated compounds *via* agar disk diffusion method against Gram positive bacteria (*Staphylococcus aureus,* and *Streptococcus pyogenes)*, and Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa).*
- To evaluate in *vitro* antioxidant activity of extracts and isolated compounds using DPPH (diphenyl-picrylhydrazyl) free radical scavenging assay.
- To evaluate sun protection potential of extracts and isolated compounds and calculate Sun Protection factor by using Mansur equation.

1.4. Significance of the Study

This research project might be expected to bring out data on identification and characterization of phytoconstituents and bioactive compounds which have therapeutic effects on various health problems and different medicinal uses. Furthermore, isolation and structural determination is very important to find biologically active compounds. Therefore, when isolation of bioactive compounds is accompanied by structural elucidation, it is useful for pharmacological action and allows complete synthesis of other useful analogue products. Since there is no previous research has been done on detail investigation on phytochemistry and biological activities of *Spirulina* in Ethiopia it will used as a reference for other researchers in the areas of studies.

In general, the present study will fill the research gap listed under the statement of the problems. By investigating its phytochemicals and potential therapeutic effects, we hope to contribute to the development of safe and effective natural medicines for antibiotic resistance, oxidative stress, and sun damage-related diseases.

1.5. Justification of the Study

The availability of raw materials is necessary to produce biologically active compounds for preclinical evaluation. *Spirulina* does not impose a severe impediment to mass production, and *Spirulina* species have been commercially cultivated worldwide. A cultivated source of *Spirulina* ensures a consistent and environmentally responsible supply of valuable phytochemicals. The diverse array of bioactive compounds found in *Spirulina* makes it a promising candidate for pharmaceutical and nutraceutical development. Investigating its phytochemical composition allows for the identification of lead compounds for drug development or the formulation of functional products with specific health-enhancing properties. Overall, the statement of the problem clearly justifies the research by highlighting the urgency, relevance, scientific merit, and potential impact of investigating the phytochemical profile, antioxidant, antibacterial, and sun protection properties of *Spirulina* in Ethiopia.

2. LITERATURE REVIEW

2.1. *Spirulina*

Spirulina, also named *Arthrospira*, are one-celled form of blue-green algae that thrives in warm, alkaline freshwater bodies. It has gained considerable popularity in the healthy food industry and is considered a food supplement for humans, livestock, poultry, and aquaculture diets. *Spirulina* is a free-floating microalga in which the filaments have spiral properties. *Spirulina* was first classified in the plant kingdom due to its photosynthesis ability and the richness of plant pigments (Deng & Chow, 2010). Later on, because of its biochemical, genetic and physiological characteristics, it was placed on the bacteria kingdom based on a new understanding (Vonshak, 1997). *Spirulina* is widely used as a dietary supplement throughout the world. It is considered a potential solution for combating malnutrition/anemias, oxidative stress, cancer, viral and bacterial infections, hyperlipidemia, diabetes, obesity, inflammatory allergic reactions, heavy metal/chemical-induced toxicity, and radiation damage in clinical and preclinical research.

Spirulina grows in water and can be harvested and processed easily. It has very high macronutrient and micronutrient contents, such as proteins, amino acids, unsaturated fatty acids, minerals, and vitamins. It has long been used as a dietary supplement for centuries by people living close to the alkaline lakes where it is naturally found, for instance, those living adjacent to Lake Chad in the Kanem region and Lake Texcoco in Mexico (Sotiroudis & Sotiroudis, 2013).

2.2. General characteristics of *Spirulina*.

2.2.1. Morphology

Spirulina gets its name from the way it grows in microscopic spirals that stick together, and this makes it easy to harvest through a filtration process. They are photosynthetic, and therefore autotrophic, and reproduced by binary fission. *Spirulina* trichomes (filaments), which contain cylindrical cells, aligned together in spirals or in straight lines. The helical shape of the filaments is characteristic of the genus and is maintained only in a liquid environment or culture medium. The presence of gas-filled vacuoles in the cells, together with the helical shape of the filaments, results in floating mats. These filaments have variable length (usually 50–500μm) and a diameter close to 3–12μm, but the cell dimensions, degree of coiling, and length of filaments varies with species. The cell organization of *Spirulina* is typical of a prokaryote Gram-negative bacterium with a lack of membrane-bound organelles. The cell wall constitutes a weak envelope that is composed

of several layers, mostly of a peptidoglycan and lipopolysaccharide nature. The *Spirulina* cells have a few inclusions, such as thylakoid membranes with phycobilisome, carboxysomes, ribosomes, DNA fibrils, and gas vacuoles, as well as polyglycan, polyphosphate, and cyanophycin granules. The body surface of *Spirulina* is smooth and without covering, so it is easily digestible by simple enzymatic systems (Ahsan *et al.*, 2008; Ciferri & Tiboni, 1985).

2.2.2. Taxonomic classification of *Spirulina*

In 1827, Turpin isolated *Spirulina* from a freshwater sample. In 1844, Wittrock and Nordstedt reported a spiral-septate blue-green microalga near the city of Montevideo, then called *Spirulina platensis.* But it was not until 1852 that the first taxonomic report written by Stizenberger appeared. He gave this new genus the name *Arthrospira*, based on the septa presence, helical form, and multicellular structure. In 1892, Gomont discovered the aseptate form of the *Spirulina* genus and the septal form of the *Arthrospira* genus. However, because of the common helical morphology, the members of the *Spirulina* genus and *Arthrospira* genus had been reunified under the designation *Spirulina* in 1932. However, in 1989, these microorganisms were separately classified into two genera, *Spirulina* and *Arthrospira* ; this classification is currently accepted (Tomaselli *et al.*, 1996).In the various *Arthrospira* geneses, the two species *Arthrospira platensis* and *Arthrospira maxima* are the most important where in (**Figure 1)**

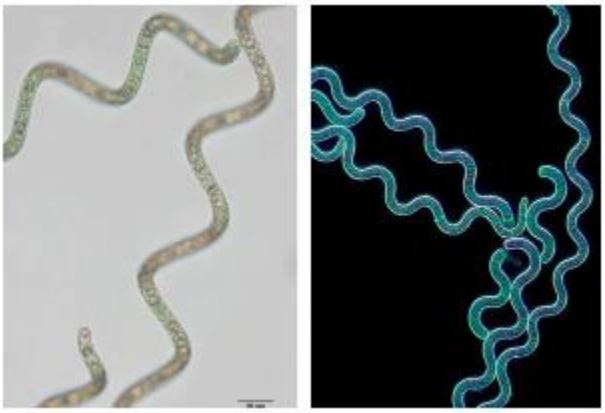


Figure 1. *Spirulina* (*Arthrospira platensis* and *Arthrospira maxima*) *(Small, 2011)*.

As for the historic reason, most of the worldwide investigation on these microalgae has been performed under the name "*Spirulina."* Therefore, the name *"Spirulina*" is the common designation among scientists, manufacturers, and consumers. However, most recently, the reports on *Spirulina* in the peer-reviewed journals have been corrected to "*Arthrospira"* instead,

considering both the taxonomy of this microalgae and the historic designation. Specifically, "*Arthrospira"* is used when referring to the taxonomy (**Table 1**) and "*Spirulina*" is used when referring to the composition of this microalga.

Table 1. Taxonomic classifications of microalgae *Spirulina*

Domain	**Bacteria**
Phylum	*Cyanobacteria*
Class	*Cyanophyceae*
Order	*Spirulinales*
Family	*Spirulinaceae*
Genus	*Spirulina (Raghuraman et al., 2022)*

2.3. *Spirulina* Species

Spirulina are filamentous, gram negative, non-toxic species of cyanobacteria belonging to two separate genera, namely *Spirulina* and *Arthrospira* , consisting of about 15 species (Ahsan *et al.*, 2008). Among the many varieties of *Spirulina,* the most commonly studied species are *Spirulina platensis* (*Arthrospora platensis*), *Spirulina maxima* (*Arthrospora maxima*) and *Spirulina fusiformis* (*Arthrospora fusiformis*); while the most common species used for human consumption and pharmaceutical industries are *Spirulina maxima* and *Spirulina platensis (Ahsan et al., 2008; Gonçalves et al., 2016; Guiry, 2013; Khan et al., 2005). Spirulina platensis* is generally found in Africa, Asia, and South America, whereas *A. maxima* is confined to Central America (Abdulqader *et al.*, 2000).

2.3.1. *Spirulina plantesis*

Spirulina platensis is a filamentous, Gram-negative cyanobacterium. This bacterium is non-nitrogen-fixing photoautotroph (Fujisawa *et al.*, 2010). It has been isolated in Chenghai Lake, China, soda lakes of East Africa, and subtropical, alkaline lakes (Masojídek & Torzillo, 2014). *Arthrospira platensis* is filamentous, motile bacterium. Motility has been described as a vigorous gliding without a visible flagella (Fujisawa *et al.*, 2010)

As a photoautotroph the major carbon source is carbon dioxide and water are a source of electrons to perform CO_2 reduction. *S. platensis* has been found in environments with high concentrations of carbonate and bicarbonate. It can also be found in high salt concentrations because of its alkali and salt tolerance. The temperature optimum for this organism is around 35°C. Based on environmental conditions, culture medium often has a pH between 9-10, inorganic salts, and a high bicarbonate concentration (Fujisawa *et al.*, 2010)

S. platensis has found wide applications in the areas of agriculture, food, pharmaceuticals, perfumery, medicine, and science. Nowadays, this organism is used as a food supplement and is marketed in the form of pills, capsules and powder or incorporated into various types of food like cakes, biscuits, noodles, and health drinks, etc. Various countries are developing strategic programs for the production and use of *S. platensis*. In China, the *S. platensis* industry is developing rapidly with several factories producing hundreds of tons of *S. platensis* in the form of dried powder that is used as food, fodder and medicine (Ravi *et al.*, 2010). *S. platensis* has been orally consumed for thousands of years by humans among the Mexican, African, and Asian societies and is a popular food and nutritional supplement in Japan and the United States. Among several occurring species of *Spirulina*, the most used in nutritional supplements are *S. platensis.*

2.3.2. *Spirulina* maxima

Spirulina maxima is a planktonic photosynthetic filamentous *Cyanobacterium* that forms massive populations in tropical and subtropical bodies of water which have high levels of carbonate and bicarbonate. For centuries, native peoples have harvested *Spirulina maxima* from Chad Lake in Africa and Texcoco Lake in Mexico for use as a source of food (Vonshak, 1997) a fact which means that *Spirulina* deserves special attention both as a source of single cell protein (SCP) and because of its nutraceutical properties. *Spirulina maxima* extract comes from blue-green algae, which is a type of bacteria that thrives in water and can photosynthesize. These organisms are simple, they're plant-like, and they live in both salt water and fresh water. *Spirulina* maxima are rich in chlorophyll, which is why it has a deep green color. It has the power to relief inflammation and eliminate toxins. It is use full for the treatment of any kind of skin especially acne. It also provides proteins and vitamins A and E.

2.4. Review on cultivation and harvesting of *Spirulina*.

Algae cultivation can be carried out in either open systems, such as ponds, lakes, or lagoons, or in closed systems, as mentioned (Toyoshima *et al.*, 2015). Currently, there are two main technologies being explored for *Spirulina* cultivation: closed photobioreactors and open ponds. Both methods are utilized in commercial settings to yield valuable products.

2.4.1. Open pond system

Open ponds can be classified into two categories: natural bodies of water, such as lakes, lagoons, ponds, and artificial ponds or containers. Commonly used types of open ponds include shallow large ponds, circular ponds, tanks, and raceway ponds. These open systems are favored due to their easy construction and operation, resulting in lower production and operational expenses(Ugwu *et al.*, 2008). However, there are various limitations associated with open ponds, including reduced light absorption by cells, evaporation losses, and carbon dioxide diffusion into the atmosphere, and the requirement of extensive land areas. Additionally, inefficient aeration often leads to low biomass productivity in open cultivation systems. Successful growth in open ponds is influenced by factors such as location, season, temperature, pH level, and the availability of nutrients and carbon dioxide(Cuaresma *et al.*, 2011). Another significant disadvantage of open pond systems is the potential contamination by fauna and other rapidly growing heterotrophs. To overcome these challenges, researchers have explored closed systems as alternative solutions(R. Singh *et al.*, 2012).

2.4.2. Closed system.

A photobioreactor is a specialized container created to cultivate biomass under controlled conditions. It is typically enclosed and illuminated to support optimal growth. Unlike open systems, photobioreactors are sealed off from the external environment, preventing the exchange of gases or contaminants. These closed systems, also known as photobioreactors, offer several benefits including precise control over carbon dioxide and water supply, regulation of temperature, optimal light intensity, culture density, pH levels, gas exchange, aeration, and overall productivity of algae. Illumination in these systems can be achieved using artificial or natural light sources, or a combination of both. Photobioreactors have several advantages over open systems despite being more expensive(Tsoglin *et al.*, 1996). They minimize contamination and enable hygienic cultivation of a single species. In addition, photobioreactors provide better control over various

conditions like pH, temperature, light intensity, and carbon dioxide concentration. They also reduce carbon dioxide loss and prevent water evaporation. Furthermore, photobioreactors allow for higher cell concentrations and enhance the production of complex biopharmaceuticals. Their design enables the cultivation of different types of microalgae. By ensuring uniform illumination of the culture surface and efficient exchange of carbon dioxide and oxygen, photobioreactors minimize the non-illuminated area. Consequently, photobioreactors offer numerous advantages compared to open systems.

Several well-known companies worldwide cultivate *Spirulina* in the form of tablets, powder, flakes **(Figure 2)**, a popular ingredient in healthcare products. These companies include Earthrise Nutritional's from California, USA (earthrise.com), DIC Lifetec *Spirulina* from Japan (dlt-spl.co.jp/business/en/*Spirulina*/), Cyanotech *Spirulina* from Hawaii, USA (cyanotech.com), Boonsom *Spirulina* Farm from Thailand (boonsomfarm.com), FEBICO (Far East Bio-Tec Co.) from Taiwan (febico.com), *Spirulinea* from France and Laos (spirulinea.com), Spiruline de Burkina from Burkina Faso (spirulineburkina.org), Green Valley from Germany (greenvalley.de), Natesis *Spirulina* from France (natesis.com), *Spirulina*.PL from Poland (*Spirulina*.pl), All Seasons Health from the United Kingdom (allseasonshealth.com), NaturKraftWerke *Spirulina* from Switzerland (naturkraftwerke.com), Sanatur *Spirulina* from Germany (sanatur.de), Marcus Rohrer *Spirulina* from the Netherlands (*Spirulina*.nl), Taiwan Chlorella from Taiwan (taiwanchlorella.com), and RBC Life Sciences from the USA (rbclifesciences.com).

Gerophyta Nutraceuticals Company in Tamil Nadu, India offers a wide range of products, as *Spirulina* Powder, *Spirulina* Capsules, *Spirulina* tablets, Spiruvita-C, Dr. *Spirulina* Diavita-C, *Spirulina* herbal face pack. Other companies also offer a wide range of products such as *Spirulina* bar, *Spirulina* green tea, *Spirulina* personal care products, *Spirulina* chocolates, *Spirulina* drinks, *Spirulina* honey etc.

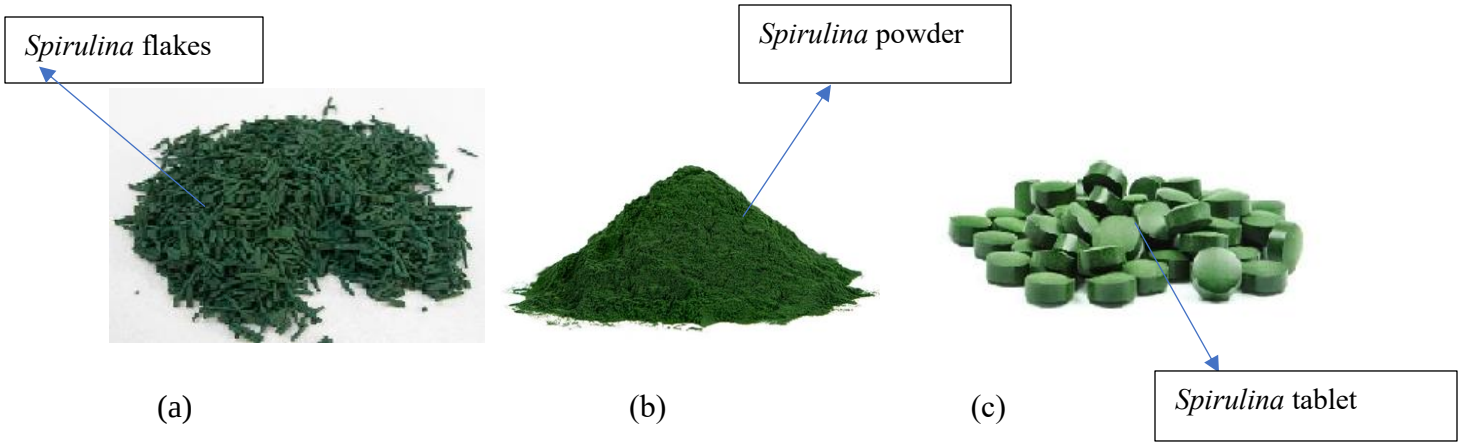


Figure 2. Dried *Spirulina* (a) *Spirulina* flakes (b) Powdered *Spirulina* (c) *Spirulina* tablet.

2.4.3. Harvesting system of *Spirulina*

The optimal time for harvesting *Spirulina* is early in the morning due to several reasons. Firstly, during this time, the *Spirulina* contains the highest percentage of proteins. Additionally, the cool temperature in the morning makes the harvesting process easier for workers. Moreover, there are more hours of sunlight available for drying the harvested product. Various techniques are used for harvesting, including filtration, flotation, centrifugation, precipitation, ion exchange, electrolytic, and ultrasonic vibration. To efficiently collect *Spirulina* from its medium, a filter or mesh cloth with a minimum size of 50 microns is used in the harvesting process.

2.5. Biochemical compositions of microalgae *Spirulina*

Spirulina has drawn the attention of nutritional scholars for decades due to its high micro- and macronutrient composition. Because of its concentrated nutrients, it is suitable for people of all ages and lifestyles. *Spirulina* is a fantastic natural nutrient source that has been used since ancient times. It is a good source of proteins, vitamins, fatty acids, minerals, and photosynthetic pigments. **(Figure 3).**

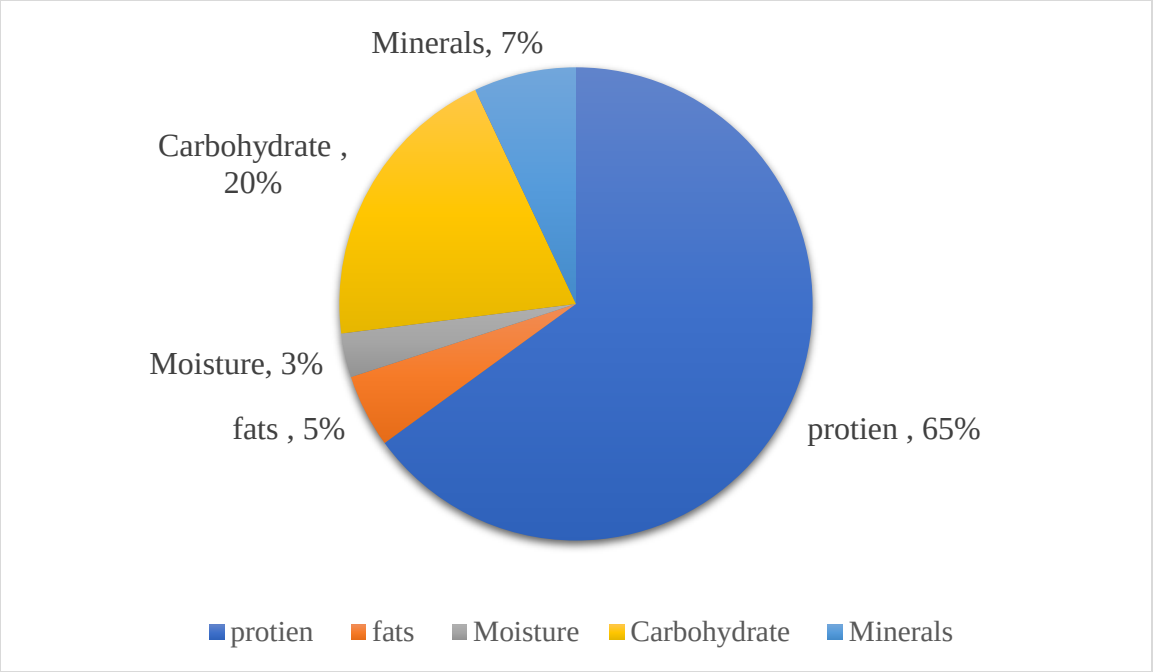


Figure 3. Biochemical compositions of microalgae *Spirulina*

2.5.1 Primary metabolites of *Spirulina.*

2.5.1.1. Proteins

Proteins are the building blocks of life; they are required for a healthy lifestyle. Proteins are used by the body to generate and repair muscles and other tissues. Proteins are degraded to amino acids, which are essential components of enzymes and hormones. Proteins are an essential component of every cell in the human body; hence they must be included in one's diet. *Spirulina* has a high protein content that ranges between 55 and 70% by dry weight(Teoh *et al.*, 2004). *Spirulina* consists of essential amino acids mainly leucine, valine, isoleucine, tryptophan, methionine, phenylalanine, L- theanine, and lysine(Colla *et al.*, 2007) as described in **(Table 2).** *Spirulina*, unlike other plant-derived proteins, is a complete protein, including all essential amino acids. Because it is a plant-based source of complete protein, it is an excellent dietary supplement choice for vegetarians. *Spirulina* cells do not have cellulose walls but relatively fragile envelope of murein which is one of its kinds in plant kingdom. This explains the very high digestibility of its proteins(Ciferri, 1983).

Table 2. Protein composition of *Spirulina*

No.	Amino Acids	Concentration ($g100g^{-1}$)
1	Leucine	4.94
2	Isoleucine	3.20
3	Valine	3.51
4	Tryptophan	0.93
5	Theanine	2.97
6	Lysine	3.02
7	Methionine	1.15
8	Phenylalanine	2.78
9	Total	22.5

2.5.1.2. Vitamins and Minerals

Spirulina sp. contains a variety of vitamins (B_1, B_2, B_3, B_6, B_9, and B_{12}), minerals (Ca, Cr, Cu, Fe, Mn, Mg, P, Se, Na, and Zn). The body requires minerals, which are vital inorganic nutrients, to carry out several activities and functions necessary for healthy functioning. Numerous nutrients like iron, zinc, sodium, potassium, phosphorus, manganese, magnesium, copper, and calcium are all adequately supplied by *Spirulina* shown table (3). *Spirulina* is significant economically because of its high iron concentration (**Table 3**). Magnesium, phosphorus, and calcium are present in quantities comparable to those in milk(Pyne *et al.*, 2017).

Table 3: Minerals composition of *Spirulina.*

No	Minerals	Concentration ($mg100g^{-1}$)
1	Iron	100
2	Copper	1.2
3	Calcium	700
4	Zinc	3
5	Sodium	900
6	Potassium	1400
7	Phosphorous	800
8	Manganese	5
9	Magnesium	400
10	Total	4309.2

2.5.1.3. Carbohydrate and Essential Fatty Acids of *Spirulina*

Carbohydrates make up about 15–25% of the dry weight of *Spirulina*. It contains mannitol, sorbitol, glycerol, sucrose, glucose, and fructose. A carbohydrate named mesoinositol is a top supplier of organic phosphorous. Mesoinositol content in *Spirulina* is eight times higher than in beef and significantly higher than in vegetables. According to recent studies, the *Spirulina* compound calcium *Spirulina* (Ca-SP) is a new sulfated polysaccharide(Pyne *et al.*, 2017) was summarized in (**Table 4).**

Table 4. Carbohydrate composition of *Spirulina*

No	Carbohydrate	Concentration (mg100g-1)
1	Glucose	54.4
2	Rhamnose	22.3
3	Mannose	9.3
4	Xylose	7
5	Galactose	3
6	Total	96

Microalgae are a source of long-chain polyunsaturated fatty acids (LCPUFAs) especially of the omega-6 family (ω6) such as γ-linolenic acid (GLA) and arachidonic acid (AA), and omega-3 family (ω3) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Omega 3 and 6 families are essential fatty acids - they cannot be synthesized *de novo* by humans; and therefore, must ingest from food (Goodhart & Shils, 1980; Martelli *et al.*, 2014). EFA aids in lowering triglyceride and total cholesterol levels, which are linked to arteriosclerosis and heart disease. Lipids make up a major portion of their cell membranes. They shield the cell membranes from antioxidant and free radical attacks when combined with vitamins E and A. Such attacks can change how nutrients are absorbed through cell membranes. The immune system could malfunction because of membrane injury because it may change the antigens.

In fact, the *Arthrospira spp.* is rich in γ-linoleic acid (**1**) (GLA; 30–35% of total PUFAs) and provides stearidonic acid (**2**), eicosapentaenoic acid (**3**), docosahexaenoic acid (**4**), and arachidonic acid (**5**) in figure 3. GLA (**1**), a unique fatty acid found in *Spirulina*, has apparently been proved to be an efficient immune protector. GLA - ω6 fatty acid is an essential fatty acid presenting anti-inflammatory properties. Although the human organism is capable of producing very long chain fatty acids from linoleic (AL) and alpha-linolenic acids (AAL), its synthesis is affected by several factors, which makes the ingestion of these fatty acids essential for the maintenance of a healthy condition (Goodhart & Shils, 1980; Sayeda *et al.*, 2015). During breastfeeding, arachidonic acid can be found in breast milk and the placenta. This acid is responsible for the development of babies, some studies indicate that the low intake of arachidonic acid from the diet of premature babies, can cause health problems for these children. For those who practice physical exercise, arachidonic acid is also important, it can be one of the keys to muscle development. Studies have shown that this acid has increased performance levels (Trappe & Liu, 2013). Lower levels of AA can contribute to neurological diseases such as Alzheimer (Rapoport, 2008) and Autism (Bell *et al.*, 2010; Vancassel *et al.*, 2001).

EPA (eicosapentaenoic acid), an omega-3 fatty acid, acts as a precursor of the prostaglandin-3, thromboxane-3, and leukotriene-5 groups and thus has anti-inflammatory effects in the body. This is a substance that is part of the defense against inflammation. By helping to neutralize the pro-inflammatory activity of other similar molecules. One of the main benefits of EPA is that it

supports heart health and blood circulation, prevents blood clots from forming in the blood, and reduces the risk of thrombosis and cerebrovascular events (stroke).

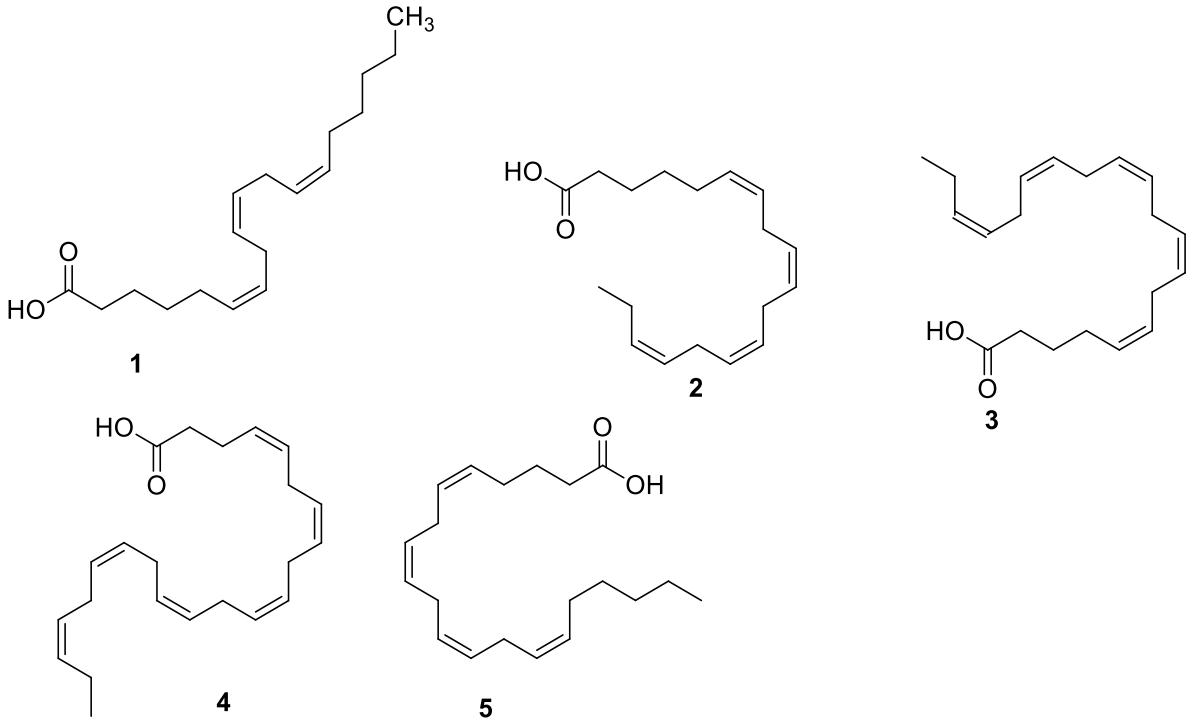


Figure 4. Some chemical structures essential fatty acid of *Spirulina*

However, the chemical characteristics of the species belonging to the same microalga category differ according to specific source, culture condition, harvest time, and extraction method, even if their appearance is similar. (Vonshak *et al.,* 1996) reported that salt-adapted cells had a modified biochemical composition with a reduced protein and chlorophyll content and increased carbohydrate content. *A. platensis* is one of the most promising sources of GLA which can be enhanced by cultivation under light–dark cycles in the laboratory or outdoors (Tanticharoen *et al.,* 1994). Therefore, the quality of the commercial *Spirulina* powder varies in different sources.

2.5.2. Secondary metabolites of *Spirulina*

2.5.2.1. Phenolic compounds

Phenolic compounds (PCs) also known as polyphenolics are bio compounds chemically composed of one or more phenolic rings that can be halogenated. These halogenations are responsible for the different biological activities (Cabrita *et al.*, 2010; La Barre *et al.*, 2010). Phenolic compounds are

considered as one of the most important classes of natural antioxidants. They protect both the cells and other natural body chemicals from the damage caused by free radicals. These are reactive atoms that contribute to tissue damage in the body.

The production of PCs from *Spirulina maxima* was studied. The authors have found mainly linotroban(**6**), gallic acid(**7**), cinnamic acid(**8**), *p*-OH-benzoic acid(**9**), chlorogenic acid(**10**) and vanillin acid(**11**), showing concentrations of 33.61, 19.82, 15.77, 14.18, 13.70 and 7.12% (based in the relative area), respectively (Custódio *et al.*, 2014).

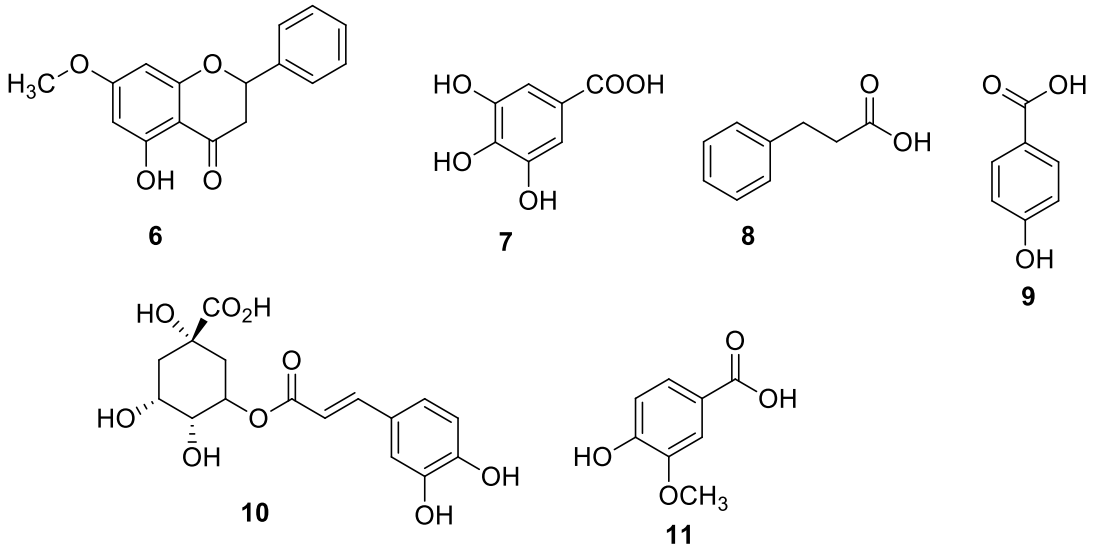


Figure 5. Some chemical structures of phenolic acid isolated from *Spirulina.*

A new extraction technique based on a combination of solid-phase/supercritical-fluid (SPE/SFE) to obtain phenolic compounds from *Spirulina platensis* was studied (Klejdus *et al.*, 2009). They have found 3,4-dihydroxybenzaldehyde (**12**) (853.85 ng/g of dried cell) and *p*-hydroxybenzoic acid (**9**) (604.94 ng/g of dried cell) as main compounds; and protocatechuic acid (13) (137.98 ng/g of dried cell), vanillic (**11**) (254.29 ng/g of dried cell), syringic (**14**) (3.45 ng/g of dried cell), caffeic acid (**15**) (169.52 ng/g of dried cell), chlorogenic acid (**10**) (72.11 ng/g of dried cell) and 4-hydroxybenzaldehyde (**16**) (107.35 ng/g of dried cell) as minority compounds.

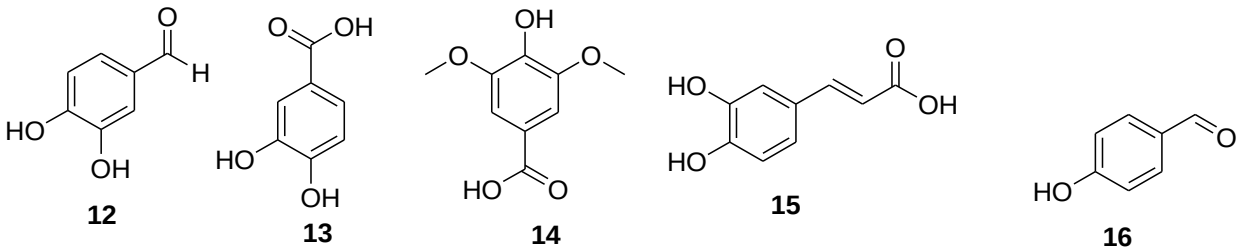


Figure 6. Phenolic compounds isolated from *Spirulina.*

Spirulina microalgae have more content of *p*-coumaric acid, which has antioxidant properties (Ferguson *et al.*, 2005) and ferulic acid that also has antioxidant properties were explained in (**Table 5).** Thus, *Spirulina* microalgae are rich sources of phenolic compounds that have antioxidants properties, among others interesting nutritional and health properties.

Table 5. Phenolic compounds in *Spirulina*

Phenolic compounds	***Spirulina*(ng/g)**
Phloroglucinol	51,000
***p*-coumaric acid**	920
Ferulic acid	0.97
Apigenin	6

2.5.2.2. Sterols

Sterols are lipids containing a ring formation in their chemical structures. Sterols play a fundamental role in the membrane integrity of microalgae (Caballero *et al.*, 2003). Cholesterol and sitosterol were found in *Spirulina maxima* (Nadal, 1971). The authors studied the correlation between sterols and antimicrobial activity. Similarly, three sterols produced by *Spirulina platensis* were identified: campestral(**17**), stigmasterol(**18**) and *β*-sitosterol(**19**); 8.7, 25.4 and 29.4% respectively(Hetta *et al.*, 2014).

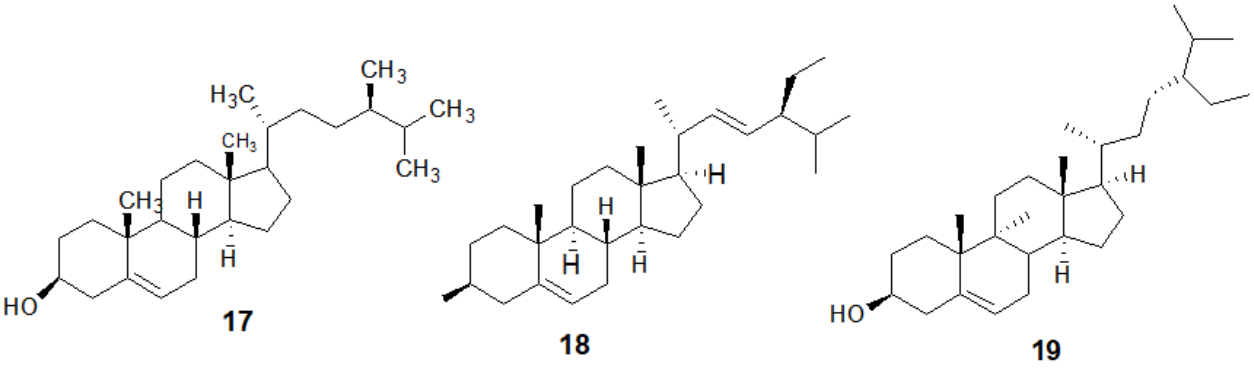


Figure 7. Some chemical structure of isolated sterols from microalgae *Spirulina*

2.5.2.3. Volatile compounds (VOCs)

In the last years the interest in microalgae volatile compounds (VOCs) has increased, mainly due to the diverse structural features and interesting biological activities of VOCs. However, VOCs can cause musty, fishy, and mud-like odors and produce harmful toxins. Besides microalgae VOCs, usually are not related to musty, fishy, and mud-like odor. VOCs present antibacterial, antifungal, antiviral and anticayj.uhni8jn/.ij,.hggcrnncer activities (Borowitzka, 1997; Mathew *et al.*, 1995)

Recently chemical profile of VOCs of *Spirulina platntesis* was determined and then, applied those VOCs in food products (Milovanović *et al.*, 2015a). As result, they obtained mainly (93.19%) hydrocarbons (medium length alkanes and alkenes) and in small quantities, odorous compounds, such as 2-methylisoborneol, 2-pentylfuran, β-cyclocitral, and β-ionone, as can be shown in (**Table 6)** and **(Figure 8)**.

Table 6: Composition of VOCs produced *by Spirulina platensis* (Milovanović *et al.*, 2015a)

Class	Compounds	Composition (%)
Hydrocarbons	2-pentylfuran (**20**)	3.03
	Tetradecane (**21**)	0.27
	Pentadecane (**22**)	6.48
	Hexadecane (**23**)	5.12
	6,9-heptadecadiene (**24**)	0.62
	Heptadecane (**25**)	77.67
Alcohols	2,6-dimetrylcyclohexanol **(26**)	2.41
	2-ethyl-1-hexanol (**27**)	0.38
	2-methylisoborneol **(28**)	0.98
Ketones	1,1,3-trimetryl-2-cyclohexanone (**29**)	0.38
	β-ionone (**30**)	1.59
Esters	1-tert-butyl-2,2-dimethyl-propyl ester (**31**)	0.45

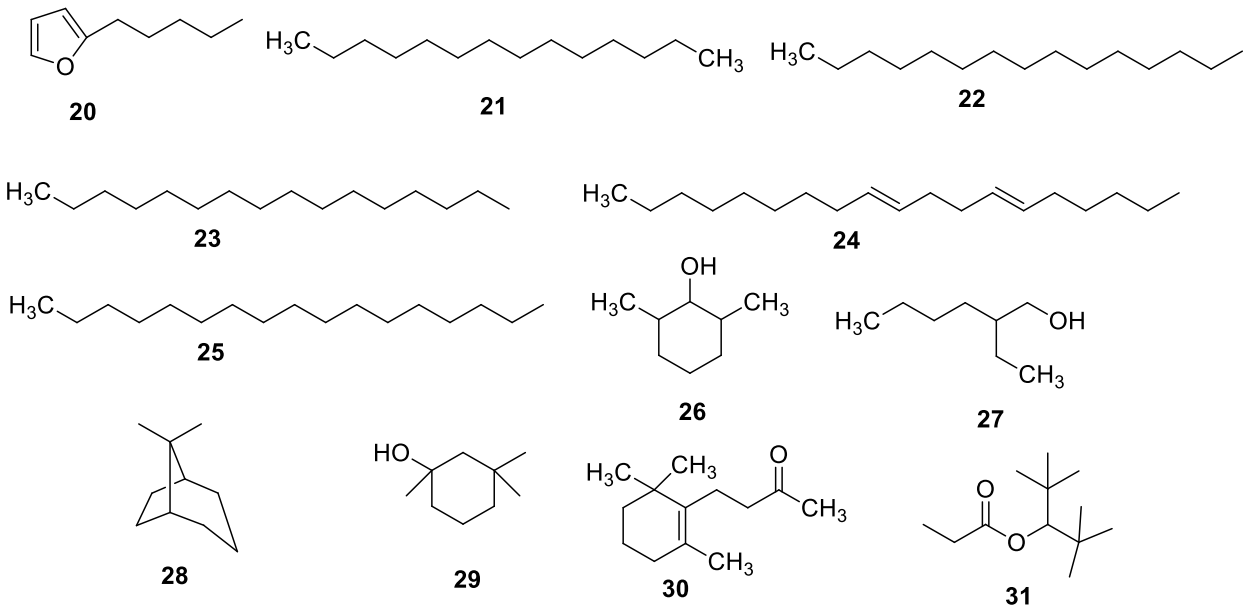
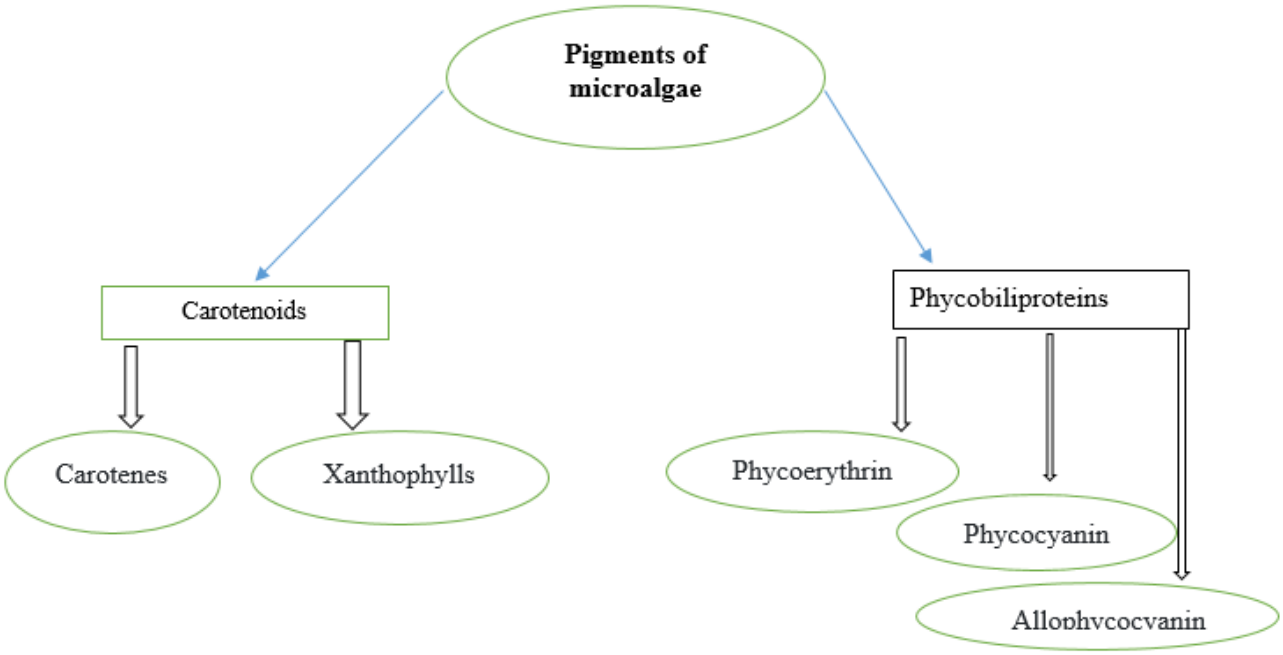


Figure 8. Some volatile compounds isolated from *Spirulina.*

2.5.2.4. Pigments, carotenoids and phycobiliproteins

Pigments are fundamental for photosynthetic algae metabolism. In other metabolisms such as human metabolism, pigments can act as antioxidant, anti-carcinogen, anti-inflammatory, anti-obesity, anti-angiogenic and neuroprotective. Carotenoids and phycobiliproteins are the two major classes of microalgae pigments. Carotenoids are subdivided into two groups: carotenes and xanthophylls (oxygenated derivatives of carotenes) as shown in (**scheme 1)**. Carotenoids are also widely used in the food industry as dietary supplements, fortified foods, food dyes, and animal feed. Phycobiliproteins, on the other hand, are a class of colored proteins with linear tetrapyrrole prosthetic groups (bilins). Phycobiliproteins are typically divided into three categories: phycoerythrin, phycocyanin, and allophycocyanin. Phycobiliproteins have the following potential applications: antioxidant, anti-inflammatory, anti-tumor, immunomodulating, radical scavenging, antiviral, and antifungal. (da Silva Vaz *et al.*, 2016; Gayathri *et al.*, 2016; Gong & Bassi, 2016; Manirafasha *et al.*, 2016).



Scheme 1. Classification of pigments in microalgae

Phycocyanin

Phycocyanin is the most active bioactive substance in *Spirulina* which has a content of 10%–20% of the dry biomass (Han *et al.*, 2021). The anti-oxidation activity of phycocyanin is related to its specific structure, and its deep blue color is due to the open-structured tetrapyrrole chromophore, and phycocyanobilin covalently linked to the apoprotein was shown Figure 8 (Hirata *et al.*, 1999; Hirata *et al.*, 2000). Phycocyanobilin can transform from the ground state to the excited state through electron transfer in redox reactions. The chemical structure of phycocyanobilin is like that of bilirubin, a highly specific inhibitor of triphosphopyridine nucleotide (NADPH) oxidase (Lanone *et al.*, 2005; Matsumoto *et al.*, 2006). This may be why phycocyanobilin inhibited NADPH oxidase Figure 8 shows the structures of phycocyanin (**32**), allophycocyanin **(33**), and bilirubin (**34**). As a natural blue pigment, phycocyanin is also one of the primary photosynthetic pigments and it can improve the immunity of the body (Takagi & Yoshida, 2006). It is challenging to effectively obtain phycocyanin from other food sources than *Spirulina.* It is worth noting that, the promising anti-oxidation and free radical scavenging capabilities of *Spirulina* may be depend on its phycocyanin content, and phycocyanin attributes its anti-oxidation activity to phycocyanobilin (Hirata *et al.*, 2000; Sagara *et al.*, 2015).

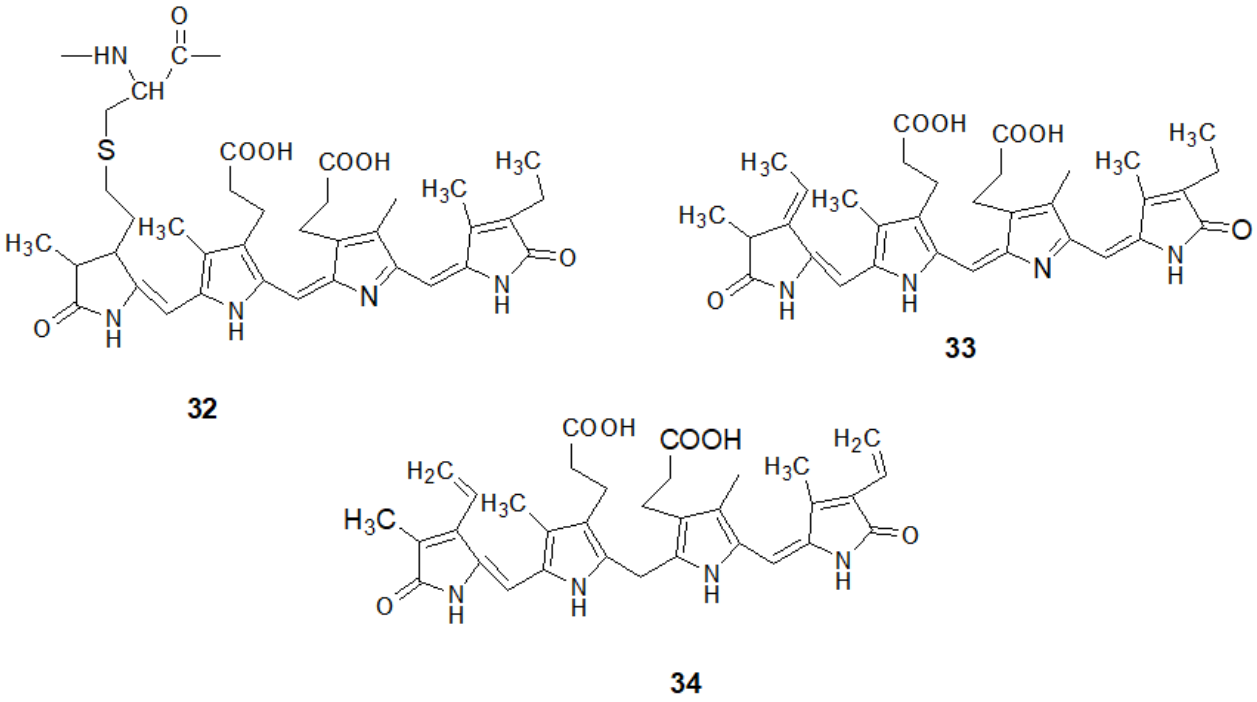


Figure 9. Structures of phycocyanin, allophycocyanin, and bilirubin (Wu *et al.*, 2016a).

2.6. Pharmacological Study of micro algae *Spirulina*

2.6.1. Anti-bacterial activities of *Spirulina*

Micro algal cultures of *Spirulina* have displayed significant anti-bacterial activity against six *Vibrio* strains: *Vibrio parahaemolyticus, Vibrio anguillarum, Vibrio splendidus, Vibrio scophthalmi, Vibrio alginolyticus, and Vibrio lentus* (Kokou *et al.*, 2012). Moreover, purified C-phycocyanin from *S. platensis* markedly inhibited the growth of some drug-resistant bacteria such as *Escherichia coli, Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*(Sarada *et al.*, 2011).

2.6.2. Antioxidant activities

Antioxidants can be defined as "natural or synthetic substances that may prevent or delay oxidative cell damage caused by physiological oxidants having distinctly positive reduction potentials, covering ROS/reactive nitrogen species (RNS) and free radicals" (Apak *et al.*, 2016). The antioxidant properties of *Spirulina* and its extracts have attracted the attention of many researchers. According to Miranda and colleagues, the antioxidant activity of a methanolic extract of *Spirulina* was determined *in vitro* and *in vivo*(Miranda *et al.*, 1998). Aqueous extract of *Spirulina* has a protective effect against apoptotic cell death via scavenging free radicals(Chu *et al.*, 2010). In addition, *Spirulina* has been shown to exert protective effects against oxidative stress caused by lead acetate in the rat liver and kidney(Ponce-Canchihuamán *et al.*, 2010). Notably, the c-phycocyanin from *Spirulina* has been found to be a strong free radical scavenger(Huang *et al.*, 2007). Phycocyanin was able to scavenge hydroxyl (IC_{50} ¼ =0.91 mg/mL) and alkoxyl (IC_{50} ¼ =76 mg/ mL) radicals with activity equal to 0.125 mg/mL of dimethyl sulfoxide and 0.038 mg/mL of trolox. In addition, the hepatoprotective effect of phycocyanin was exhibited due to its radical scavenging activity, and thus reducing the hepatoxicity caused by carbon tetrachloride and R-(þ)-pulegone(Vadiraja *et al.*, 1998). Studies *in vitro* and *in vivo* have shown that the antioxidant components produced by *Spirulina*(Reddy *et al.*, 2000) can prevent or delay oxidative damage by reducing the accumulation of ROS(Zhang *et al.*, 2011). Through the activation of the antioxidant enzyme systems of catalase, SOD, and glutathione peroxidase(Thaakur & Jyothi, 2007). Recently, Bhat and Madayastha reported that c-phycocyanin from *Spirulina* effectively inhibited CCl_4-induced lipid peroxidation in rat liver *in vivo* with an IC_{50} of 11.35 mM(Bhat & Madyastha, 2000)

3. MATERIALS AND METHODS

3.1. Chemicals and instruments

3.1.1. Chemicals

Organic solvents used in the present study include methanol, ethanol, petroleum ether, ethyl acetate, dichloromethane, chloroform, and acetone. Reagents such as K_2HPO_4, $NaNO_3$, K_2SO_4, $MgSO_4$. $7H_2O$, $CaCl_2.2H_2O$, $FeSO_4.7H_2O$, EDTA, $NaHCO_3$, Na_2CO_3, NaCl, sodium hydroxide, boric acid, Ammonium solution, ferric chloride, sulfuric acid, hydrochloric acid, ciprofloxacin , silica jell, and DPPH were used.

3.1.2. Apparatus and instruments

Beakers, conical flasks, capillary tube, measuring cylinders (10, 100, 1000, 2500 ml in capacity), sample bottles, funnel, TLC tank, glass rod, glass columns of diameter 30/32mm and 19/26mm for chromatography. Analytical balance weighing, mortar and pestle for grinding dried samples, A rotary evaporator, digital measuring balance, 500 1000, 2000 mL Erlenmeyer flask, TLC plate (pre-coated aluminum sheet, 20 × 20 cm), silica gel with a particle size of 230-400 mesh, UV lamp (254 nm and 365 nm) , pipette, spatula, test-tube with a test-tube rack, vials, filter paper, refrigerator, Micro pipette, Glass aquarium tank (capacity 20L or more), aeration pump, fluorescent light, Oven for drying sample and cotton wool, P^H meter and light microscope , ciprofloxacin for positive control were used.

NMR-spectroscopy- ^1H and ^{13}C NMR spectra were recorded on 600 MHz Bruker NMR spectrometer using $CDCl_3$ and CD_3OD solvents. The chemical shifts were reported in δ unit (ppm).

IR-spectroscopy. FT-IR spectra were obtained on a Perkinelmer apparatus. The spectra were measured in KBr pellets and presented in cm^1.

UV-Vis spectroscopy. λ max for each purified compounds were recorded on PG instrument UV-Vis double beam spectrometer (T80+ model) having scanning range from 190-2100 nm.

3.2. Description of the sample collection

The species of *microalgae Spirulina fusiform* used in this study was obtained from its natural habitat - Lake Chitu which is a shallow freshwater lake located at south point of Abijata-shalla national park (7° 23′N 38° 24′ E), 287 km south of Addis Ababa, Ethiopia **(Figure 10).** The area

is found at an altitude around 1650m above sea level with annual rainfall ranging between 500 mm and 700mm.

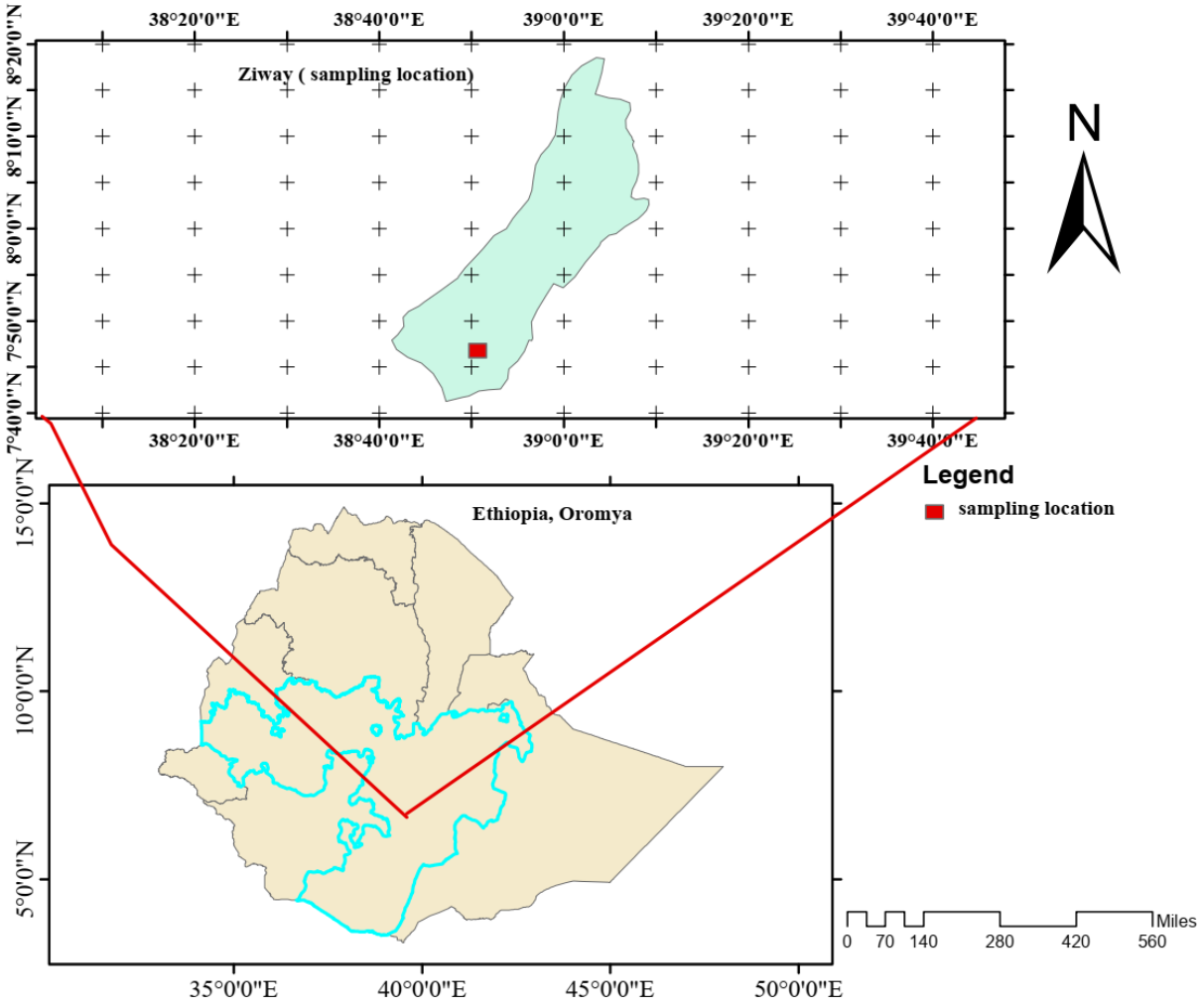


Figure 10. Map of study areas drawn with GIS software drawn by Mr. Diginet

3.3. Experimental procedures

3.3.1. Collection of Algal Samples, Isolation and Mass culture of *Spirulina*

3.3.1.1. Collection of algal samples

Spirulina dominated water bloom samples were collected by professionals from three sites within the water body of the lake on March 14/2023. The water samples were taken from a surface depth of 10 to 50 cm using plankton net with mesh sizes between 64µm. The amount taken was 1000 mL for each sample collected in a plastic bottle and then labeled. Measurements of some physical and chemical parameters, which are believed to have a bearing effect on the species composition

and production of *Spirulina*, were carried out *in situ.* pH and conductivity were measured in situ using digital pH meter (model Jenway 370) and conductivity meter (model Elmeirron CC-505) respectively.

3.3.1.2. Isolation of *Spirulina*

Trichome of *Spirulina* was isolated from sampled water collected from Lake Chitu by the serial dilution method which was developed in the Institute of Pharmaceutical Science (IPS). The samples were transferred to containers. The flasks were examined for algal growth using an optical microscope every two days, with serial dilutions made in Zarrouk's medium from flasks showing growth. The analytical grade ingredients for Zarrouk were purchased from a laboratory equipment and chemical supplier in Addis Ababa. Zarrouk medium was prepared in 2 parts (stock solution I and Stock solution II) as shown in **(Table 7)** and **(Table 8)** to increase solubility when autoclaving. For each part, components were added to around 900 ml distilled water and then was filled up to 1L with distilled water. 10 mL of micronutrient was added to stock solution II. Micronutrient stock solutions are prepared in composition as listed in **(Table 9)** and stored frozen at -20 °C. Then part 1 and 2 were autoclaved separately at 121°C and 15 psi for 15min. After being allowed to cool then the parts were mixed in 1:1 ratio aseptically.

Table 7: Preparation of stock solution I

Inorganic Compound	Mass used in gram	Total Volume
$NaHCO_3$	27.12	In 1000 mL of distilled water
Na_2CO_3	8.06	
K_2HPO_4	1	

Table 8: Preparation of stock solution II

Inorganic compounds	Mass used in gram	Volume	Total volume
$NaNO_2$	5		
K_2SO_4	2		
NaCl	2	In 990 mL of distilled	
$MgSO_4.7H_2O$	0.097	water	1000mL
$CaCl_2.7H_2O$	0.08		
$FeSO_4.7H_2O$	0.02		
Na_2 EDTA	0.16		
Micronutrient Solution		10ml	

Table 9. Preparation of micronutrient solution

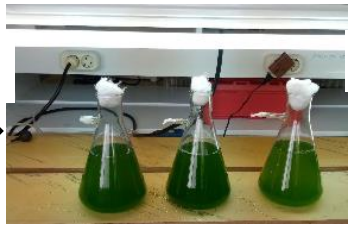
Compounds	Concentration in 100ml of distilled water	Volume taken	volume	Total volume
$ZnSO_4.7H_2O$	0.1 g	1 ml	19ml plus 881ml of distilled water	1000 ml
$MnSO_4.4H_2O$	0.1 g	2 ml		
H_3BO_3	0.2 g	5 ml		
Co $(NO_3)_2.6H_2O$	0.02 g	5 ml		
$Na_2MoO_4.2H2O$	0.02 g	5 ml		
$CUSO_4.5H_2O$	0.005 g	1 ml		
$FeSO_4$	0.7 g		In 100 ml of distilled water	
Na_2EDTA	0.4 g			

3.3.1.3. Mass culture of *Spirulina*

After isolation of pure *Spirulina* with the help of serial dilution 100ml of *Spirulina* culture (0.038 g dry biomass) was transferred in three 500 ml flasks and inoculated with 400ml zarrouk media. The culture was incubated at a temperature ranging from 25 to 30 °C under continuous fluorescent light (36Watt) and a photoperiod of 12/12 hr. light/dark cycle. The pH of the culture before inoculation was recorded. The culture was continuously agitated using aerators fixed on the air pump daily to facilitate gas exchange and avoid settling. The culture was checked under the microscope for contamination detection and was purified by raising the pH and serial dilution

techniques to obtain the uni-algal culture. After attaining enough cell density in the 500ml flaks inoculums (exponential growth phase) were transferred to 1000ml flasks. The same protocol was used to get enough cell density in both 1000ml and 2000ml. For biomass indoor culture each 2000ml *Spirulina* cultures were transferred to aquarium tanks, (119 cm height, and 216 cm diameter). The cultures were exposed to an artificial light source from fluorescent lamps (two 36 watt each), providing a photon flux density of about 40 μmol photon $m^{-2}s^{-1}$ on the surface of the culture. The purity and the overall maintenance of isolated cells in the cultures were conducted by frequent follow up light microscopic investigation. The growth of *Spirulina* was noticed every day by observing the intensity of the green color. During the process of their growth fresh sterilized Zarrouk media was also added to the cultures to improve nutrient availability for their growth. Several successive transfers have been also carried out using Zarrouk media to purify the cultures and refresh them to increase their concentration for further analysis and treatment. Biomass harvesting cells of *Spirulina* were harvested from log phase cultures by suspending on a silk cloth for filtration and the cell pellets were washed three times The collected biomass of *Spirulina* was then dried in oven at 40°C for 48hrs, powdered by manual mortar and then stored in room temperature for future steps.

21 days



21 days

2000 mL Flasks

Spirulina in 500 mL

Occasional Shaking

Microscope investigation

Media addition every 4/5 days

1000 flasks

21 days

Spirulina in aqua

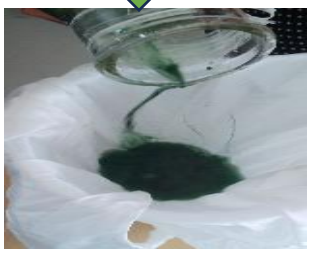
Occasional Shaking

Microscope investigation

Media addition every 4/5 days

Aqua

21 days



Harvesting process

Drying Process

Powdered spirulina

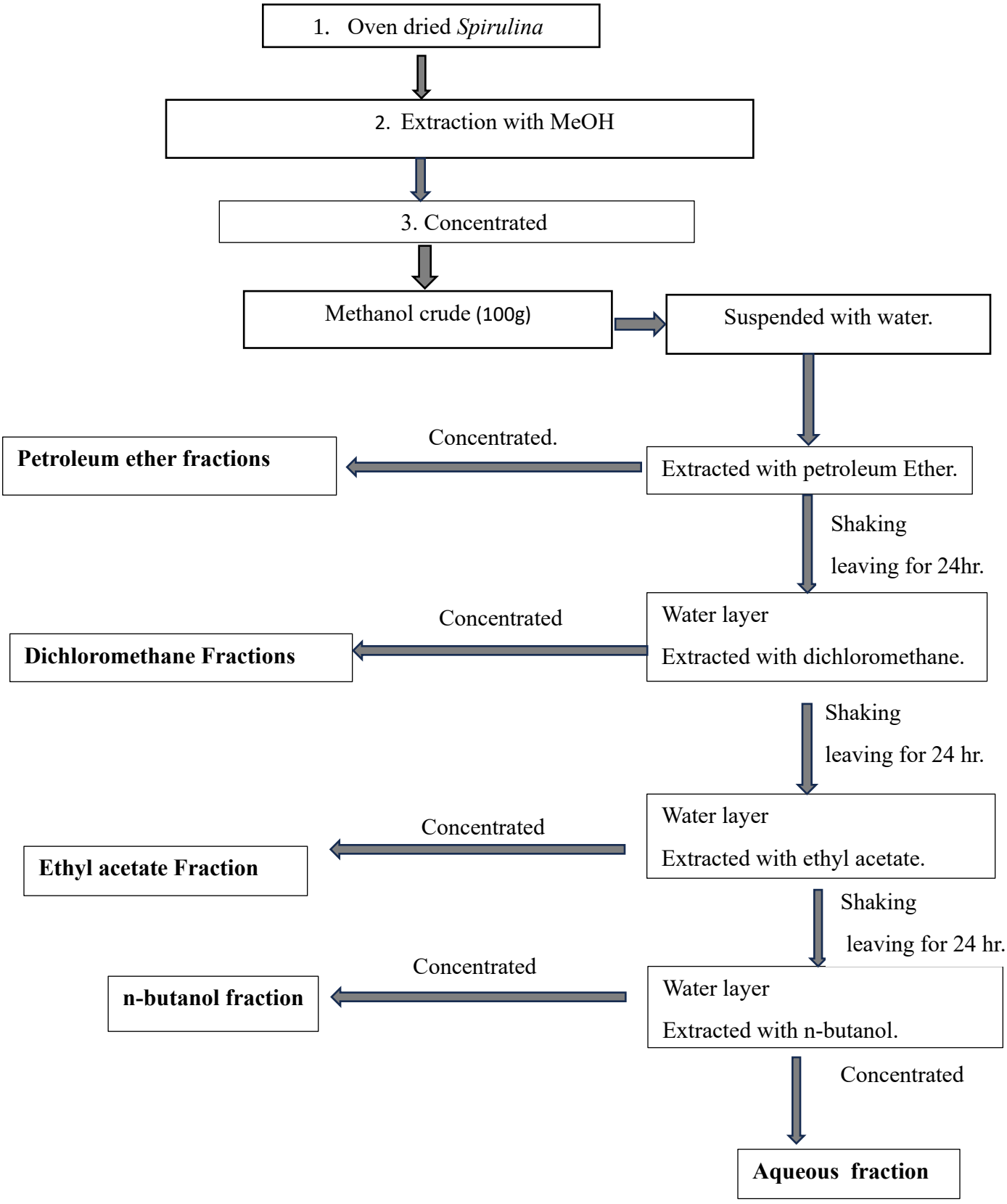
To ensure the ongoing fertility of the culture medium, the nutrients that were taken out by the harvested biomass were replaced. Throughout the entire duration of the experiment, we maintained the availability of nutrients and other growth conditions to avoid any contamination and ensure the successful cultivation of Spirulina.

3.3.2. Extraction

3.3.2.1. Preparation of microalgae extracts via liquid-liquid partitioning.

The dried samples were pounded to coarse powder using mortar and pestle. 180 g of the powdered sample were soaked with two liters methanol in conical flask and subjected to a sonicators to disrupt the cell wall of *Spirulina* for 2 hrs. The flask was then placed on an orbital shaker and shaken for 24 hrs at a speed of 120rev/min. The solution was filtered using Whatman No. 1 filter paper. To exhaust the *Spirulina*, the above procedure was repeated two times. The filtrates were combined and dried with anhydrous sodium sulphate, and concentrated using Büchi Rotavapor (Rotavapor R114, Büchi Labortechnik, Flawil, Switzerland) at a reduced pressure and a temperature of at or below 40ºC.

The dried MeOH extract(100g) was dissolved in distillated water (5 x mass) and consecutively partitioned with equal volumes of petroleum ether (PE), Dichloromethane (DCM), Ethyl acetate (EtOAc) and butanol (BuOH). Each solvent fraction was filtered and concentrated with rotary evaporator at 35º C. All extract was stored in refrigerator at 4 ºC until required for further analysis. The extracts were analyzed with TLC for further fractionation. This has been summarized in **(Scheme 2)**.



Scheme 2. General outline for the extraction of microalgae *Spirulina Fusiformis*

3.3.2.2. Extraction of essential oils

Powdered *Spirulina* (61 g) was subjected to hydro-distillation with a Clevenger-type apparatus for 3 hr. Heat was supplied from the heating mantle and the essential oil was extracted until no more essential oil was obtained. The essential oil from microalgae was extracted with diethyl ether solvent. The organic layer is dried under anhydrous sulphate concentrated and stored at 4ºC until used.

3.4. Qualitative analysis of the chemical constituents of extracts

Phytochemicals are naturally occurring chemicals found in nature and the term is often used to describe the large number of secondary metabolite compounds found in nature. Screening the phytochemical profile of a crude extract is a valuable tool for understanding the chemical composition of the extract and its potential benefits and risks. It can be used to guide the further development and use of the extract. Phytochemical screening of the classes of compounds was carried out first to establish the presence of secondary metabolites. The phytochemicals test to screen the presence of the following phytochemicals were carried out using the standard procedures as described by (Falodun *et al.*, 2006) (Banu & Cathrine, 2015; Ekwueme *et al.*, 2015).

***Test for flavonoids*:** 3mg of extracts was treated with conc. H_2SO_4 solution. The formation of a yellowish orange color indicates the presence of flavonoids.

***Test for saponins (Foam test)*:** 3mg of extracts was diluted with 5mL of distilled water, shake vigorously and was observed for a stable persistent froth.

***Test for tannins*:** To a 3mg of extracts, a few drops of 10% ferric chloride solution were added. The appearance of a green or blue color indicates the presence of tannins.

***Test for steroids*:** 3mg of extract was mixed with 1mL of chloroform and 2-3 drops of conc. H_2SO_4 was added to it. The appearance of a pink or red color indicates the presence of steroids.

***Test for Terpenoids (Salkowski test)*:** 3mg extracts were mixed with 2mL of chloroform and 3mL of conc. H_2SO_4 solution. A reddish-brown color at the interphase indicates the presence of terpenoids.

***Test for alkaloids (Wagner' test)*:** 3mg of the extract was treated with diluted HCl, filtered followed by addition of Wagner's reagent (1.27 g of iodine and 2g of KI along with 100mL of distilled water) and observed for the formation of reddish-brown colored precipitate.

Test for Phytosterols (Libermann-Burchard's test): A small amount of the extract (50 mg) is dissolved in 2 ml of acetic anhydride and few drops of concentrated sulphuric acid is added. An array of color change shows the presence of phytosterols.

Test for coumarins: 1.5ml of the extract was mixed with few drops of alcoholic sodium hydroxide in the watch glass; the appearance of yellow colour indicated the presence of coumarin.

3.5. Isolation of compounds

Thin layer chromatography (TLC) was performed using precoated aluminum-backed supported silica gel 60 F254 (0.3mm thickness). The bands separating on the TLC plate indicate the separation of compounds which were detected under UV absorbance at 254 and 366 nm UV light source. Chromatograms were further visualized by immersing the TLC plates with vanillin reagent and subsequently heating them with a heat plate until colored spots appeared. The TLCs were repeatedly improved by changing the solvent systems until a system that gives the best separation.

Based on the TLC profile dichloromethane extract was chosen for column chromatography. In this experiment, a glass column with a stopcock at the bottom is used for column chromatography. The column (30/32 mm in diameter and 40 cm long) was mounted vertically at a retort stand. 360 g of silica gel was mixed with petroleum ether HPLC grade, the slurry was stirred thoroughly to ensure all the air bubbles were removed. The prepared stationary phase was poured carefully into the column to about 28cm height of the column. At each addition, the solvent level was above the silica gel, and a clean long glass rod was used to remove any stained gas bubbles by continued stirring. 2g of the dichloromethane crude extract was adsorbed on 2g of silica gel loaded on the top of the column and subjected to column chromatography and eluted with a gradient solvent system such as **(Table 10)** of hexane - EtOAc and EtOAc – MeOH (100:0 to 50:50 v/v). A small piece of cotton wool was fixed just above the silica gel to protect the column. A total of 385 fractions of each 10 mL-100ml (Table 10) were collected and concentrated using a rotary evaporator. Each fraction was analyzed by TLC and visualized using an Ultraviolet (UV) lamp at 254 and 365 nm.

Table 10 . Column chromatography fractionation of crude extracts

No	Solvent System	Ratio (%)	Total Volume (ml)	Ranges of fractions	Remarks
1	Petroleum ether	100	100	Not collected	
2.	Petrolium ether /Ethyl acetate	95/5	100	F1-F6	sugar like
3	Petrolium ether /Ethyl acetate	90/10	200	F7-F20	Colorless
4	Petrolium ether /Ethyl acetate	85/15	100	F21-24	colorless
5	Petrolium ether /Ethyl acetate	80/20	300	F25-F51	Yellow
6	Petrolium ether /Ethyl acetate	75/25	300	F52-F84	Green
7	Petrolium ether /Ethyl acetate	70/30	100	F85-F90	Green
8	Petrolium ether /Ethyl acetate	65/35	200	F91-F107	Light brown
9	Petrolium ether /Ethyl acetate	60/30	200	F108-F124	Brown
10	Petrolium ether /Ethyl acetate	55/45	300	F125-F151	Deep green
11	Petrolium ether /Ethyl acetate	50/50	200	F152-F175	Green
12	Petrolium ether /Ethyl acetate	45/55	100	F176-F180	Light green
13	Petrolium ether /Ethyl acetate	40/60	100	F181-F201	Color less
14	Petrolium ether /Ethyl acetate	30/70	200	F202-F222	Colorless
15	Petrolium ether /Ethyl acetate	20/80	200	F223-F240	Yellow
16	Petrolium ether /Ethyl acetate	10/90	200	F241-F250	Colorless
17	Ethyl acetate	100	200	F251-292	Yellow
18	Ethyl acetate/Methanol	99.5/0.5	100	F-293-296	Orange color
19	Ethyl acetate/Methanol	99/1	100	F287-F308	Red
20	Ethyl acetate/Methanol	98/2	100	F309-F314	Dark Red
21	Ethyl acetate/Methanol	95/5	100	F315-F320	Brown
22	Ethyl acetate/Methanol	90/10	100	F321-F333	Colorless
23	Ethyl acetate/Methanol	80/20	100	F334-F337	Colorless
24	Ethyl acetate/Methanol	70/30	200	F-338-F351	Colorless
25	Ethyl acetate/Methanol	60/40	200	F352-F361	Colorless
26	Ethyl acetate/Methanol	50/50	200	F362-F385	Colorless

The fraction with the same TLC profile were mixed and purified further using column chromatography and preparative thin layer chromatography (PTLC). The same protocol was used to pack a smaller column (19/26 mm in diameter and 50 cm long) to a height of 30 cm with silica gel for re chromatography.

3.6. GC-MS analysis

GC-MS analysis of essential oils and fractions was performed by a GC (7890B, Agilent Technologies, USA) coupled with an MS (5977A Network, Agilent Technologies) in the central analytical lab in Andong National University. The GC had an HP- 5MS column (30 m × 250 μm internal diameter and 0.25 μm). Helium was used as a carrier gas (flow rate 1 mL/min). The injector temperature was 230 °C and the injection mode was split mode (4:1). The initial oven temperature was 40 ° C for 3 min and raised up from 40 to70 ºC at the rate of 4 ° C/min and held at isothermal for 3 min and raising with no holding time to 80 º C at 1 ºC/min, 100 °C at 4 °C/min then to 180 ºC at 10 ºC/min and then 20 °C/min until it reached 280 ºC and held at this temperature for 2 min with the total run-time of 60 min. Mass spectra were recorded in electron-impact mode, with ionization energy of mode at 70 eV, scanning the 33-500 m/z range.

Since retention time in GC depends on experiment condition, retention index was introduced to reduce such dependency. The retention index (RI) was calculated using the method proposed by kovaat,(van Den Dool & Kratz, 1963) a generalization of the retention index system including linear temperature programmed gas—liquid partition chromatograph (Eq. 1), where *I* is the retention index calculated by substituting the retention times of the sample (Chen *et al.*) and the earlier and later eluting reference molecules (*M*) with the number of carbon atoms (*n*) and n+1, respectively. Usually, this is based on the retention times of the homolog row of n-alkanes (in our case C7-C40). Identification of the compounds was achieved by comparing their Kovats retention indices and mass spectra with those reported in the literature and supplemented by the NIST Standard Reference Database 69: *NIST* chemistry *Webbook*. (1)

$$I = 100i\ \frac{X-M(n)}{M(n+1)-M(n)} + 100n \qquad \text{.....eq. (1)}$$

3.7. Biological Activity assay

3.7.1. Paper disk diffusion assay

The antibacterial activity test of methanol extracts and isolated compounds of *Spirulina* against two gram-positive (*Staphylococcus aureus* and *Streptococcus pyogenes*) and two Gram negative bacteria (*Pseudomonas Aeruginosa* and *Escherichia coli)* were carried out by disc diffusion method. Twenty milliliters of sterile Mueller Hinton agar (maintained at 45-500ºC in a molten state), poured into sterilized Petri dishes. After solidifying Muller Hinton agar, 0.1ml of test

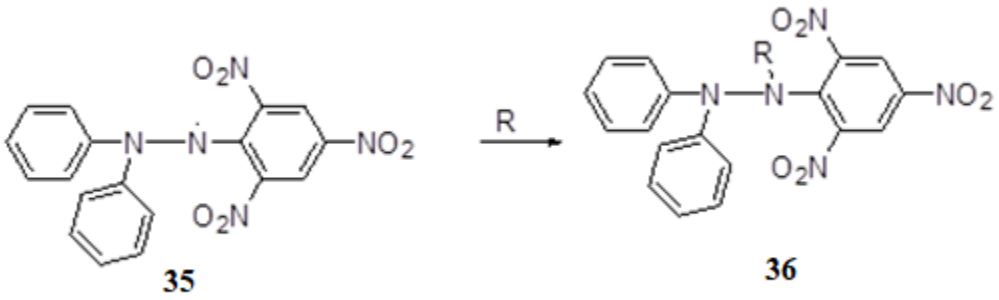
organism were inoculated with the help of sterile cotton swab. On MHA plate. Sterile agar-disc diffusion previously soaked in a known concentration (2 mg/100 µL, 1.5 mg/100µL and 1.25 mg/100 µL) of methanol extract, isolate compounds and standard drug ciprofloxacin were prepared in DMSO and carefully placed on the surface of the agar plate at equal distance from each other and 15 mm from edge of the plate as described by (Miller *et al.*, 2012). Each disc was pressed down to ensure complete contact with the agar surface and each plate was inverted and placed in an incubator set at 37 ºC for 18-24hrs. The antimicrobials susceptibility was evaluated after 18hrs. By measuring zone of inhibition using plastic ruler in mm and the result were compared with the standard drug ciprofloxacin as a reference standard and DMSO soaked discs were placed as negative control.

3.7.2. Antioxidant activity

In vitro antioxidant activity of the extract, and isolated compounds were measured based on their free radical scavenging activity, which was determined by the 1, 1diphenyl-2-picryl-hydrazyl (DPPH) method. A DPPH solution (0.1 mM) was prepared by dissolving 0.0394 g DPPH in 1000 mL methanol. The solution was kept in the dark for 30min. To complete the reaction methanol crudes and isolate compounds were dissolved in DMSO to give 1 mg/mL; the solutions were serially diluted to give concentration of 10, 5, 2.50 and 1.25 µg/mL. To 1 mL of each concentration, 1 mL DPPH was added. The mixture was shaken vigorously and incubated at 37°C for 30. Then absorbance was recorded at 517 nm using double beam UV-Vis spectrophotometer(Al-Dhabi & Valan Arasu, 2016). Ascorbic acid was used as standard. The percentage inhibition was calculated using the following formula:

$$\%radical\ scavenging\ activity = \frac{Ab\ control - Ab\ sample}{Ab\ control} \times 100 \ldots\ldots\ldots eq.2$$

Where Ab control was the absorbance of the DPPH solution and Ab sample was the absorbance of a tested sample. The analysis was performed in triplicate. The DPPH radical reacts with suitable reducing agent producing new bond, thus changing the color of solution. The solution loses color with the increase in the concentration of antioxidant as the electrons taken up by DPPH radical from the antioxidant(C.-A. Calliste *et al.*, 2001; C. Calliste *et al.*, 2010). DPPH (35) is a stable free radical that can accept an electron or hydrogen radical to become a stable diamagnetic molecule **(Scheme 3).**



Scheme 3. Reaction of DPPH (-35) radical with other radicals (•R = •H, alkyl radical etc.)

In the presence of the sample, the bleaching rate of the stable free radical, DPPH, will be measured at a specific wavelength. DPPH absorbs at 570 nm in its radical form, but not in its reduced form. Its absorption is reduced by an antioxidant or a radical species.

IC_{50} was determined from excel curve after plotting % radical scavenging activities and concentration (µg/ml) in excel. The % radical scavenging activity of isolated compounds data were entered into excel sheet in adjacent columns and then the graph was plotted with a scatter diagram. From the plotted curve, IC_{50} was determined by using the equation (3).

$$Y = mx \quad \text{.....eq. 3}$$

Where, Y is % of inhibition, x is Concentration of compound and m is slope of line.

3.7.3. Sun Protection Potential

The sun protection factor was determined using a modified method reported by (Namukobe *et al.*, 2021). Each crude extract (0.1 g) was dissolved in 50 ml of methanol to make a solution of concentration 2 mg/ml without ultra-sonication. The absorption data of each sample was measured using a Shimadzu UV-VIS double beam spectrophotometer against methanol as a blank. The absorption data were obtained for every 5 nm interval between the ranges of 290 to 320 nm, the observed absorbance values at 5 nm intervals (290-320 nm) were calculated by using the equation (4).

$$SPF = CF\ X \sum_{\lambda=290}^{\lambda=320} \mathrm{EE}(\lambda)\ \mathrm{X}\ \mathrm{I}(\lambda) X\ abs \ldots\ldots\ldots\ldots eq.(4)$$

Where CF is the correction factor (=10), EE is the erythemal effect spectrum, I is the solar intensity, and Abs is the absorbance. The values of EE × I are constants. They were determined(Mishra *et al.*, 2012)

3.8. Data analysis

The antimicrobial analysis data generated by triplicate measurements were reported as mean ± standard deviation. The collected data for the antibacterial activity of the extracts, and isolated compound against the tested bacteria were analyzed by using SPSS software, version 22.

4. RESULTS AND DISCUSSION

4.1. Indoor Cultivation of *Spirulina*

In this study, the indoor cultivation of *Spirulina* was successful. The indoor cultivation method helped to minimize contamination risks and maintain the desired characteristics of *Spirulina*, although it faced numerous obstacles. The main challenge was to maintain optimal growth conditions, including the appropriate temperature and light. On the 13th day, the cultures pH was gradually increasing from 9.46 to 10.35 in indoor testing. (Belay 2013) stated that *Spirulina* is usually cultivated in a medium in which the pH ranges between 9 and 10. In this study, *Spirulina* kept growing, even at a pH above 11 (maximum pH was 11.3).

4.2. Identification of *Spirulina*

The *Spirulina* contained water sample collected from Lake Chitu was observed under light microscope at 400x magnification power with ocular micrometer and most of the *Spirulina* in the lake were tightly coiled (H- type), with a few loosely coiled ones (**Figure 11**).

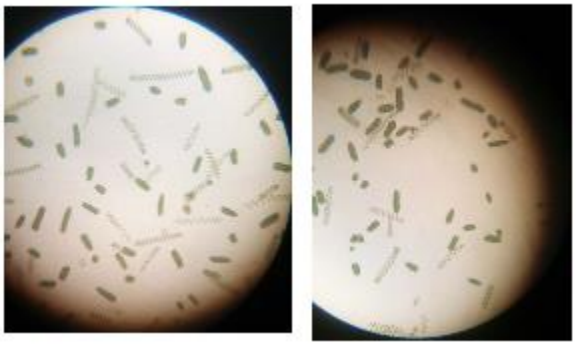


Figure 11. Lake Chitu water bloom samples (wild *Spirulina*) under light microscope

However, after cultivated *Spirulina* in the laboratory, there were three distinct morphotypes: tightly coiled (H-type), loosely coiled (S-type), and intermediately coiled (C-type) (**Figure 12**). The observed morphotypes weren't fixed, but rather flexible. Growing conditions, particularly light and nutrient availability, played a key role in shaping their form. For example, tightly coiled H-types could transform into loosely coiled S-types, and S-types could further evolve into intermediately coiled C-types. This suggests that the environment in which *Spirulina* grows can influence its morphology

a) b) c)

Figure 12. Cultured *Spirulina's* different morphotype (a) tightly coiled, (b) loosely coiled and (c) intermediately coiled.

As shown above the morphotypes were mainly explained by the difference in degree of coiling as highly coiled (H-type), loosely coiled (S-type) and intermediately coiled (C-type). Several studies using *Arthrospira* isolates from Indian freshwaters (Jeeji Bai & Seshadri, 1980) and saline–alkaline lakes of Kenya (Ballot, 2004) revealed three different morphotypes with tightly coiled, loosely coiled and intermediate coiled comparable to the morphotypes observed in our study. We found the same result as (Ogato & Kifle, 2014) did, three morphotypes of *Arthrospira* were identified from a sample collected from Lake Chitu, Ethiopia. In a more recent study by (Li *et al.*, 2001) two morphotypes of *Arthrospira* were observed in samples and subcultures from Lake Chitu. These morphotypes were characterized as tightly and loosely coiled trichomes. However, the intermediately coiled type was not reported in their study. The present study collected samples over an extended period of time and observed the intermediately coiled type.

In samples and subcultures of *Arthrospira* from Lake Chitu, (Li *et al.*, 2001) observed two morphotypes with tightly and loosely coiled trichomes. However, they found that the morphotypes belonged to the same species on the basis of 16S rRNA (100% similarity). (Jeeji Bai & Seshadri, 1980) studied three distinct morphotypes of isolates from Indian waters and concluded that they all belong to the same species, despite their morphological differences, particularly in the degree of coiling. Similarly, (Ballot, 2004) found different morphotypes in their phylogenetic study of *Arthrospira* from Kenyan saline-alkaline lakes and Indian freshwater bodies, but all showed 100% similarity in 16S rRNA. A more recent phylogenetic study on *Arthrospira* from Africa, Asia, and America by (Dadheech *et al.*, 2010)also found no habitat-dependent phylogenetic differences among the strains. These studies, along with others, have shown that different morphotypes of

Arthrospira species have 99.7-100% similarity in 16S rRNA gene sequences (Ballot, 2004; Dadheech *et al.*, 2010; Li *et al.*, 2001; Nelissen *et al.*, 1994) supporting the idea that different morphotypes could be genetically identical species, and vice versa. Furthermore Reports indicated that Lake Chitu is known for its monoalgal population of *Arthrospira* fusiformis (Kebede, 1997; Kebede *et al.*, 1994). Therefore, based on this genetic evidence, it is likely that the various morphotypes of *Arthrospira* observed in this study belong to a single species, *A. fusiformis,* which exhibits the unique morphology of the East African morphotypes.

4.3. Extraction yield

The extraction yield measured the solvent efficiency to extract specific components from the original material. The first extraction with methanol achieved a high yield of 63.89%, indicating it efficiently extracted a large portion of the compounds. This initial extract was then further separated into three parts using different solvents: petroleum ether, dichloromethane (DCM), and ethyl acetate. These secondary extractions yielded light-yellow oil (1.3 g), reddish-brown crude (2.41 g), and brown crude extract (2.3 g), respectively. The percentage yields of crude extract in respective solvent were listed in (**Table 11**). The DCM and EtOAc fractions yields were comparable and greater than those of petroleum ether extract which indicated *Spirulina* contains medium polarity compounds.

$$\%yeild = weight\ of\ extract \frac{1}{weight\ of\ the\ sample}\ x\ 100\% \text{.....eq.5}$$

Table 11. Percentage yield of fractions obtained from the crude methanol extract.

No	Organic extract	Mass of crude extract	Yield in %
1	PE	1.3g	1.44
2	DCM	2.41g	2.67
3	EtOAC	2.3g	2.55

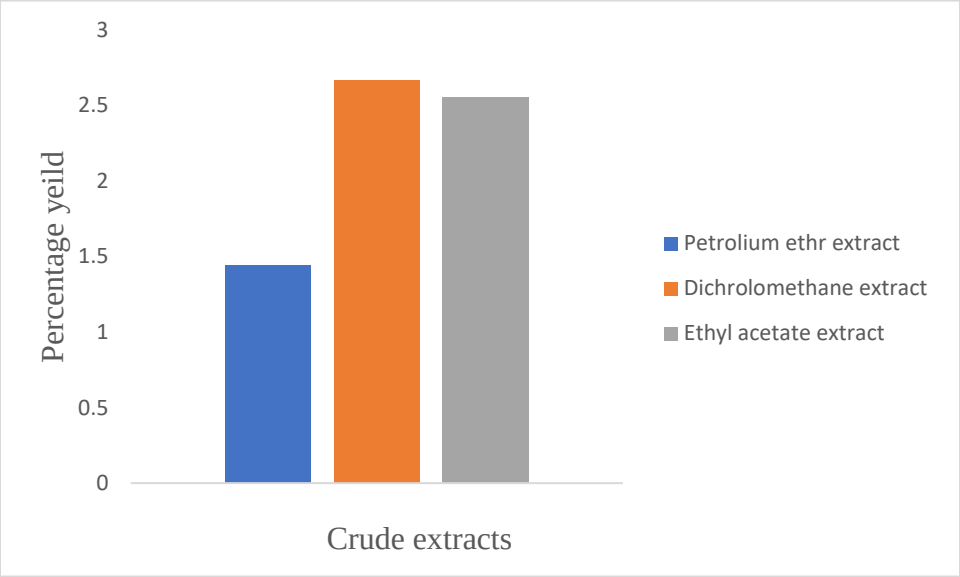


Figure 13. Bar graph of fractions obtained from the crude methanol extract.

4.4. Preliminary Phytochemical Analysis

The distribution of different phytochemical constituents in petroleum ether, dichloromethane, ethyl acetate and methanolic extracts of *Spirulina* were evaluated qualitatively and shown in **Table 12.**

The phytochemical analysis of the extract from *Spirulina* revealed the presence of several bioactive compounds including phenols, flavonoids, terpenoids, coumarins, and saponin as shown in (**Table12**). Notably methanol extracts showed the presence of all the phytochemicals analyzed as it is polar solvent that can dissolve a wide variety of phytochemicals, including tannins, alkaloids, steroids, terpenoids, saponins, flavonoids, and phenols (**Appendix 1**). On the other hand, the dichloromethane extract of *Spirulina* exhibited a high presence of tannins and flavonoids, with moderate amounts of saponins, terpenoids, phytosterols, steroids, and coumarin and showed absence of alkaloid and saponins. Similarly, the ethyl acetate extract of *Spirulina* showed a significant presence of flavonoids, with moderate amounts of alkaloids, phytosterols, terpenoids, and coumarin. Lastly petroleum etheric extract of *Spirulina* predominantly contained phytosterol, with moderate amounts of coumarin and flavonoids, while lacking alkaloids, terpenoids, saponins, steroids, and tannins. These findings provide valuable insights into the potential health benefits and therapeutic properties of *Spirulina*.

Table 12. Phytochemical analysis of *Spirulina fusiformis* extract

Phytochemical screening	Methods		Extract			
		Observations	Petroleum ether extract	Dichloromethane Extract	Ethyl acetate extract	Methanol extract
Test for alkaloids	Wagner test	Brown color	_	_	+	+
Test for terpenoids	Salkowski's test	red color at lower layer	+	+	+	++
Test for saponins	Froth test	Persistent frothing	_	_	_	-
Test for phytosterols	Liebermann-Buchas test	color changes to light yellow	++	+	+	++
Test for steroids	Salkowski test	reddish-brown	_	+	++	++
Test for tannins	Gelatin Test	dirty precipitate	_	++	_	+
Test for coumarins	Alcoholic sodium hydroxide test	Light yellow	+	+	+	++
Test for flavonoids	Shinoda test	Redish color	+	++	++	++

Notice that: + indicates moderate presence, ++ indicates highly present, - indicates absence.

The methanolic extract of a *Spirulina* sample was found to contain alkaloids, sterols, triterpenes, phenols, tannins, coumarins, and flavonoids, which aligns with the findings of (Tepal, 2016) and Mane and Chakraborty(Mane & Chakraborty, 2018). However, saponins were not detected, a result that is consistent with the studies of Tepal (Tepal, 2016) and Mane and Chakraborty (Mane & Chakraborty, 2018), but contradicts the research of Ali et al(Ali & Doumandji, 2017) and Boubeker et al(Boubeker *et al.*, 2018).

4.4. GC-MS analysis

GC followed by MS analyses was performed to identify major compounds in various fractions and essential oil isolated from *Spirulina fusiformis*. In the phytochemical analysis of essential oils, fr-98, fr-99, fr-100, fr-101 the total number of identified compounds were 13, 7, 1, 1, and 11 respectively.

4.4.1. GC-MS data analysis of essential oils

The retention indices and percentage composition of the compounds identified in the *Spirulina fusiformis* essential oils are presented in (**Table 13)**. A total of 13 compounds were identified in the essential oil of the plant **(Appendex 3).** Among the identified compounds in essential oil, eicosane, heptacosane and heptadecane were found in highest percent composition of 37.35, 26.89 and 27.46 % respectively (**Table 13**). In comparison to the results previously reported in the literature there were considerable differences qualitatively and quantitatively. The difference may be related to microalgal cultivation techniques and the conditions of extraction.

(Ozdemir *et al.*, 2004) reported that fifteen volatiles' compounds were identified from *Spirulina* originated from Turkey, the major components were heptadecane (39.7 %) and tetradecane (34.6 %). In our *Spirulina* the percentage of heptadecane was found to be lower percentage composition 27.46 % compared with those reported by these workers. These authors also mentioned four volatiles which were not found in our essential oils namely, phytol, isophytol, alpha-lonene, tetradecane.

(Milovanović *et al.*, 2015b) identified fourteen constituents in essential oil of *Spirulina* originated from Serbia and reported that the main components were heptadecane (73.1 -82.2 %), hexadecane (4.7-5.4 %) and pentadecane (4.1-8.8 %). The percentage composition of heptadecane reported by these workers is higher than those found in the EO of our sample. In our essential oil's samples, the percentage of heptadecane was only 27. 46 %. In addition, the authors mentioned the presence of six constituents which were not found in our essential oil's samples such as the 2-pentylfuran, 2-ethyl-1-hexanol, 2, 2, 6-trimethylcyclohexanone, 2, 6 dimethylcyclohexanol, beta-cyclocitral, beta-Ionone.

Table 13. GC-MS analysis of essential oils of *Spirulina*

No	RT	Compound	Molecular formula	Composition	Kovat Index (KI)	Reported kovat index	Reference
1	3.196	8-heptadecane	$C_{17}H_{34}$	2.306	768.67		
2	7.864	Ethylbenzene	C_8H_{10}	0.408	868	868	(Leffingwell & Alford, 2005)
3	8.186	p-xylene	C_8H_{10}	1.082	899.71	888.1	(Pérez-Parajón *et al.*, 2004)
4	8.956	Benzene 1,3-dimethyl-		0.350	870	872	(Pérez-Parajón *et al.*, 2004)
5	10.873	p-nitro benzaldehyde valerhydrazone		1.349	799.8	NR	
6	11.191	Hexadecoinic acid, methyl easter (palmitic acid)	$C_{17}H_{34}O$	0.892	799.6		
7	11.555	Cyclohexane, ethyl-		0.275	826.7	824	(Rembold *et al.*, 1989)
8	19.246	Benzenepropanal, 4-(1,1-dimethylethyl)-	$C_{13}H_{18}O$	0.338	1400	1212	(Guignard *et al.*, 2020)
9	28.751	Hexadecane	$C_{17}H_{36}$	0.719	1700	1700	
10	37.574	Heptacosane		26.897	2743	NR	
11	46.076	Eicosane	$C_{20}H_{42}$	37.353	2036	NR	
12	47.618	2,3,4,6-Tetrafluorophenyl isothiocyanate	C_7HF_4NS	0.568	793		
13	56.234	Heptadecane	$C_{17}H_{36}$	27.46			

Notice: NR- not report

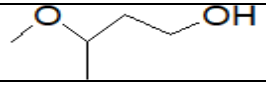
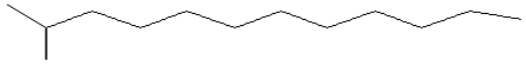
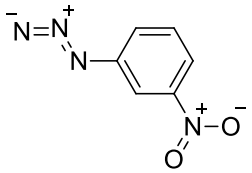
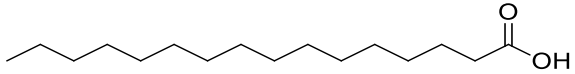
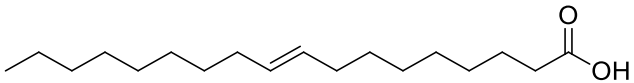
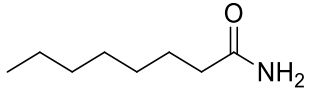
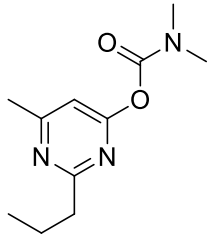
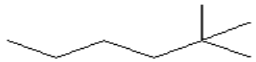
4.4.2. GC-MS Analysis of Fractions

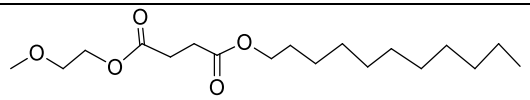
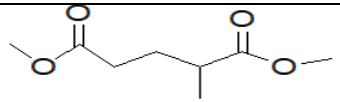
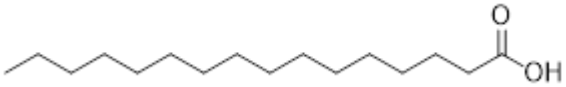
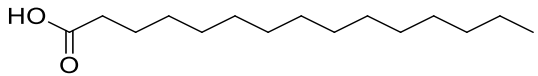
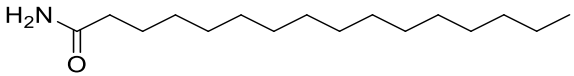
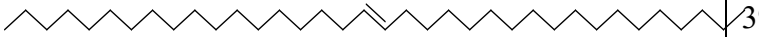
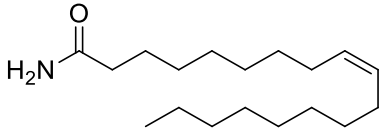
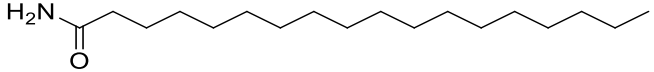
The GC–MS of *Spirulina* fractions (fr-98, fr-99, fr-100, and fr-101) collected by column chromatography were also analyzed. A total of 20 compounds were identified from these fractions corresponding to mass spectral fragmentation patterns to that of the known compounds in the database of the National Institute of Standards and Technology (NIST) library. The compounds identified in these fractions were tabulated in (**Table 14**)

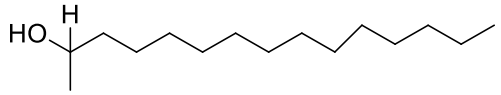
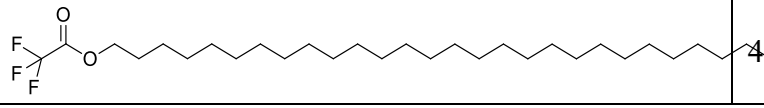
Hexadecanoic acid was the most reoccurring predominant compound and was identified in two fractions anlyzed (fr-98 and fr-101) of the assayed fractions. In terms of percentage composition, n-Hexadecanoic acid 97.218%, 83.55% were the highest in fr-98 and 83.55% respectively **(Appendix 4 and 5).**

Only one compound each was present in each fr-99(2-Hexanol, 2-methyl-) and fr-100(Butane, 1-ethoxy-). Generally, among the identified compounds most were reported to have antimicrobial and antioxidant properties.

Table 14.GC-MS analysis of fractions obtained by column chromatography.

Sample code	N	Compound name	CSBC	RT	Area	MF	MW
Fr.98	1	1-Butanol, 3-methoxy-		7.258	0.31	$C_5H_{12}O_2$	104.15
	2	Dodecane, 2-methyl-		31.256	0.126%	$C_{13}H_{28}$	184.36
	3	Benzene, 1-azido-3-nitro-		32.631	0.402%	$C_6H_4N_4O_2$	164
	4	n-Hexadecanoic acid		33.399	97.218 %	$C_{16}H_{32}O_2$	256.42
	5	Oleic Acid		34.873	0.455%	$C_{18}H_{34}O_2$	282.5
	6	Octanamide		36.627	0.261%	$C_8H_{17}NO$	143.23
	7	Pyramat		39.211	1.222%	$C_{11}H_{17}N_3O_2$	223.27158
Fr. 99	1	2-Hexanol, 2-methyl-		7.263	100%	$C_7H_{16}O$	116.201

Fr.100	1	Butene, 1-ethoxy-		7.258	100%	$C_6H_{14}O$	102.1748
Fr. 101	1	Succinic acid, 2-methoxyethyl undecyl ester		7.257	0.44	$C_{18}H_{34}O_5$	330.46
	2	Pentanethioic acid, O-methyl ester		32.631	0.42	$C_8H_{14}O_4$	174.19
	3	n-Hexadecanoic acid		33.379	83.55	$C_{16}H_{32}O_2$	256.42
	4	Pentadecanoic acid		34.254	0.34	$C_{15}H_{30}O_2$	242.39
	5	Hexadecanamide		36.623	1.47	$C_{16}H_{33}NO$	255.43
	6	18-Pentatriacontene		39.125	0.46	$C_{35}H_{70}$	490.930
	7	9-Octadecenamide, (Z)		39.212	10.09	$C_{18}H_{35}NO$	281.4766
	8	Octadecanamide		39.571	1.24	$C_{18}H_{37}NO$	283.4925

	9	2-Pentadecanol		39.808	0.97	$C_{15}H_{32}O$	228.41
	10	Octacosyl trifluoroacetate		40.821	0.52	$C_{30}H_{57}F_3O_2$	506.76

4.5. Characterization of the Isolated Compounds

The methanol-extracted powdered *Spirulina* was fractionated using petroleum ether, dichloromethane, ethyl acetate, and butanol. The Dichloromethane and ethyl acetate fraction were were then subjected to silica gel chromatography, employing a gradient elution from petroleum ether to ethyl acetate. This process produced numerous fractions, each containing distinct spots on thin layer chromatography (TLC). Further purification of the fraction collected was done using repeated column chromatography and preparative thin layer chromatography techniques. Consequently, two compounds, coded as compound **37** (palmitic acid) and compound **38** (C-5 reduced Diester astaxanthin), were isolated from the dichloromethane fraction. Additionally, compound **36** (steroid) was obtained from the ethyl acetate fraction.

4.5.1. Steroid (36)

Compound **36** was obtained as a pale-yellow solid from the fraction of ethyl acetate and isolated using column chromatography with an Rf value of 0.55 (using a mixture of ethyl acetate and hexane in a ratio of 1:3 as the eluent). Although it does not exhibit sensitivity to UV light at a wavelength of 254 nm, it undergoes a color change to brown when exposed to iodine vapor. (**Figure 14).**

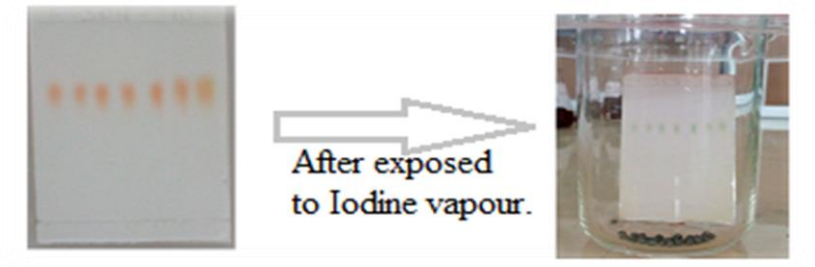


Figure 14: TLC spot of compound **36**

^{1}H NMR spectrum (**Table 15**) revealed a one-proton singlet at δH 3.67 for methoxy hydrogen on carbon number 3. The typical signal for olefinic H-6 of the steroidal skeleton was evident from a multiplet at δH 5.30 integrating for one proton. Furthermore, the ^{1}H NMR spectrum also indicated the presence of two olefinic protons (H-22 and H-23) which appeared as characteristics downfield signals at δH 5.3 and 5.36, respectively. Each of the signals was observed as multiplet which indicated coupling with the neighboring methine protons. The spectrum further revealed signals

for six methyl at δH [0.99 (s), Me-18), (0.85, s, Me-19), (0.89, d, Me-27), (0.85, d, Me-26), (0.87, t, Me-29) and (0.99, d, Me-21)] (**Appendix 3).** (Kim *et al.*, 2014). On this basis, the structure of compounds **36** was proposed as steroid shown in the following structure. However further spectral data such as ^{13}C NMR, DEPT 135 , 2D NMR and MS are needed to fully elucidate the structure of the compound.

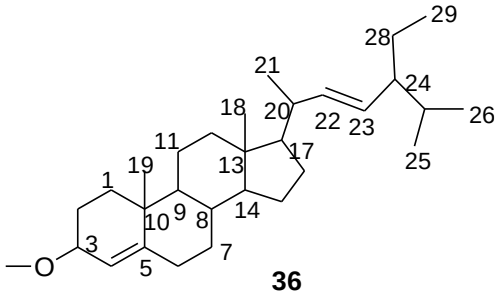


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Table 15: ^{1}H NMR ($CDCl_3$) (600 MHz) spectral data of compound **36** and literature data

POSITION	^{1}H NMR (δ ppm)	(Kim *et al.*, 2014)	
		^{1}H NMR(*δ*ppm)	^{13C} NMR(*δ*ppm)
1	0.99, 2H	0.81	31.9
2	1.67(m, 2H)	1.65	36.5
3	4.1 (m, 1H)	3.53	71.8
4	2.31(ddd, J = 13.1, 5.1, 1.9 Hz)	2.01	40.5
5	-		140.7
6	5.3, (1H, d, *J* = 5.2 Hz)	5.36	121.7
7	2.08(m, 2H)	2.08	31.6
8	1.29	1.23	31.9
9	0.98(m, 2H)	0.99	50.1
10	-	-	37.2
11	1.61(t, 2H)	1.60	21.2
12	2.01(m, 2H)	2.02	39.7
13	-	-	42.3
14	1.61(m, 1H)	1.63	56.8
15	1.67(m, 2H)	1.7	28.9
16	1.67(q, 2H)	1.7	25.4
17	1.25(m)	1.7	55.9
18	0.99(s, 3H)	0.96	21.2
19	1.25(m, 3H)	1.23	19.4
20	2.05(m, 1H)	2.05	44.2
21	0.99(d, J = 6.5 Hz, 3H)	0.89	19.0
22	5.07(*dd*, *J* = 15.2, 8.5 Hz)	5.02	129.3
23	5.05(H-23, dd, J = 15.2, 8.5 Hz)	5.02	138.3
24	1.67(t, J = 7.4 Hz, 3H)	1.65	51.2
25	1.61(d, 3H)	1.65	31.9
26	0.89 (d, J = 6.9 Hz, 3H)	0.89	12.2
27	0.87(d, J = 7.5 Hz, 3H)	0.88	21.1
28	1.25(t, 3H)	1.24	24.4
29	0.84(m, 2H)	0.88	12.0
30	3.67(MeO)		

4.5.2. Diester astaxanthin (37)

Compound **37** was obtained from dichloromethane fraction and was observed to have an orange-red color. Its Rf value of 0.59 (8PE: 2EtA) is determined through preparative thin layer chromatography (**Figure 15**).

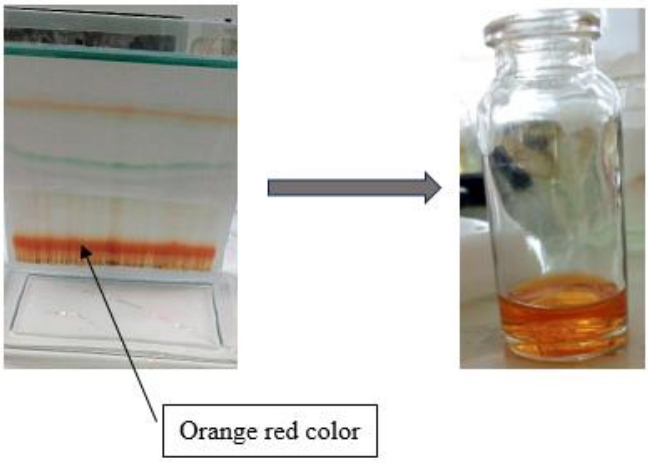


Figure 15: Preparative thin layer chromatography of compound **37**

The ^{13}C NMR spectrum displayed 46 carbon signals for 76 carbon that include a solvent peak ($CDCl_3$ 76.9ppm). The compound contains 12 primary carbons six attached to ring are 22.59(C_{16}, C16'), 27.22(C17, C17) while 29.34(C18, C18), 14.08(C19, C19; C29, 20), 14.13(C38, C38) are attached to the carbon chain. 24 secondary methylene carbons shifts are 29.11(C23, C23), 29.16(C24, C24'), 28.93(C25, C25), 27.22(C26, C26), 29.11(C 28,228), 29.71(C 31, C31), 30.36(C32, C32), 31.56(C33, C33), 31.94(C34, C34),29.37(C 35, C35), 31.99(C 36, C36), 22.59(37, C37) for the fatty acid side chain. The tertiary methine carbons shifts are 127.91(C5, C5), 128.41(C7, C7), 128.08(C 14, C14), 129.81(C 29, C29'), 128.25(C13, C13), 128.08(C9, C9). The carbon shift 61.80(C2, C2), and 70.33(C1, C1) is due to secondary methylene carbon in the ring and secondary methylene carbon linked to acyl C1 (70.33). The quaternary carbon shifts on the ring 38.72(C3, C3) and tertiary methene carbons shift 68.16(C4, C4) in the beta ionine ring **(Appendix 5**).

The ^{1}H-NMR spectrum of the compound, recorded in deuterated chloroform solvent at 25 °C, provides valuable insights. Specifically, the region between 0.86 and 1.01 ppm and the region at 2.80 ppm indicate the presence of CH_3 and CH_2 hydrogens, respectively, of fatty acid substituents. Sharp peaks within the chemical shift range of 1.22 to 1.34 ppm imply the existence of protons 16/16′, 17/17′on the carbons of the CH_3 group, which are attached to the aromatic ring at both ends of the aliphatic chain. Hydrogens 18/18′, 19/19′, and 20/20′, which overlap each other, can be attributed to the area between 2.04 and 2.09 ppm. Furthermore, the 13 (−CH) methine protons on the structure of the molecule are represented by signals within the range of 6.0 and 7.8 ppm.

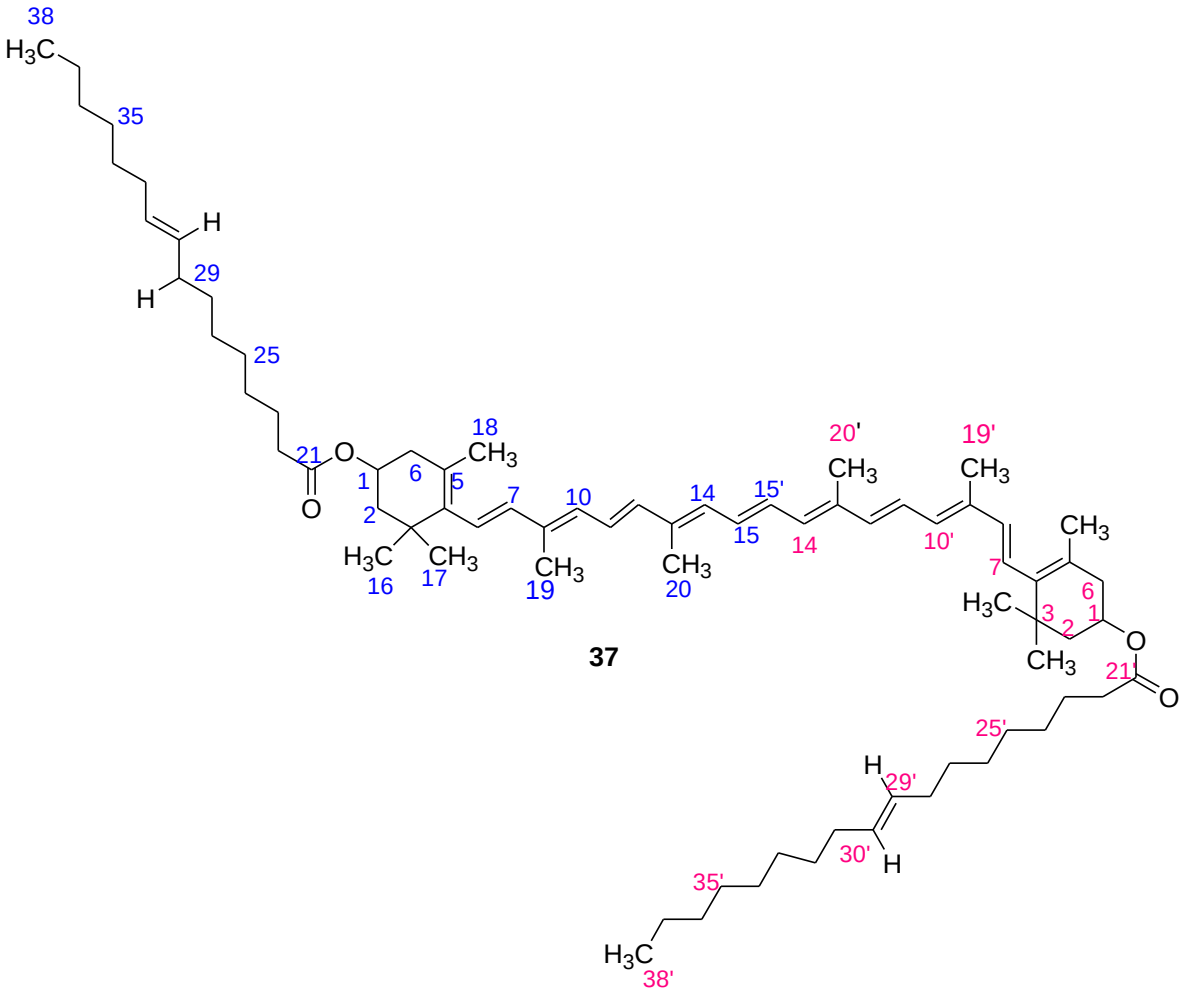
Additionally, the presence of unsaturated fatty acids is indicated by multiplets between 5.2 and 5.4 ppm, reflecting the methine protons (**Appendix 4**)

Table 16: ^{13}C NMR and ^{1}H NMR ($CDCl_3$) (600 MHz) spectral data of compound **37** and literature data.

Position	δ ^{1}H	δ ^{13}C	Reference(Ranga Rao, 2011)		position	δ ^{1}H	δ ^{13}C	Reference(Ranga Rao, 2011)	
			1H	13C				1H	13C
1	5.36	70.33	5.10	71.64	19	2.03	14.08	2.04	13.76
1'	5.66	70.33	5.10	68.69	19'	2.03	14.08	2.04	13.76
2	1.32	61.8	1.37	61.79	20	2.04	14.08	2.05	13.76
2'	1.32	61.8	1.36	61.79	20'	2.04	14.08	2.04	13.76
3	1.56	38.72	-	37.22	21	-	175	-	37.22
3'	1.56	38.72	-	37.22	21'	-	167	-	37.22
4	1,24	68.16	1.26	68.81	22	2.30	29.37	2.33	29.37
4'	1.31	68.16	1.31	68.50	22'	2.30	29.37	2.33	29.37
5	-	127.91	-	127.75	23	1.40	29.11	1.38	29.02
5'	-	127.91	-	127.75	23'	1.41	29.11	1.37	29.02
6	-	38.71	-	-	24	2.01	29.16	2.00	29.17
6'	-	38.71	-	-	24'	2.01	29.16	2.01	29.17
7	5.38	128.41	5.40	126.8	25	1.45	28.93	1.48	28.84
7'	5.38	128.41	5.39	126.8	25'	1.45	28.93	1.48	28.84
8	5..38		5.38	131.63	26	1.31	28.23	1.31	28.39
8'	5.38		5.31	131.63	26'	1.32	28.23	1.35	28.39
9	-		-	133.38	27	2.01	28.93	2.00	28.84
9'	-		-	133.38	27'	2.01	28.93	2.00	28.84
10	5.31		5.28	127.44	28	2.01	29.11	2.01	29.02
10'	5.31		5.33	127.44	28'	2.01	29.11	2.01	29.02
11	5.41	127.55	5.40	127.58	29	2.03	129.81	2.03	129.89
11'	5.41	127.55	5.40	127.58	29'	2.03	128.81	2.03	129.89
12	5.38	130.32	5.38	129.89	30	2.05	129.82	2.04	129.85
12'	5.38	130.32	5.37	129.89	30'	2.05	129.82	2.04	129.85
13	-	128.25	-	-	31	2.07	29.71	2.07	30.15
13'	-	128.25	-	-	31'	2.07	29.71	2.07	30.15
14	5.41	127.55	5.40	127.29	32	1.27	31.36	1.27	31.20
14'	5.41	127.55	5.40	127.29	32'	1.27	31.36	1.27	31.20
15	5.31	127.83	5.38	127.44	33	1.29	31.56	1.29	31.46
15'	5.31	127.83	5.37	127.44	33'	1.29	31.56	1.29	31.46
16	2.01	22.59	2.00	20.22	34	1.27	31.99	1.27	31.59
16'	1.01	22.59	2.01	20.22	34'	1.27	33.99	1.27	31.59
17	1.27	24.93	1.27	26.37	35	1.31	29.37	1.31	29.37
17'	1.27	24.93	1.27	26.37	35'	1.31	29.37	1.31	29.37
18	1.29	25.7	1.29	26.70	36	1.32	31.94	1.35	31.59
18'	1.29	25.7	1.29	26.70	36	1.32	31.94	1.35	31.59
					37	1.64	22.11	1.62	20.22
					37'	1.64	22.11	1.62	20.22
					38	0.99	14.13	0.99	13.92
					38'	0.97	14.13	0.97	13.92

The analysis of the peaks obtained from the FTIR analysis of the compound isolated from *Spirulina* confirms the presence of functional groups related to astaxanthin esters. The absorption spectrum of the compound, as shown in (**Appendix 6)** exhibits multiple peaks ranging from 500 to 4000 cm−1 due to molecular vibrations. Specifically, the absorption peaks at 2922 and 2854 cm−1 correspond to symmetric and asymmetric stretching vibrations of methyl and methylene groups (CH_3 and CH_2), respectively. Moreover, the absorption peak at 1738 cm−1 indicates the existence of an ester functional group (R'COOR) in the compound's structure, which is characteristic of astaxanthin esters. The peaks IR bands observed in the range of 1459 cm−1 represent the stretching bonds of C-CH_3 in the spectra. Additionally, the peaks bands at 1364 cm−1 suggest the symmetric deformation of the CH functional group, and the peak at 1266 cm−1 signifies C-OH stretching vibrations present in the A spectrum. Furthermore, a peak at 965 cm−1 corresponds to C-H stretching vibration in the C=C conjugate system within the absorption spectrum (Subramanian *et al.*, 2015).

These results from the nuclear magnetic resonance (NMR) analysis, in conjunction with the FTIR data, we proposed the structure of the compound to be 6-dihydrodiesterastathantin . But further elucidation of the structure of the compound needs analysis of 2D NMR and HRMS data.



37

4.5.3. Hexadecanoic acid (38)

Compound **38** was isolated from the dichloromethane crude extract as color less oil with RF value 0.34 with solvent system of 8PE/2EtOAc.

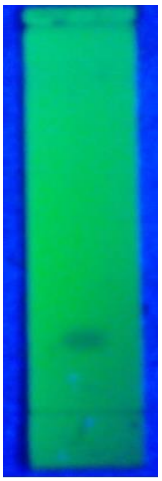
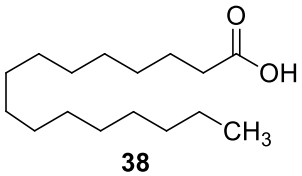


Figure 16. TLC spot of compound **38** under short Uv lamp (8PE/2EtOAc) solvent system

The proton NMR spectrum ($CDCl_3$) of the compound (**Appendix 8**) showed signals (ppm) at: δ 0.87 – 0.94 (m, 1H), 1.29 (d, J = 16.7 Hz, 5H), 1.65 (t, J = 7.4 Hz, 1H), 2.36 (t, J = 7.6 Hz, 1H). According to the proton NMR the triplet peak at 2.35 ppm is assigned to the CH_2 protons that are alpha to the carboxylic acid group. The multiple peaks at 1.65 ppm are assigned to the CH_2 protons of C3. The multiplet peak at 1.29-1.41 ppm is assigned to the CH_2 protons of C4-C15. The triplet peak at 0.88 ppm is assigned to the CH_3 protons of the terminal methyl group.

In the ^{13}C NMR of the compound showed signals (ppm) at δ 14.45, 23.04, 25.04, 29.71, 32.27, 34.42, and 180.41. According to the ^{13}C the peak around 180.41 ppm in the ^{13}C-NMR spectrum (**Appendix 9**) corresponds to the carbon atom of the carboxylic acid group. The presence of the alpha carbon atom of the carboxylic acid group is reflected by the peak around 34 ppm. Carbon atoms of middle methylene groups appear between 23 and 32 ppm. The peak at 14.45 ppm represents methyl carbon. The GC–MS analysis of Compound **38** also confirmed the proposed structure for the compound. The NMR data of the compound was also compared with those reported.



38

Table 17. ^{1}H and ^{13}C NMR spectroscopic data for hexadecenoic acid (sp1) compared against literature values. Literature(Sekandi *et al.*, 2023).

Carbon no	^{13}C NMR (600 MHz, $CDCl_3$) δc, type	^{13}C NMR (400 MHz) Literature	^{1}H NMR (600 MHz, $CDCl_3$)	1H NMR (400 MHz) Literature
1	180.41 quat.C	180	-	
2	34.42 CH_2	34	2.35	2.34
3	32.27 CH_2	32.1	1.65	1.64
4-15	23-32 CH_2	24-32	1.29-1.41	1.26–1.59
16	14.45 CH_3	14.3	0.88	0.87

4.6. Biological activity of extracts and isolated compounds of *Spirulina*

4.6.1. Antibacterial activity

The *in vitro* antibacterial activity of the extracts (methanol and water) and isolates compound of laboratory-grown cultures of *Spirulina* were tested for their antibacterial activity in different concentrations against two Gram positive and two Gram negative. Ciprofloxacin as the positive control and DMSO was used as a negative control. The zones of inhibition diameters of extracts and isolates compounds are shown in **(Table 18)**. The range of the inhibition zone was between 8 and 23 mm among the samples screened.

Methanol extract showed a maximum zone of inhibition (18±.33mm) against *S. aureus* at concentration 200 mg/ml compared to the ciprofloxacin (16±.33) at a concentration of 10µ/ml followed by *P. aeruginosa, E. coli* and *S. pyogenes* (17±.57, 16 ± .57, 11±.33) at concentration 200mg/ml **(Figure 17).** Water extract showed a maximum zone inhibition (19+.57) on *P. aeruginosa* at concentration 200 mg/ml). However, the water extract showed no inhibitory activity against all screened organisms at concentration of 25mg/ml. The methanol extract showed more potent activity compared to water extract. These findings are in accordance with previous studies which have reported the antibacterial activity of *Spirulina platensis* extracts against various bacterial pathogens(Hamad *et al.*, 2023). Our findings were in alignment with the study conducted by (Usharani *et al.*, 2015). They discovered that the crude methanol extract of *Spirulina platensis* had the greatest average inhibition zone (20± 0.4 mm) against the Gram-positive *S. pyogenes*, followed by *S. aureus* (19± 0.3 mm). The same results were also obtained by (Ali & Doumandji, 2017; Kaushik *et al.*, 2009; Sudha *et al.*, 2011). These findings highlight the potential of *Spirulina* as a natural source for developing effective antibacterial agents.

Among the compounds isolated compound **37** showed a maximum zone of inhibition against *S. pyogenes* (23±.88mm) while compound **36** showed high inhibition against *S. aureus* (17±.33) and compound **38** showed high activity in *E. coli* which is 16±.33 mm at concentration 200mg/mL compared to positive control (23±.88, 19±.33 and 18±.33mm respectively) at a concentration of 10µg/ml. However, compound **38** showed no inhibitory activity against all tested organisms at concentration of 25mg/ml and showed less inhibition for all screened organism at concentration of 50mg/ml and 100mg/ml relatively to the standard. The study specifically found that *Spirulina* extract displayed antimicrobial effects against different pathogenic bacteria, particularly when used in high concentrations.

(Abdel-Moneim *et al.*, 2022) reported that *Spirulina* had stronger antimicrobial activity against *S. aureus, E. coli, P. aeruginosa*, *Salmonella* spp. (Abdel-Moneim *et al.*, 2022)also found that *Spirulina* methanolic extract showed the highest efficacy against the tested microorganisms. This strong antimicrobial activity might be a result of its elevated content of total phenolics. According to a study by (Kaushik & Chauhan, 2008), they found that extracts derived from *Spirulina platensis* were effective in inhibiting the growth of various bacteria such as *Staphylococcus aureus, Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, and *Klebsiella pneumoniae*. They used hexane, ethyl acetate, dichloromethane, and methanol to obtain the phenolic extracts and the methanolic extracts had the best results. According to (V. Kumar *et al.*, 2011) they discovered that *Spirulina platensis*, when extracted with methanol, contains phenolic compounds that exhibit strong antimicrobial properties against Gram-positive bacteria such as *Staphylococcus aureus*. In another study by (Parisi *et al.*, 2009), the antibacterial effects of algal extracts were tested in vitro against *Staphylococcus aureus* and *Salmonella typhimurium* using the Agar well diffusion method and Paper disc diffusion method. Various concentrations ranging from 250 ppm to 7000 ppm were examined, and it was observed that all these extracts inhibited the growth of these bacteria.

The present study results revealed that *Spirulina* extracts had a higher potential to inhibit gram-positive bacteria's growth than gram-negative bacteria. The variability in the inhibition zone values observed in Gram positive and Gram-negative bacteria is primarily due to the distinctive chemical composition and morphology of their cell membranes. The notable antibacterial shown by Gram positive bacteria can be attributed to the presence of a peptidoglycan layer, which allows the penetration of hydrophobic compounds found in the extract (A. Singh *et al.*, 2020). Conversely,

Gram negative bacteria show resistance due to the presence of a lipopolysaccharide layer in their cell membrane. This lipopolysaccharide bilayer, along with embedded porins and uptake channels, forms a robust outer membrane that efficiently prevents the entry of antibacterial substances(Marshall & Bavro, 2019).

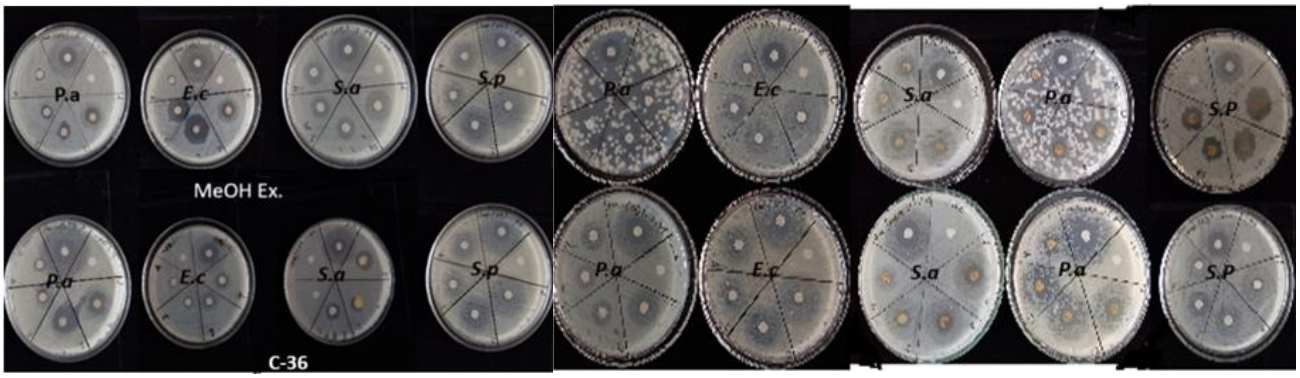


Figure 17: Antibacterial activity of *Spirulina* extracts and isolated compound

Table 18. Antibacterial activity of compound isolates and crude extract

Test path	Con.	Extracts						Isolated compounds								
		MeOH			H_2O			Compound **36**			Compound **37**			Compound **38**		
		SEM	SD	Var	SEM	SD	Var	SEM	SD	Var	SEM	SD	Var	SEM	SD	Var
E. coli	200	16 ± .57	1.00	1.00	10±.33	0.57	0.33	14±.33	.57	.33	19±.55	1.00	1.00	16±.33	.57	.33
	100	13±.88	1.52	2.33	7±.33	0.57	0.33	13±.57	1.00	1.00	18±.33	0.57	.33	14±.33	.57	.33
	50	13±.72	1.25	1.58	7±.43	0.57	0.33	10±.33	.57	.33	16±.33	0.57	.33	11±.33	.57	.33
	25	12±.57	1.00	1.00	NI	.00	.00	8±.57	1.00	1.00	15±.33	0.57	.33	NI	0.00	0.00
	+Ve	18±.57	1.00	1.00	16±.33	.57	0.33	19±.33	.57	.33	20±.57	1.00	1.00	18±.33	.57	.33
P. aeruginosa	200	17±.57	1.00	1.00	19±.57	1.00	1.00	15±.57	1.00	1.00	18±.88	1.52	2.33	12±.57	1.00	1.00
	100	12±.33	0.57	.33	11±.57	1.00	1.00	14±.57	1.00	1.00	20±.57	1.00	1.00	11±.00	0.00	0.00
	50	10±.33	0.57	.33	9±.33	0.57	0.33	11±.57	1.00	1.00	16±.33	.57	2.33	8±.33	.57	.33
	25	8±.66	1.15	1.33	NI	.00	.00	9±.33	.57	0.33	9±.33	.57	0.33	NI	.00	.00
	+Ve	17±.57	1.00	1.00	17±.33	.57	0.33	16±.33	.57	0.33	23±.88	1.52	2.33	17±.00	0.00	0.00
S. pyogens	200	11±.33	0.57	.33	15±.57	1.00	1.00	14±.33	.57	0.33	23±.88	1.52	2.33	13±.57	1.00	1.00
	100	11±.57	1.00	1.00	9±.57	1.00	1.00	15±.33	.57	0.33	13±.33	.57	0.33	11±.33	.57	.33
	50	8±.00	0.00	.00	7±.00	.00	.00	13±.57	1.00	1.00	11±.33	.57	0.33	9±.33	.57	.33
	25	7±.33	0.00	.00	NI	.00	.00	8±.00	.00	0.00	11±.31	1.52	2.33	NI	.00	.00
	+Ve	18±.33	0.57	.33	18±.57	1.00	1.00	19±.33	.57	0.33	17±.33	1.52	2.33	17±.57	1.00	1.00
S. aureus	200	18±.33	0.57	.33	15±.33	.57	.33	17±.33	.57	.33	10±.57	.57	0.33	14±.57	1.00	1.00
	100	16±.57	1.00	1.00	11±.88	1.52	2.33	12±.66	1.15	1.33	8±.00	0.00	0.00	11±.33	.57	.33
	50	7±.33	.57	.33	7±.33	.57	.33	11±.88	1.52	2.33	NI	0.00	0.00	10±.57	1.00	1.00
	25	7±.33	.57	.33	NI	.00	.00	8±.33	.57	.33	NI	0.00	0.00	NI	.00	.00
	+Ve	16±.33	.57	.33	18±.57	1.00	1.00	16±.33	.57	.33	15±.33	.57	0.33	15±.33	.57	.33

Notice: SEM- Standard error of the mean

4.6.2. Antioxidant activity results

The DPPH method is a technique used to measure the ability of active components to stabilize and donate hydrogen, resulting in the formation of free radicals with reducing properties(Flieger & Flieger, 2020). DPPH radical scavenging assay is a simple method for finding antioxidant activities of samples by recording absorbance at 517 nm. The decrease in absorbance at 517 nm in addition to the change in color of the DPPH from purple to yellow indicates the antioxidant activity of the samples.

The extracts and isolated constituents of the microalgae *Spirulina* reduce the stable DPPH radical to the yellow-colored diphenyl picrylhydrazicine indicating their potential as radical scavengers. **(Table 19)** shows the percentage of scavenging activity of crude extracts (methanol and water extracts) and isolated compounds, along with their respective concentrations required to decrease the initial DPPH concentration by 50% (IC_{50}). The results indicate that the radical scavenging activities of the extracts and isolated constituents of *Spirulina* increase as their concentrations increase **(Figure 18).** This is in line with the result that the higher concentration of extract, the higher percentage of inhibition(Agustini *et al.*, 2015). This is because on high concentration of extract, the higher antioxidant present in the sample so that consequently the level of free radical inhibition by antioxidant contained in the sample is also higher. The results revealed that the antioxidant properties of the *Spirulina* samples were increased in a concentration-dependent manner.

The methanol extract of the microalgae *Spirulina* inhibited the DPPH radical by 66.66, 73.35, 76.4 and 79.5 at 25,50, 100 and 200 µg/ml respectively on the other hand, the compound 36 displayed % inhibition of 79.8, 79.9, 80.2, 83.6 at 25,50, 100 and 200 µg/ml respectively. It was also noticed that water extracts exhibited lower percentage inhibition of the DPPH radical compared with the methanol extracts at the same concentrations. This difference is likely due to the drying process of the water extracts may cause the volatility or denaturation of these bioactive constituents in water extract to some extent. To assess the extract's efficacy in scavenging free radicals, ascorbic acid (with an IC_{50} value of 9.122) was employed as a reference. The closer the IC_{50} value of the microalgae *Spirulina* extract to that of the ascorbic acid, the greater the inhibitory effectiveness.

A recent study conducted by (Gheda *et al.*, 2021)compared the antioxidant activity of different extracts from *Spirulina.* They found that the methanolic extract exhibited a greater antioxidant effect when compared to the ethyl acetate and hexane extracts. This strong antioxidant effect of the methanolic extract is believed to be attributed to its high total phenolic content. Additionally, (Chu *et al.*, 2010) assessed the anti-oxidation activity of the water extract from *Spirulina platensis* through chemical and cell-based assays. They found radical scavenging activity of the *Spirulina* platensis water extract was high. However, in this study water extracts exhibited lower percentage inhibition of the DPPH radical. This difference between the reported literature and our is likely due the methods to rich to water extract. previous research by (Wu *et al.*, 2016b) highlighted the role of bioactive metabolites from *Spirulina* in regulating antioxidant enzyme activity in a cell line model. Moreover, *in vivo* studies have shown a positive relationship between the antioxidant activity of *Spirulina* and its anti-inflammatory effect, as reported by(Wu *et al.*, 2016b). These valuable discoveries provide valuable insights into the practical and functional significance of these natural products.

Table 19. Percentage scavenging activity of the extract and isolated compounds

Con.	**Sample code**									
	MeOH Ex.		**37**		**36**		H_2O Ex.		Ascorbic acid	
	Abs.	%RSA	Abs.	%RSA	Abs.	%RSA	Abs.	%RSA	Abs.	%RSA
25	0.150	66.66	0.243	50.9	0.113	76.8	0.395	21.6	0.496	84.3
50	0.121	73.25	0.167	64.3	0.0941	79.9	0.358	23.3	0.467	89.7
100	0.107	76.40	0.132	70.9	0.153	80.2	0.225	50.8	0.45	90.5
200	0.089	79.5	0.118	72.8	0.089	83.6	0.198	56.25	0.43	95.5
IC_{50}	3.24		0.46		12.19		3.41		9.122	

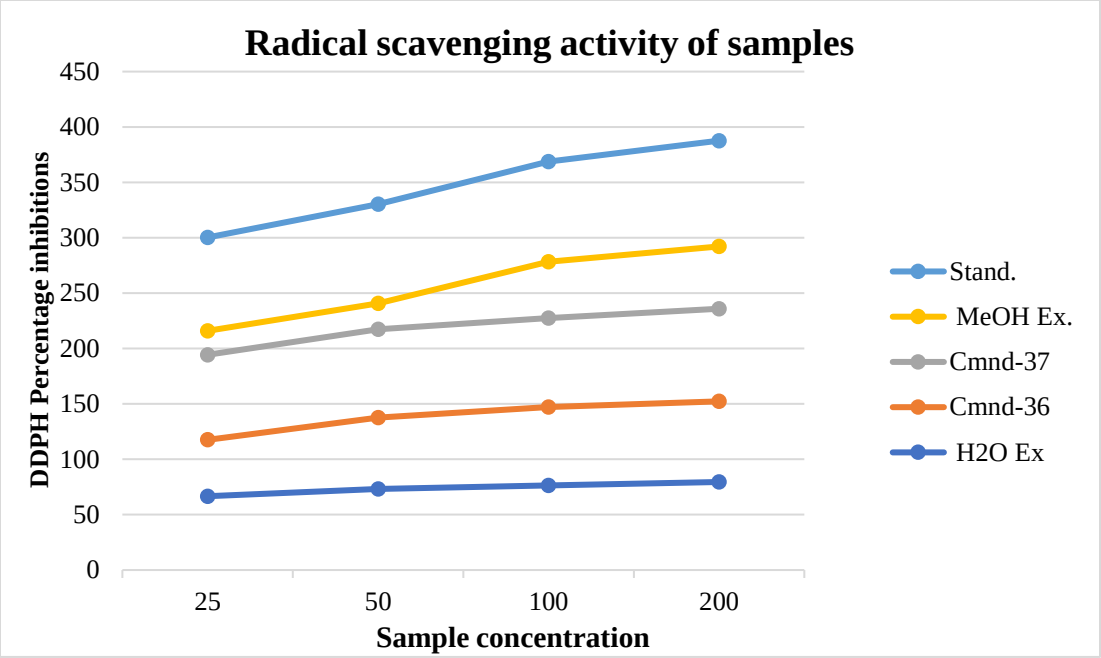


Figure 18. Antioxidant activity of extracts and isolated compounds from *Spirulina fusiformis*

The antioxidant activity of algal extract was reported to be dependent on the chemical components of the extracts that's mainly consist of carotenoids, polyphenols tocopherols, and vitamin c. This substance can act as potent antioxidant in protecting lipid peroxidation, free radical scavenging, and hydroxyl radical scavenging activities by different mode of action. Compound **37**(Diester astaxanthin) inhibited the DPPH radical by 50.9, 64.3, 70.9 and 72.8 at 25,50, 100 and 200 µg/ml respectively (**Table 19**). (Dhankhar *et al.*, 2012) reported that Diester astaxanthin as highly potential antioxidant in protecting membranous phospholipids and other lipid against peroxidation.

4.6.3. Sun protection potential (SPP)

The SPF (Sun Protection Factor) is a numerical scale used to measure the effectiveness of sun care products in providing protection against harmful UV rays. In this research, the SPF values of *Spirulina* extracts (water and methanol crude) and compound **37** were evaluated using the *In Vitro* method. The absorbance levels of all the tested samples were measured and presented in **(Table 20)**.

Table 20. Absorbance values of screened samples.

Wavelength	EE X I	Absorbance		
		MeOH Ex.	H_2O Ex.	Cmnd-37
290	0.015	1.391	1.366	2.279
295	0.0817	1.321	0.991	2.011
300	0.2874	1.223	0.741	1.867
305	0.3278	1.199	0.567	1.699
310	0.1864	1.083	0.442	1.589
315	0.0837	0.99	0.362	1.599
320	0.0118	0.869	0.331	1.498

Sun Protection Factor (SPF) values of the selected samples were calculated using Mansur equation and shown in the table (21)- and the comparison of the SPF values of screened samples were shown in (**Figure 19).**

Table 21. Sun protection factor of screened samples

No.	*Samples*	*SPF values*
1	H_2O Extract	6.19
2	MeOH Extract	11.737
3	Cmnd-37	17.4902

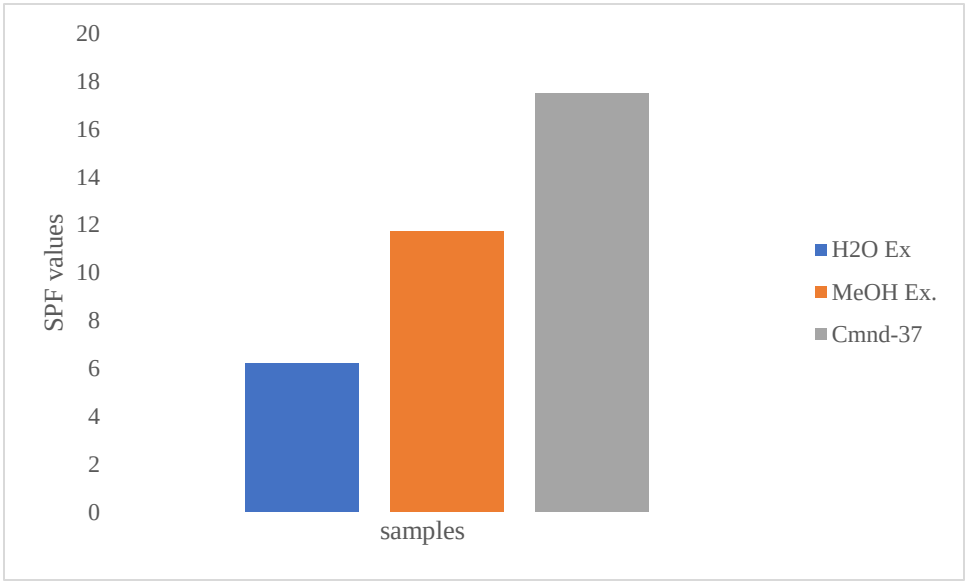


Figure 19. Bar graph for SPF values of screened samples.

All the extracts showed some SPF values. The calculated SPF values ranges between 6.19 to 17.49. Among the tested samples, compound **37** exhibits the highest SPF value, which is 17.49.

The data collected showed that the *S. fusiformis* extract had shown maximum Sun Protection Factor (SPF) with a value 17.49, as depicted in Fig. 2. This SPF value was found to be superior to those reported in earlier studies on other plant extracts. There is one study related to *Spirulina* (Mapoung *et al.*, 2020) reported ethanol extract was recorded (30.39 ± 3.37). As such, the *S. fusiformis* extract demonstrated advantageous characteristics in terms of its capacity to absorb UVB-radiation *in vitro*. An SPF of 15 or more aids in preventing skin wrinkles, contributing to a radiant, healthy, and youthful appearance of the skin(Mapoung *et al.*, 2020).

In future it is possible that microalgae *Spirulina*, might incorporated alone or in combination with other substances in different forms like creams, lotions, etc. as an alternative to chemical sunscreens. This approach could offer a safer option for sun protection and potentially be more cost effective. Scientists have recently reported the addition of resveratrol, rutin, and other natural ingredients to sunscreen formulations as an alternative for preventing photochemical damage (Souza & Campos, 2017)

5. CONCULUSION AND RECOMMENDATION

There is a rising interest in utilizing microalgae *Spirulina,* not only for food but also in the development of safe pharmaceutical products. *Spirulina* exhibits antibacterial and antioxidant properties, making it valuable in combating tumor growth, anemia, and malnutrition.

To explore deeper into its potential, we conducted laboratory cultivation of *Spirulina* specifically isolated from Lake Chitu. Through this process, we were able to isolate and analyze compounds using various spectroscopic methods and taste their biological activity.

The indoor cultivation of *Spirulina* has been highlighted as a successful and practical method in this research. This controlled environment helped to reduce the risk of contamination and preserve the desired characteristics of the algae, despite the challenges in managing factors such as temperature, light, and pH. Based on observations made with an optical microscope, *Spirulina* isolates in this study were classified into three morphotypes: H-type, S-type, and C-type. The variations in the abundance of morphotypes, their morphological parameters, and their relationships with certain environmental variables suggest that *Arthrospira* species may undergo morphological transformations in response to environmental stresses in their natural habitats, such as soda lake ecosystems. The different morphotypes observed in Lake Chitu indicate the presence of different variants of the same species, *A. fusiformis.* Despite extensive research conducted on various aspects of *Spirulina* from Lake Chitu, additional investigations are needed from a practical and biotechnological perspective. This includes exploring outdoor *Spirulina* farming and indoor laboratory cultivation methods.

This study successfully employed a range of solvents to extract and qualitatively analyze the diverse phytochemical profile of *Spirulina*. The results revealed a rich array of bioactive compounds, including phenols, flavonoids, terpenoids, coumarins, and saponins, with methanol demonstrating the most comprehensive extraction due to its polar nature.

The analysis of *Spirulina* essential oil revealed a distinct profile of 13 identified compounds, differing significantly from previously reported compositions in terms of both quantity and quality. This study successfully employed a multi-step fractionation and purification process to isolate three distinct bioactive compounds. The initial extraction followed by silica gel chromatography and subsequent re-chromatography techniques yielded Compounds namely stigmasterol (**36),**

diester astaxanthin (**37** and hexadecenoic acid **(38).** Furthermore, isolation of more bioactive compounds from the microalgae is recommended.

This study has demonstrated the promising potential of *Spirulina* extracts and isolated compounds as natural antibacterial agents. Both methanol and water extracts exhibited antibacterial activity against various Gram-positive and Gram-negative bacteria, with methanol showing greater potency. Among the isolated compounds, compounds **37** and **36** showed specific targeting towards different bacterial strains, showcasing potential for development into selective antibiotics. Additional in vitro antibacterial studies are recommended for other resistant clinical isolate pathogenic bacteria such as methicillin-resistant, *Enterococcus* and multidrug-resistant.

These findings suggest that *Spirulina* could be a valuable source of natural antioxidants capable of combating free radical damage and its associated health implications. Methanol extracts and compound **36** stand out as particularly promising candidates for further exploration and development into functional food ingredients or nutraceuticals with antioxidant properties. However, it is important to note that the observed activity in vitro may not directly translate to equivalent effects *in vivo.* Future research should examine the bioavailability and efficacy of these *Spirulina*-derived antioxidants in biological systems to fully assess their potential health benefits.

Finally, the potential of microalgae *Spirulina* as a natural source of sun protection was demonstrated in this study. All tested samples exhibited SPF values, with compound **37** showing the highest at 17.49. This suggests that *Spirulina*, alone or in combination with other substances, could be a viable alternative to chemical sunscreens. Formulating it into creams, lotions, or other topical products could offer a safer, potentially more cost-effective sun protection option. Further research is needed to optimize the extraction process, explore synergistic effects with other ingredients, and evaluate the long-term stability and efficacy of *Spirulina*-based sunscreens in real-world settings. However, this study marks a promising step towards the development of safer and more sustainable sun protection solutions.

6. REFERENCES

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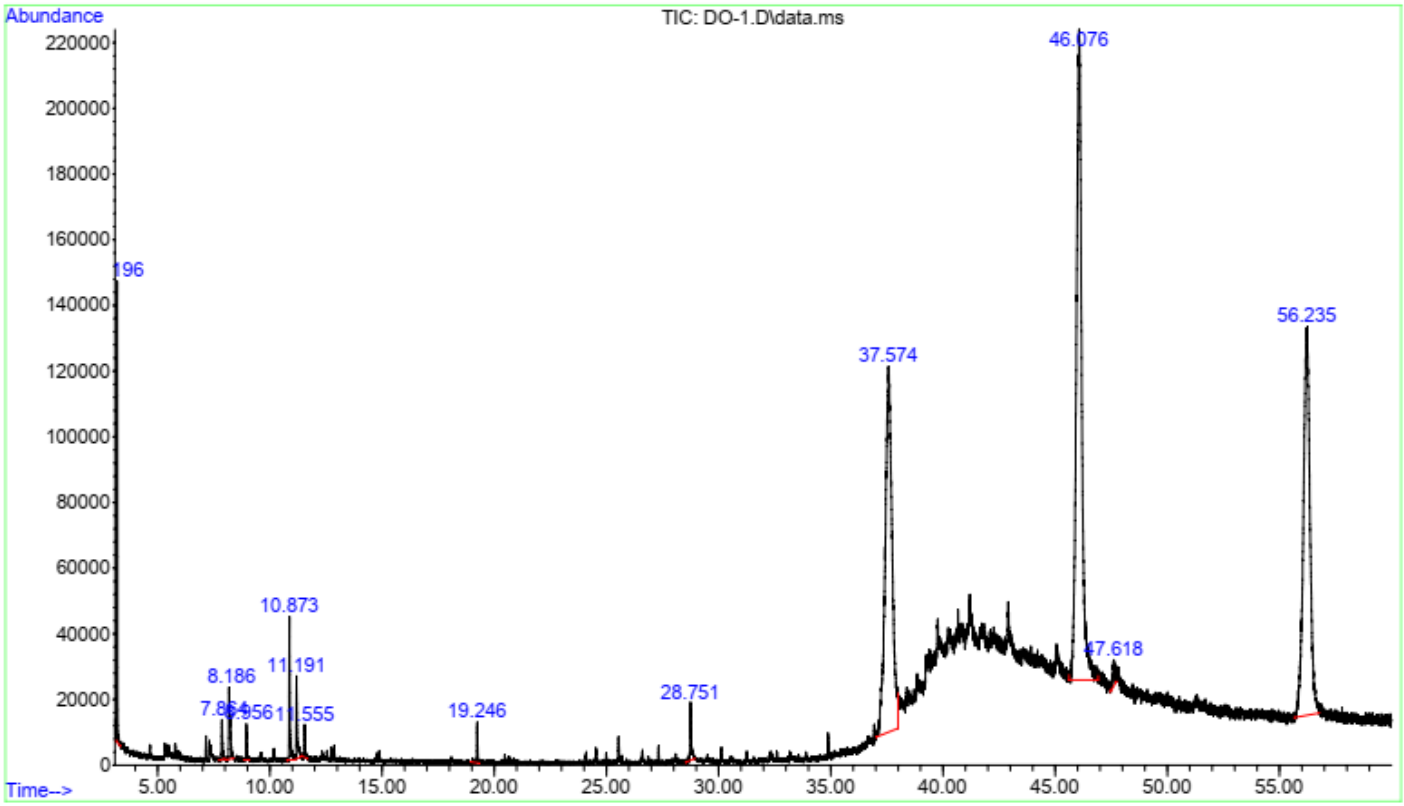
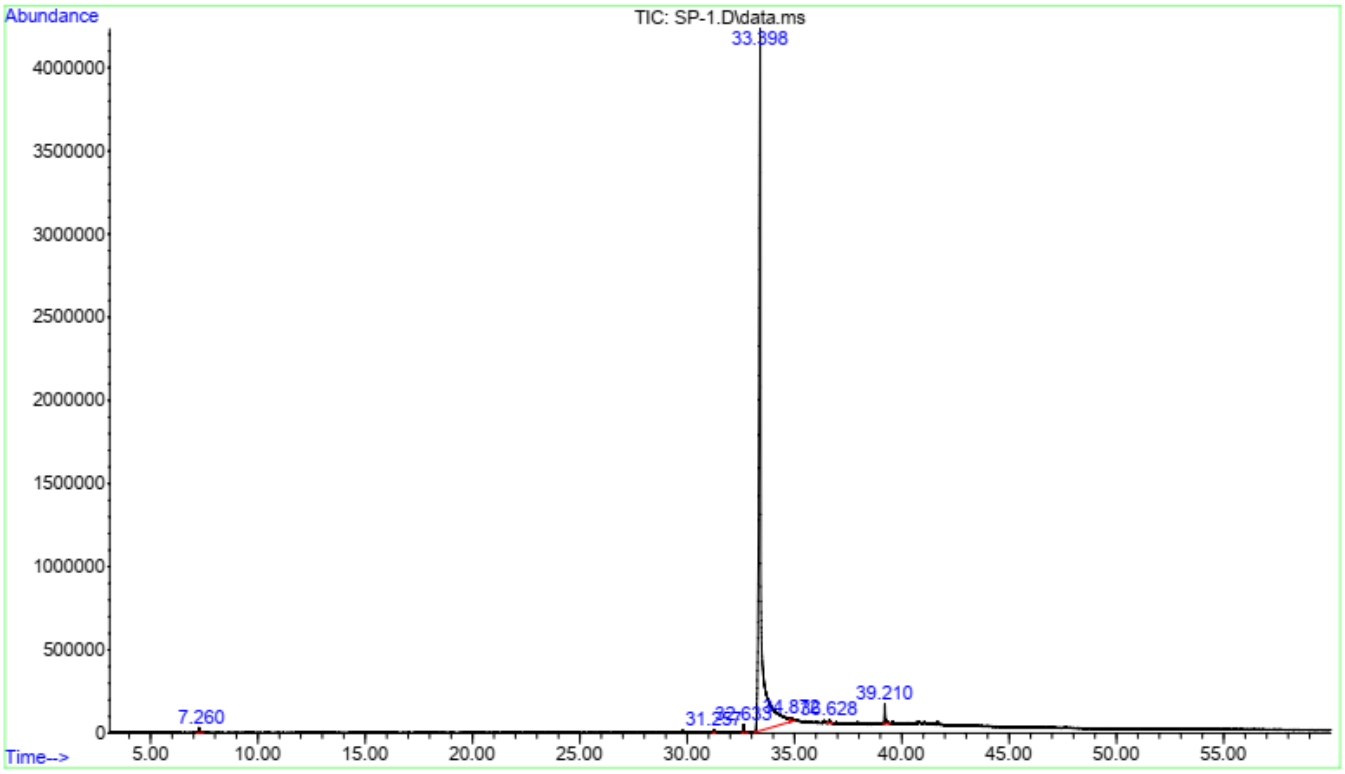
Appendix 1: Sample collecting process (picture taken by Derartu Mitiku on March 14,2023)

Appendix 2: Phytochemical analysis of *Spirulina's* extract

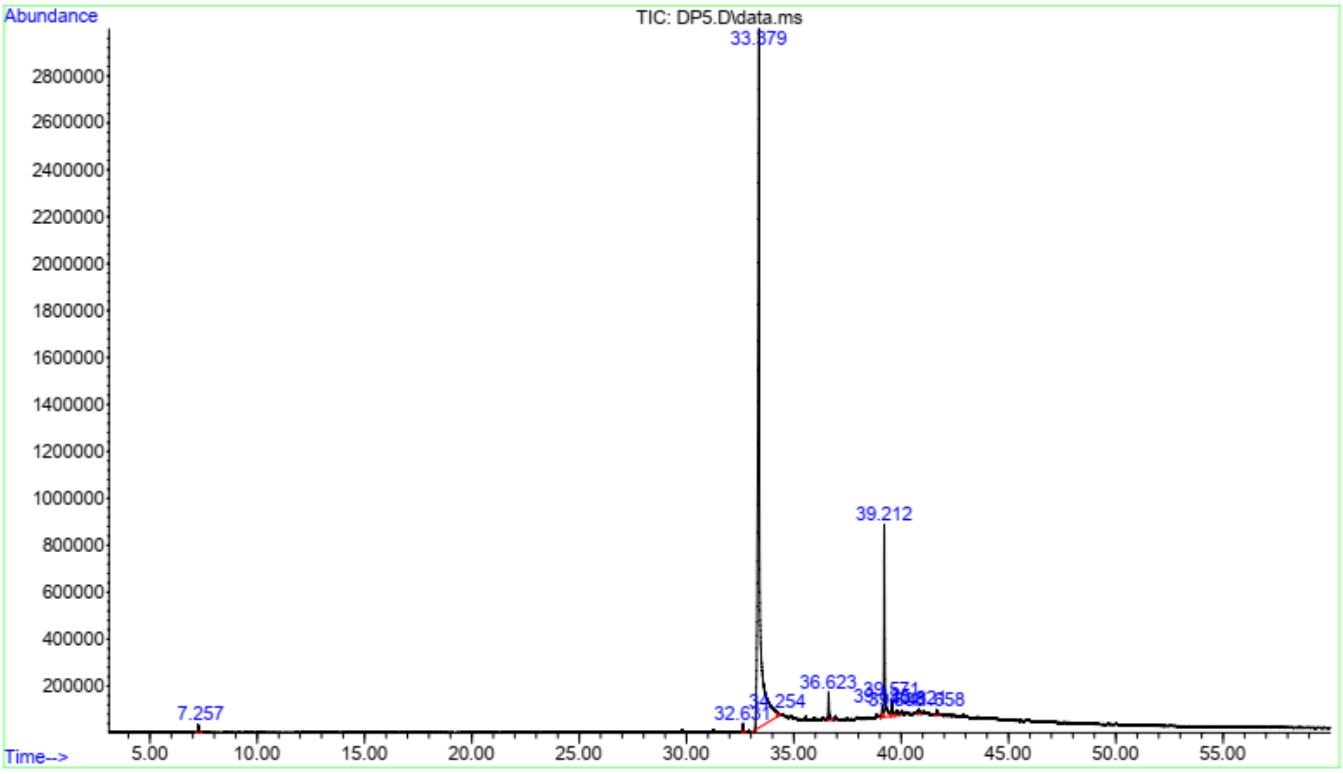
Appendix 3. GC chromatograph of essential oils of *spirulina fusiformis*.



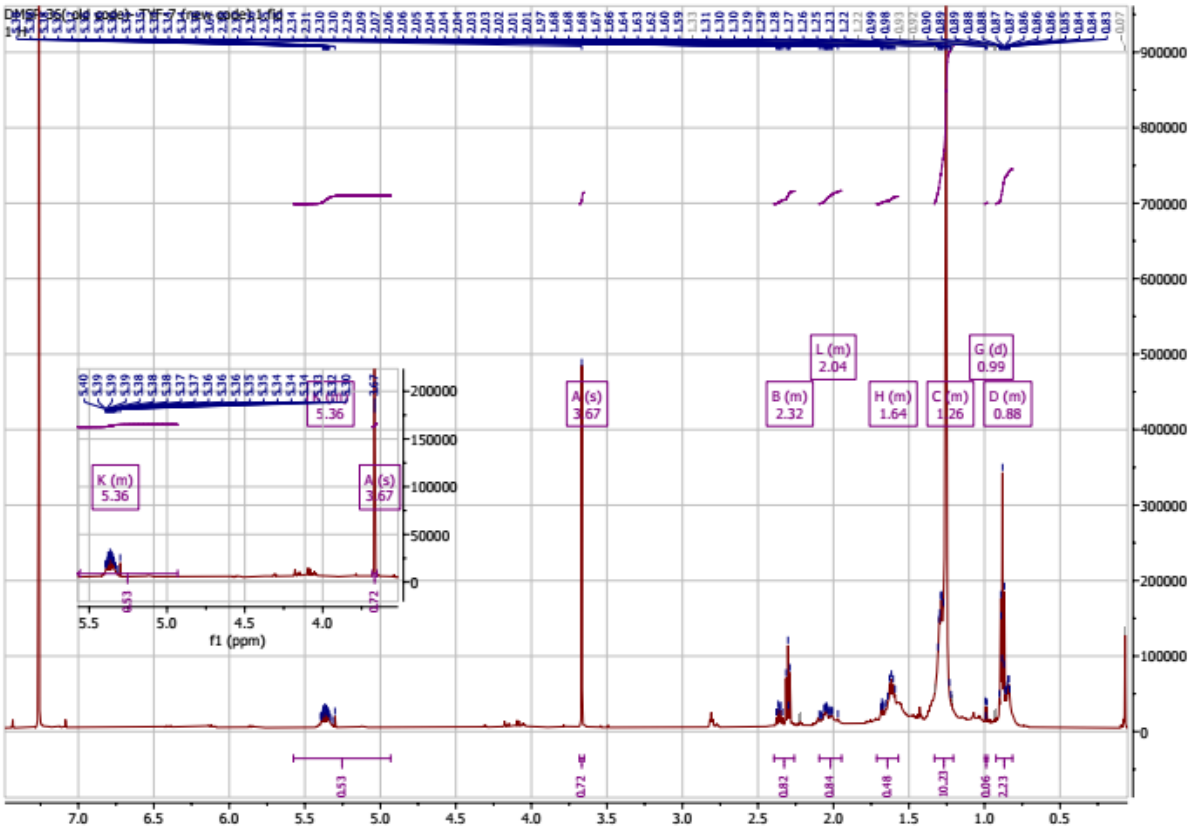
Appendix 4: GC profile of extract fraction 98



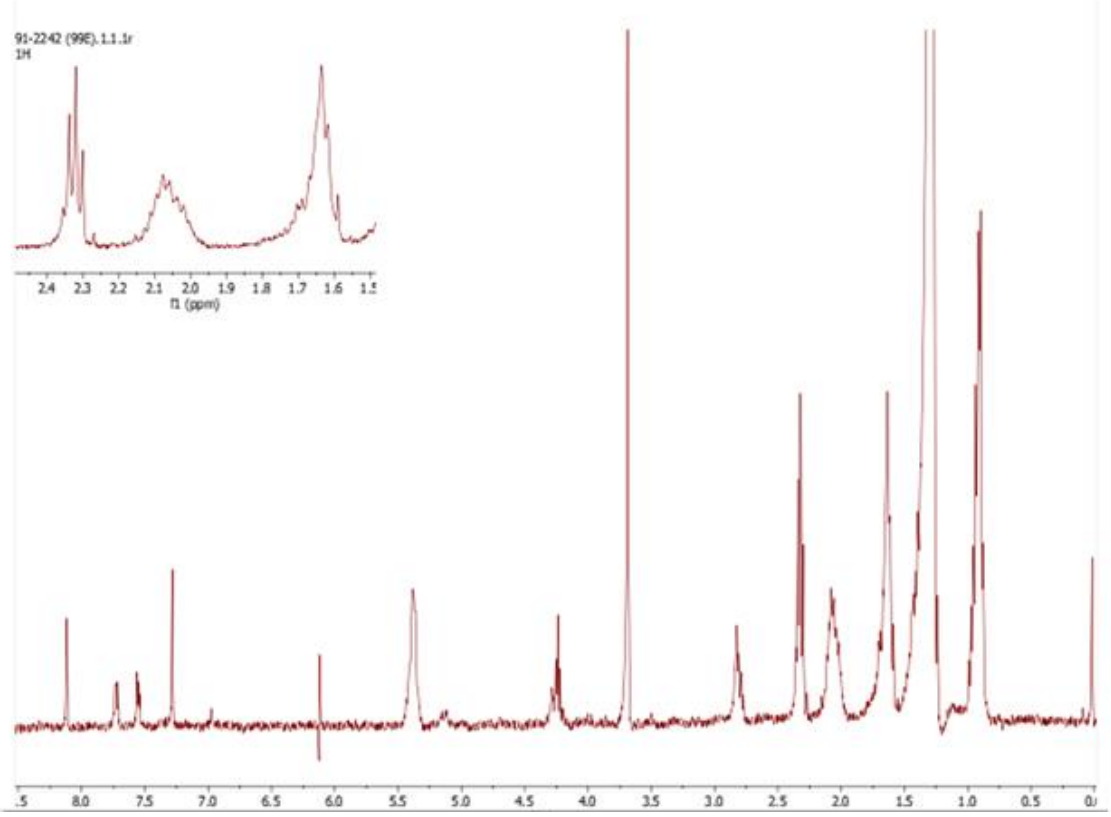
Appendex 5 GC profile of extract fraction 101.



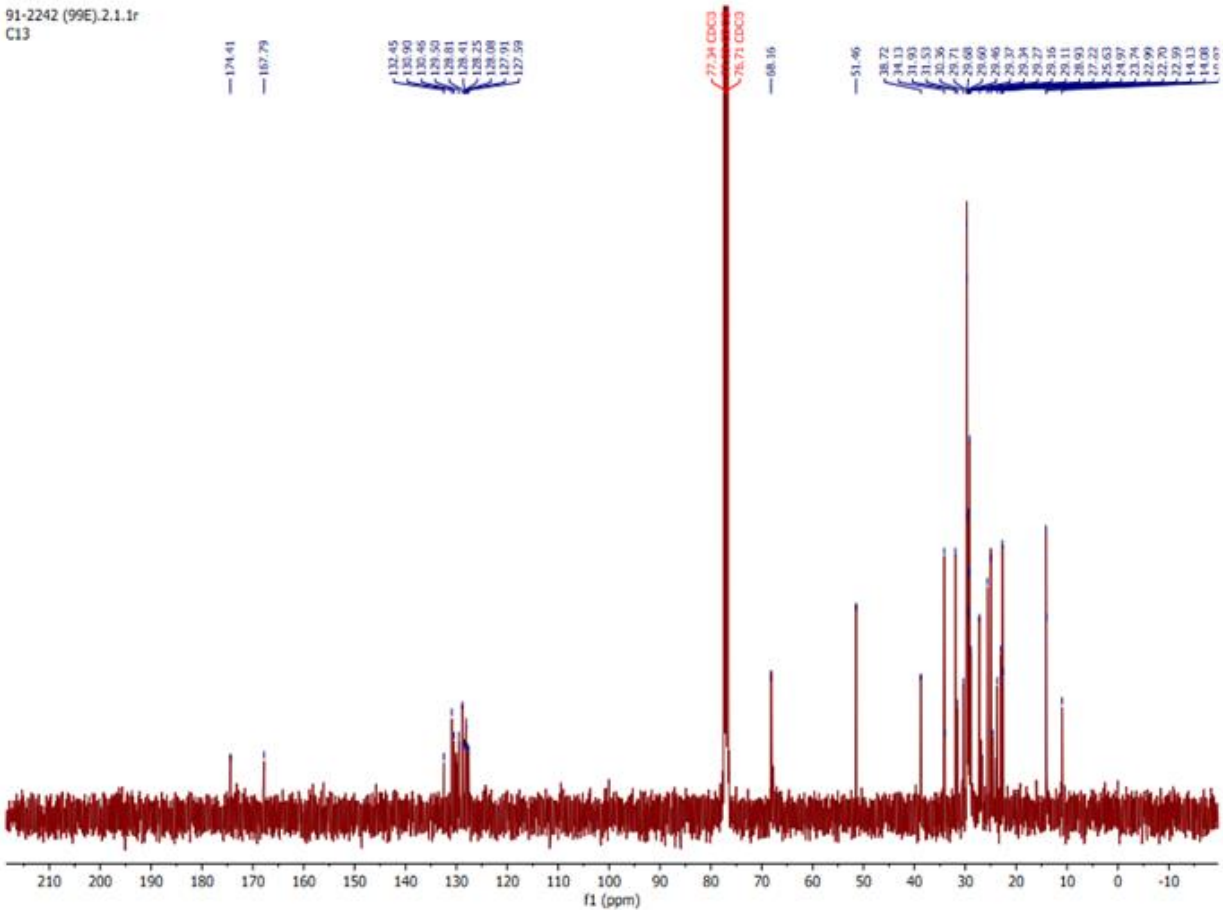
Appendix 6: ^{1}H (NMR 600 MHz, $CDCl_3$) spectrum of stigmasterol (compound **36**).



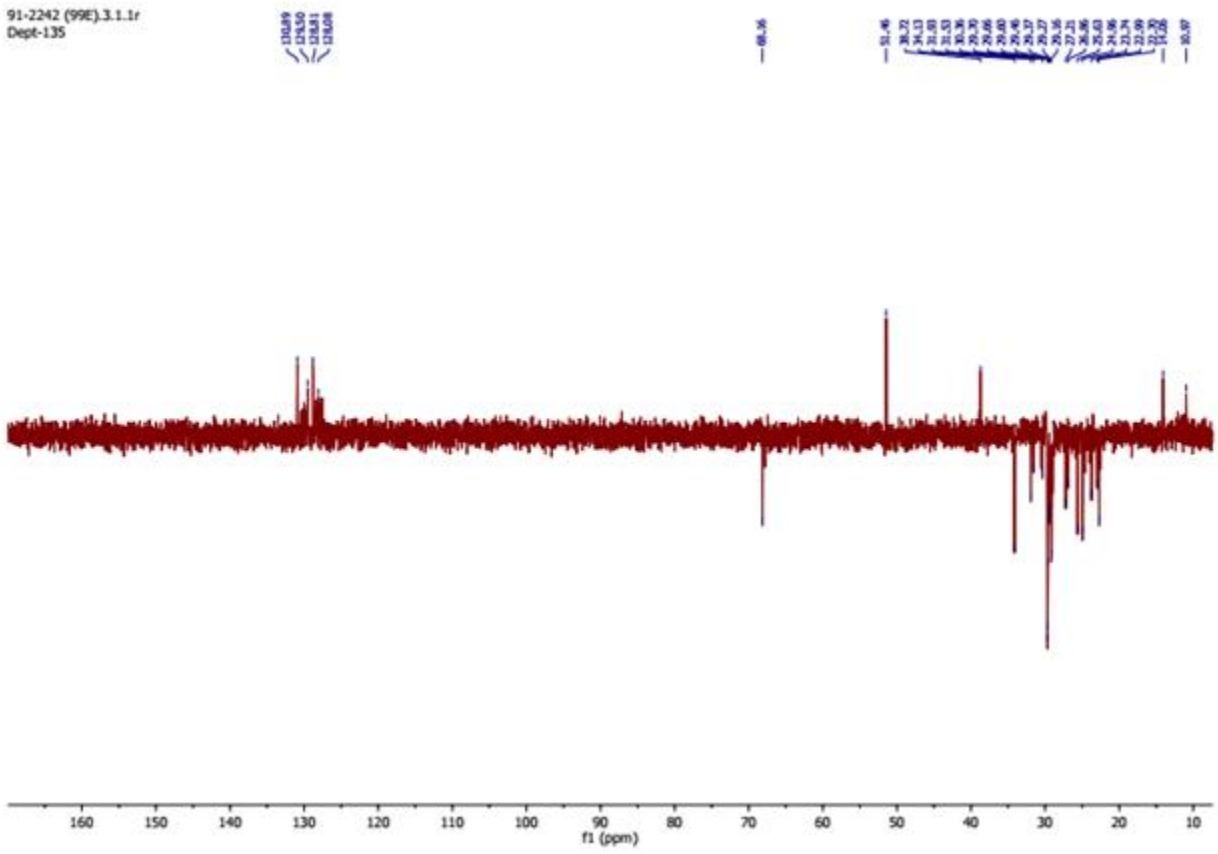
Appendix 7: 1 H (NMR 600 MHz, $CDCl_3$) spectrum of diester astaxanthin (compound **37**).



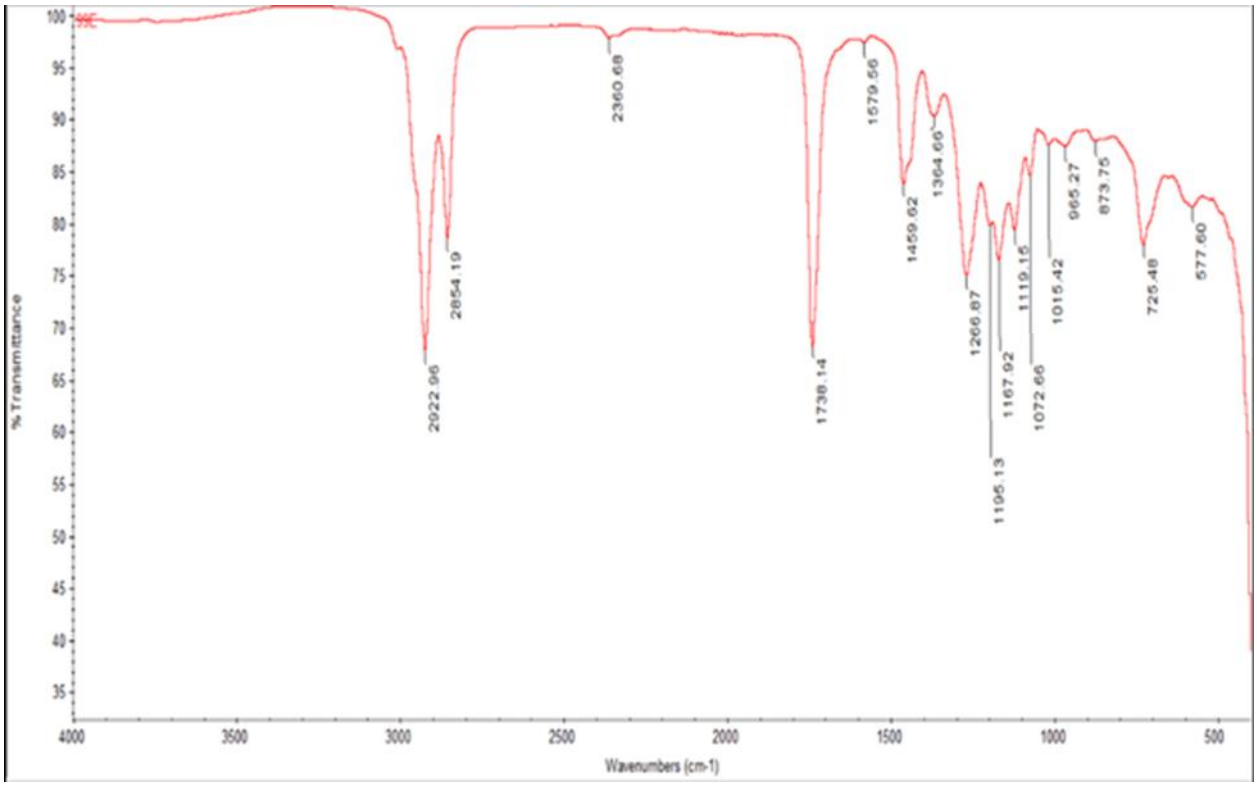
Appendix 8: ^{13}C NMR (600 MHz, $CDCl_3$) Spectrum of diester astaxanthin (compound **37**)



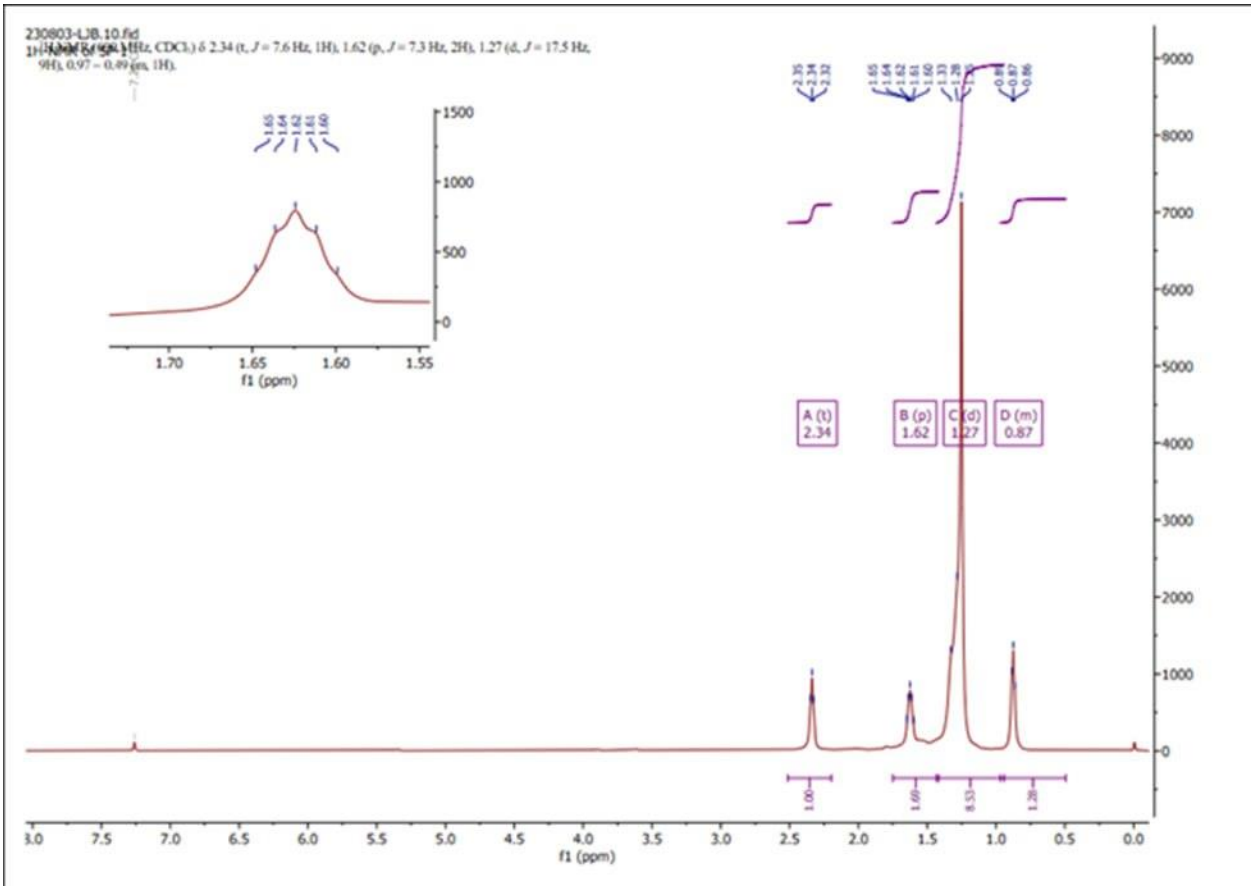
Appendix 9: Dept. 135 spectrum of Astaxanthin diester (compound **37**)



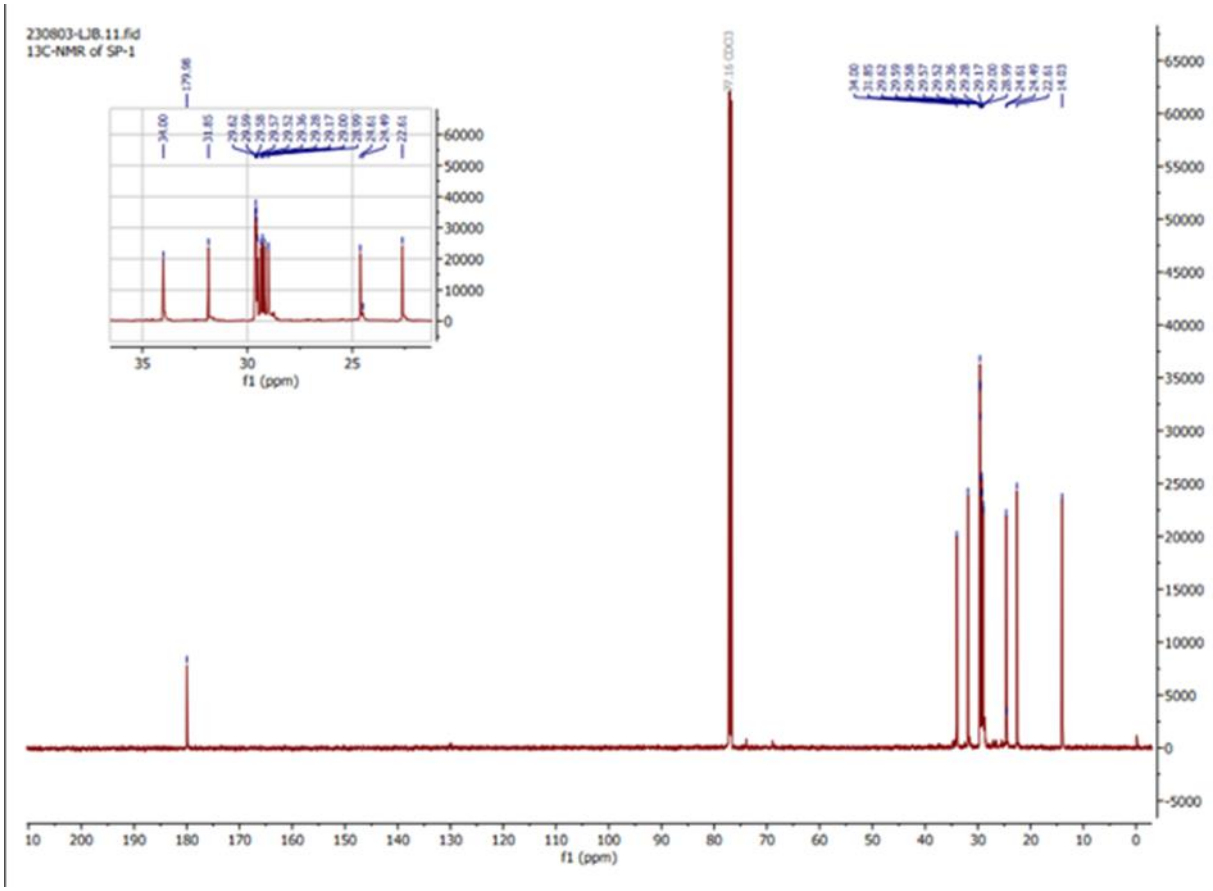
Appendix 10: IR spectrum of Astaxanthin diester compound **37**.



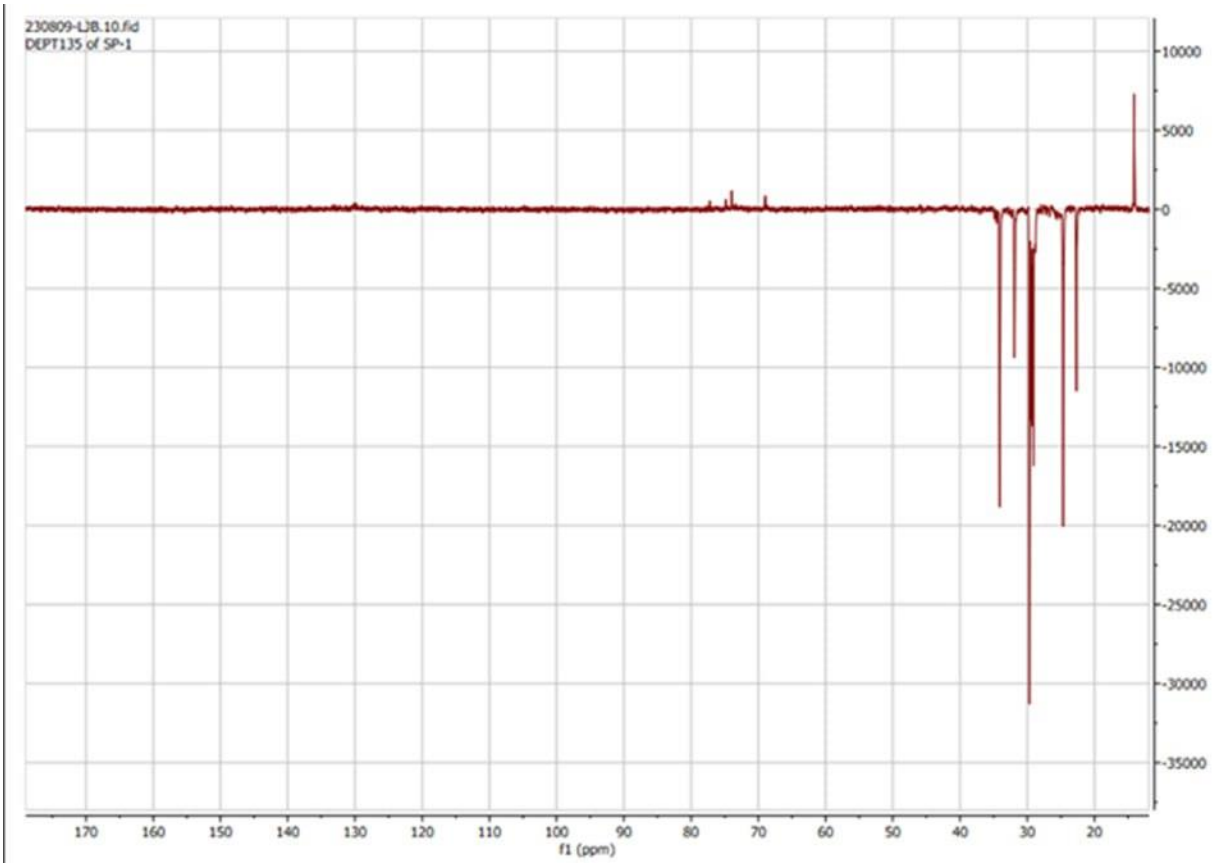
Appendix 11: [1] H (NMR 600 MHz, CDCl3) spectrum of Hexadecenoic acid (compound **38**)



Appendix 12: ^{13}C NMR (101 MHz, $CDCl_3$) Spectrum of Hexadecenoic acid (compound **38**)



Appendix 13: Dept 135 spectrum Hexadecenoic acid (compound **38**)



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