JIMMA UNIVERSITY
SCHOOL OF GRADUATE STUDIES
COLLEGE OF NATURAL SCIENCES
DEPARTMENT OF CHEMISTRY

PHYTOCHEMICAL INVESTIGATION AND ANTIBACTERIAL ACTIVITIES ON EXTRACT OF GREEN ALGAE (*Spirogyra spp.*) IN JIMMA TOWN

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PHYTOCHEMICAL INVESTIGATION AND ANTIBACTERIAL ACTIVITIES ON EXTRACT OF GREEN ALGAE *Spirogyra spp.* IN JIMMA TOWN

A Thesis Submitted to the Department of Chemistry, College of Natural Sciences, Jimma University, for Partial Fulfillment of the Requirement of the Degree of Master of Science in Chemistry (Organic Chemistry)

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ABRIVIATIONS

CFU - Colony Forming Units

DEPT - Distortionless Enhancement by Polarization Transfer

DMSO - Dimethyl Sulfoxide

IR - Infrared Spectroscopy

NMR - Nuclear Magnetic Resonance Spectroscopy

TLC - Thin Layer Chromatography

ABSTRACT

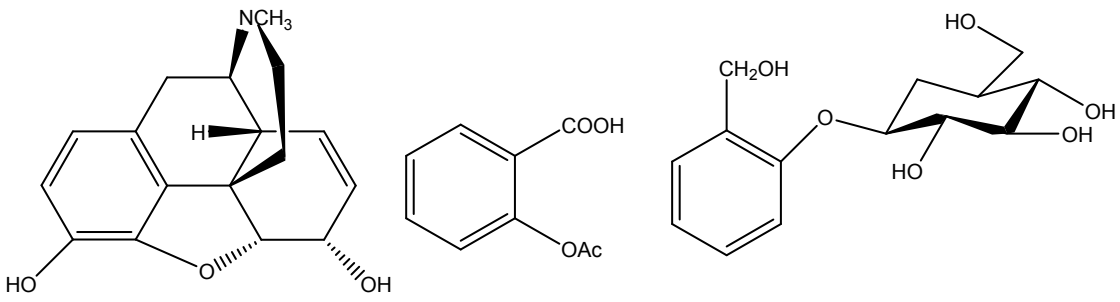
In this study, *Spirogyra spp.* was screened for its phytochemical constituents and antibacterial activity. The fine algal powder was sequentially extracted by using petroleum ether, chloroform, acetone and methanol. The resulting extracts were subjected to qualitative phytochemical analysis and for their antibacterial activity against *Escherichia coli, Salmonella thyphimurium, Pseudomonas aeruginosa and Staphylococcus aureus* by Disc diffusion method. Phytochemical analysis showed the existence of bioactive compounds such as terpenoids, tannins, flavonoids, saponins. *In vitro* screening of crude petroleum ether, chloroform, acetone and methanol extracts have shown activity in inhibiting the growth of bacterial *spp.* tested with difference in their inhibitory zone (10 to 16mm). Among the four crude extracts chloroform extract showed superior activity on bacterial growth inhibition. Four compounds (labeled as SP-1, SP-2, SP-3 and SP-4) were isolated from chloroform extract by column chromatography. Among those isolated compound SP-1 was fully characterized and SP-2 was partially characterized based on spectral data such as IR, ^{1}H-NMR, ^{13}C-NMR and DEPT-135. The fully characterized compound has structural similarity with lutein. SP-1, SP-2 and SP-4 fractions of *Spyrogyra spp.* also results in the bacterial growth inhibition. This indicates that, green algae *Spyrogyra spp.* have varied source of pharmacologically active natural products.

Key words: *Spirogyra spp.,* lutein, antibacterial, phytochemicals

1. INTRODUCTION

1.1. Background of the study

Natural products include an entire organism: plant, animal, or microorganism that has not been subjected to any kind of processing or treatment other than a simple process of preservation.[1] The ability to access natural products, understand their usefulness and drive applications, has been a major driving force in the field of natural product research. Natural products have played a great role in the development of medicinal chemistry.[2] The use of natural products as medicine has invoked the isolation of active compounds; the first commercial pure natural product introduced for therapeutic use is generally considered to be the narcotic morphine (**1**), marketed by Merck in 1826,[3] and the first semi-synthetic pure drug aspirin (**2**), based on a natural product salicin (**3**) isolated from *Salix alba*, was introduced by Bayer in 1899.[4-5]



1 2 3

Figure 1: Structures of some natural products from medicinal plants and their semi synthetic derivatives

Natural products have special selectivity to cellular targets.[6] Moreover, biologically active natural products would provide selective ligands for disease-related targets,[7] and influence the disease-related pathways and eventually shift the biological network from disease status to the healthy status. Most common bioactive compounds include secondary metabolites such as alkaloids, flavonoids, terpenoids, phenolic compounds, pigments, phytosteroles and anthroquinone.[8] These metabolites have advantages for the organisms to serve as a chemical defence against predation.[9]

Medicinal plants are useful for healing as well as for curing of human diseases because of the presence of phytochemical constituents.[10] Phytochemicals are naturally occurring compounds in the medicinal plants, leaves, vegetables and roots that have defense mechanism and protect from various diseases. Phytochemicals are primary and secondary compounds. Chlorophyll, proteins and common sugars are included in primary constituents and secondary metabolites include terpenoid, alkaloids and phenolic compounds.[11] Terpenoids exhibit various important pharmacological activities including antiinflammatory, anticancer, antimalarial, inhibition of cholesterol synthesis, antiviral and antibacterial activities.[12] Terpenoids are very important in attracting useful mites and consume the herbivorous insects.[13]

Algae have a significant attraction as natural source of bioactive molecules.[14] Secondary metabolites produced by these organisms are potentially bioactive compounds of interests in the pharmaceutical industry.[15] The macroalgae have a significant attraction as natural source of bioactive molecules with a broad range of biological activities, such as antibiotics, antivirals, antitumorals, antioxidant and antiinflammatories.[16] Evidence of phytochemical and pharmacological studies on algae are the source of amino acids, terpenoids, phlorotannins, steroids, phenolic compounds, halogenated ketones, alkenes and cyclic polysulphides.[17-18] The sterols of green algae were much more varied. The green algae contained chondrillasterol, and poriferasterol.[19] They were also reported to reduce the tendency to form a fatty liver and excessive fat deposition in the heart.[20]

Algae on synthesize a variety of compounds such as carotenoids, terpenoids, vitamins, saturated and polyunsaturated fatty acids, amino acids, acetogenins, antioxidants such as polyphenols, alkaloids, halogenated compounds and polysaccharides such as agar, carrageenan, proteoglycans, alginate, laminaran, rhamnan sulfate, galactosyl glycerol and fucoidan.[21–23] Compounds with cytostatic, antiviral, antihelmintic, antifungal and antibacterial activities have been detected in green, brown and red algae.[24] Algae produce pure forms of the fatty acids found in human milk that appear to be building blocks for mental and visual development.[25]

Numerous studies have also employed macroalgae and microalgae for the biosorption of metals and the ability of certain species of macroalgae to accumulate and tolerate high levels of metals has been demonstrated. Hence, algae represent an effective,

economically viable and environmentally friendly alternative for the bioremediation of heavy metals, especially cadmium and lead[26]. Algal biomass can be effectively applied in bioremediation because the proteins and polysaccharides of their cell walls can contain anionic carboxylate, sulfate or phosphate groups, which are optimal binding sites for metals.[27] Moreover, algae are one of the most promising sources of renewable biomass.[28] Biomass derived from algae is gaining significance for production of biodiesel.[29] Additionally, several studies have been reported on the use of dried blue green algae to inoculate soils as a means of aiding fertility.[30]

Results have also been obtained about the bioactivity of the species of *Spirogyra spp.* collected from the estuarine environment of Miani Hor.[31] Moreover, different species of *Spirogyra* were found to show promising results in their tests of antibacterial activity,[32] general bioassay[33] and allelopathic activity.[34] However, there is no report to elucidate the chemical constituents and antibacterial activities of this organism in Jimma, Ethiopia. Therefore, the aim of this study will be to conduct phytochemical screening, study on antibacterial activities of algae extract against some pathogenic bacteria, to isolate and characterization of its constituent.

1.2. Statement of the problem

Antibiotic resistance in bacteria is one of the emerging health related problem in the world nowadays. Algae are valuable natural sources effective against infectious agents. Extensive efforts for the identification of bioactive compounds derived from natural resources have been made worldwide, in order to develop safe, nontoxic and efficient anti-microbial agents of valuable practice in pharmacology. Algae of marine and terrestrial origins have been the best choice among natural resources within aquaculture and agriculture fields. Screening bioactivity of algal crude extracts is mandatory in biomedical practice, where antibacterial,[35] antifungal,[36] antiviral[37] activity have been assessed to these metabolites. Emergence concerns have been raised to establish structural and functional properties of the bioactive compounds described in algal crude extracts, up to date, over 2,400 bioactive metabolites have been isolated and identified from a diverse group of algal species.[38] However, there is no report from Jimma to elucidate the chemical constituents of these organisms. Therefore, this study was aimed to evaluate phytochemical screening and antibacterial activities of extract of green algae *Spirogyra spp.* against some pathogenic bacteria.

1.3. Objectives of the study

1.3.1. General objective

The overall objective of this study was to investigate phytochemical constituents and antibacterial activities of green algae *(Spirogyra spp.)* collected from Jimma Town.

1.3.2. Specific objectives

- To obtain gradient extracts of petroleum ether, chloroform, acetone and methanol of *Spirogyra spp.* using maceration technique.
- To assess the antibacterial activity of the crude extracts from *Spirogyra spp.* against *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa and Salmonella typhimurium*
- To assess the phytochemical constituents of crude extracts of *Spirogyra spp.*
- To isolate compounds from the crude extracts that showed relatively better antibacterial activity against the test bacterial strains.
- To elucidate the structure of the compounds using spectral techniques such as IR and NMR spectroscopy.
- To assess the antibacterial activity of the isolated compounds against *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium*

1.4. Significance of the study

Phytochemical constituents are the basic source for the establishment of several pharmaceutical industries. The constituents present in the plant play a significant role in the identification of drugs. Phytochemical screening is very important in identifying new sources of therapeutically important compounds like alkaloids, flavonoids, phenolic compounds, saponins, steroids, tannins, terpenoids etc. The treatment of infectious diseases still remains an important and challenging problem because of a combination of factors including emerging infectious diseases and the increasing number of multi-drug resistant microbial pathogens. Therefore, the importance of this research is used to identify microbial active molecules from green algae species.

2. REVIEW OF RELATED LITERATURE

2.1. Botanical description of green algae

Algae are primitive plants, that is lacking roots, stems and leaves, and have no sterile covering of cells around the reproductive cells. Algae can be classified into two main groups; Microalgae, which includes blue-green algae, dinoflagellates, bacillariophyta (diatoms) etc. and Macroalgae which includes green, brown and red algae. *Spirogyra spp.* is a filamentous green macroalgae of the family Zygnemaceae order Zygnemales, class Zygnemophyceae, phylum Chlorophycota.[39]

2.2. Phytochemical constituents of green algae *Spirogyra spp.*

Algae are represented by at least 30,000 species worldwide supplying oxygen to the biosphere, food for fish and man, medicine and fertilizers as well as being a prolific source of structurally unique natural products.[40] The terpenoids are a class of compounds predominantly isolated from marine algae in the 1970–1980s. Chemical investigations into terpenoid type structures have led to the isolation of many classes including brominated, nitrogen and oxygen heterocycles, phenazine derivatives, sterols, amino acids, amines and guanidine derivatives.[41] With respect to biological activity, green, brown and red algae have been assessed for their antibacterial and antifungal activities.[42] Study confirmed that fatty acids and sterols (like stigmasterol (4), beta-sitosterol (5), campesterol (6), clerosterol (7), avenasterol (8)), gallic acid (9), corilagin (10), tocopherol (11) of algal class, families and species are characteristics to those particular taxa.[43]

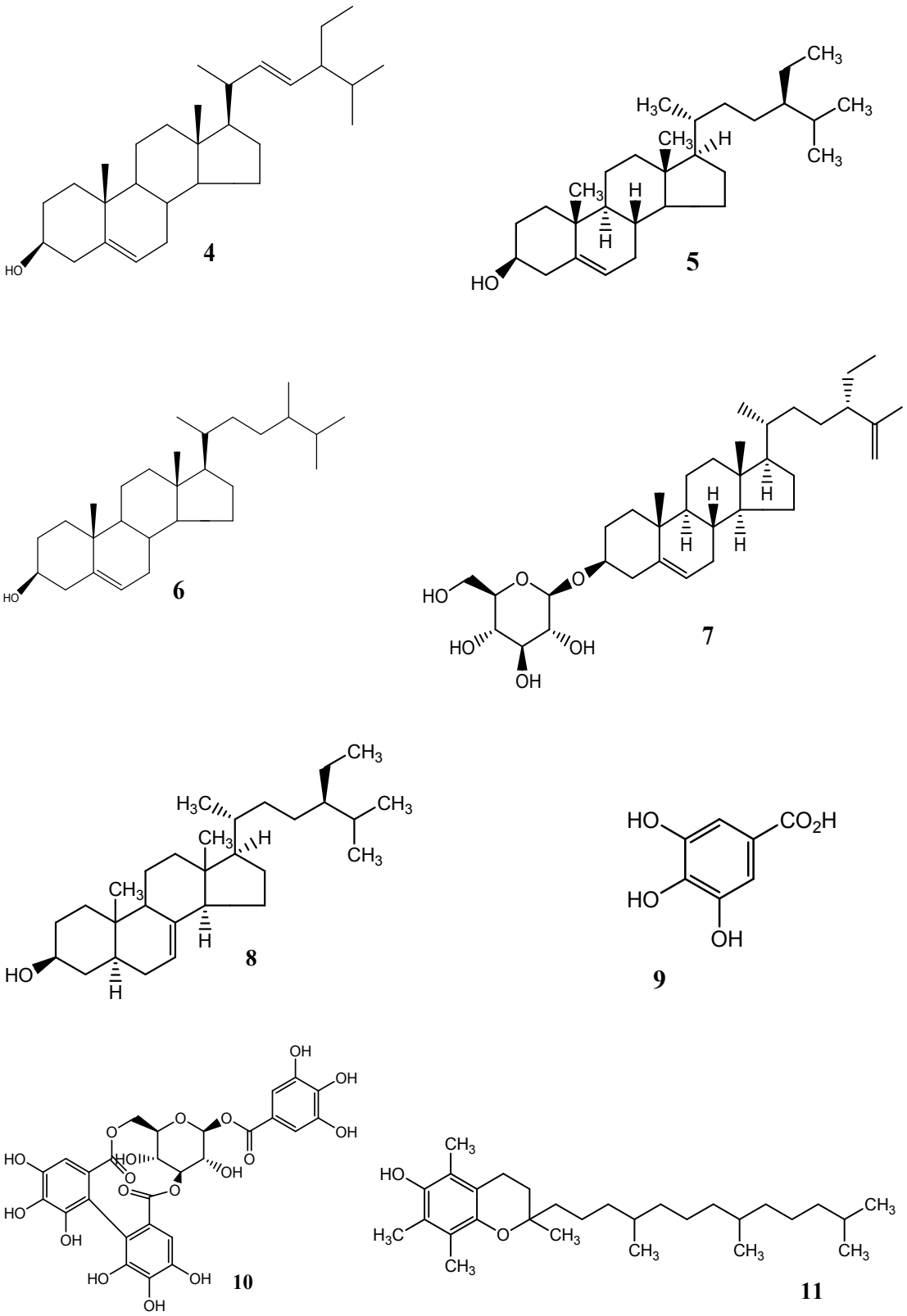


Figure 2: Structures of compounds isolated from green algae

Previously, antioxidant activity of *Spirogyra spp.* extract has been also shown *in vitro*[44] and its phytochemical compositions recently revealed the presence of phenolics, tannins, glycosides, and saponins.[45] Moreover, the study conducted on methanol extract of *Spirogyra hyaline* indicated the presence of sterols (cholesterol (12), decortinone (13), dictintriol (14), maculaniol (15) and sucrose (16)) terpenes and carbohydrates.[46] Similarly, a variety of sterols and monosaccharides like rhamnose, arabinose, xylose and galactoose *etc.* have been detected in several species of *Spirogyra* collected from lakes and ponds near Sofia, Bulgaria.[47]

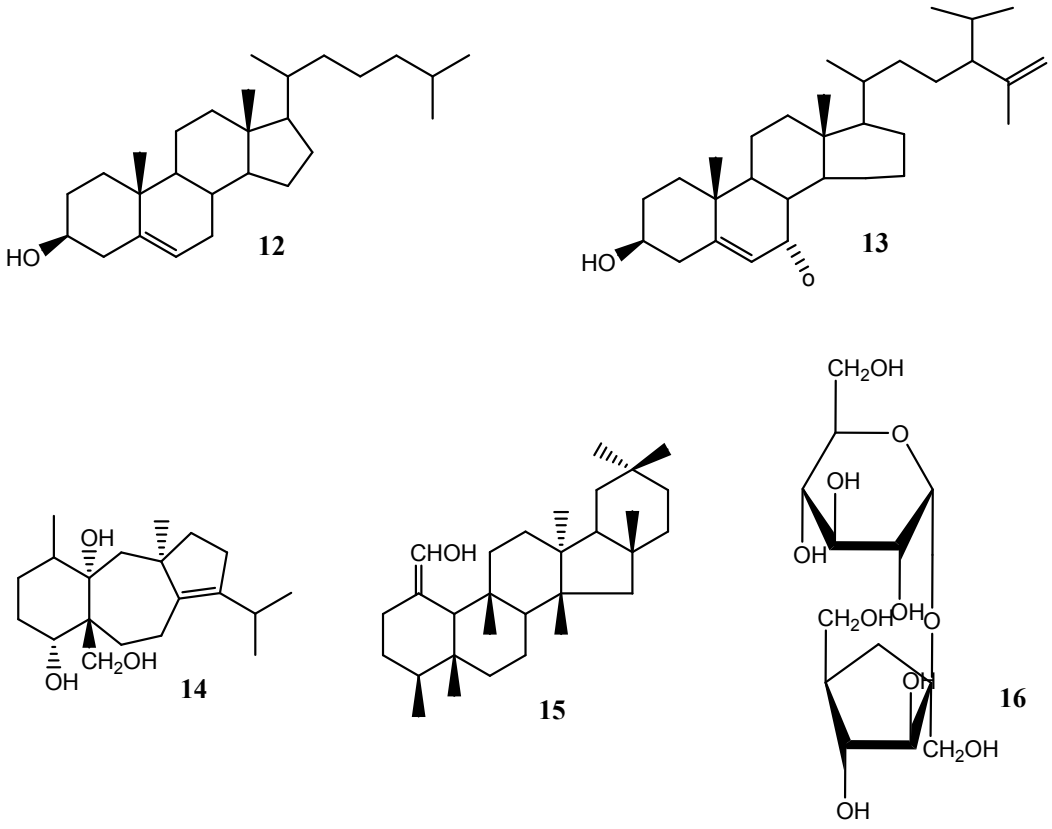


Figure 3: Natural products obtained from methanol extract of *Spirogyra hyaline*

2.3. Medicinal importance and nutritional value of green algae

The first studies concerning algae were focused on their ecological and public heath impact, due to their capacity to produce toxins with deleterious effects on plants, invertebrates and vertebrates, including humans.[48] In humans, toxins such as microcystins, nodularins and cylindrospermopsin were found to induce liver and kidney damage, cytotoxicity, neurotoxicity, dermal toxicity, gastrointestinal disturbances among others.[49] More recently, several studies have demonstrated that

algae also produce compounds with biotechnological and pharmaceutical interest such as anticancer, antiinflammatory and antibiotic activities have been described.[50] Algae are a great source of natural compounds that are widely known and consumed in Asian countries. Numerous studies have investigated compounds found in algae for their antibiotic, antiviral, antioxidant, antiinflammatory and cytotoxic activities.[51] Polysaccharides in various algae have been demonstrated to act as free radical scavengers *in vivo* and *in vitro.* [52]

The pharmaceutical industries have shown great interest in the use of algae as a source of biochemically active substances.[53] The fact that algae may produce chemical prototypes of new therapeutic agents has stimulated bioprospecting for new algal secondary metabolites and the synthetic modification of compounds with potential pharmaceutical applications.[54] In addition to novel biologically active substances, algae also provide compounds essential to human nutrition.[55] Currently, Macro algae are attracting interest in view of their low caloric content and high vitamin, mineral and dietary fiber contents[56], making them attractive to both consumers and the food industry.[57] Clinical studies have demonstrated that dietary intake of plant sterols (as part of the normal diet or as a supplement) may help to reduce blood cholesterol level.[58]

The quality of the food transferred to the higher trophic levels of the food chain is determined by the chemical composition of algae such as fatty acids, sterols, amino acids, sugars, minerals and vitamins.[59] The nutritional value of algal species depends on several characteristics such as size, shape, digestibility and toxicity.[54] In some countries like Germany, France, Japan, USA, China, Thailand food production and distribution companies have already started serious activities to market functional foods with microalgae and cyanobacteria.[60]

Freshwater green algae, *Spirogyra* spp. is filamentous algae consumed as food in northern Thailand. It contains high amounts of protein, carbohydrate, fat, sulfate and dietary fiber.[61] It has also pharmacological properties. *Spirogyra spp.* extract can inhibit gastric ulcer formation induced by physical and chemical stress in rats. It also showed hypolipidemic and hypoglycemic abilities in type II diabetic rats induced by streptozotocin and high fat diet.[62] *Spirogyra spp.* has been commonly used as ingredient in several Thai food and contains high amount of nutritional compositions,

including basic nutrients, which are carbohydrate, fat, proteins and mineral substances.[63-64] *In vivo* studies indicated that *Spirogyra* species has several beneficial effects, including antigastric ulcer and anti-inflammatory effect.[65]

In addition to the above compounds green algae contains carotenoids. Carotenoids are the tetra terpenoid organic components that are naturally occurring in the chloroplasts and chromoplasts of plants species and few other photosynthetic organisms like algae. Carotenoids usually cannot be manufactured by species in the animal kingdom.[66] Carotenoid serve as two key roles in plants and algae which absorb light energy for which is use in photosynthesis, and they are protect chlorophyll from photodamage.[67] People intake diets rich in carotenoids from natural foods, like fruits and vegetables, are healthier and have lesser mortality from a number of chronic illnesses.[68] The study establishes that the addition of both avocado fruit and oil considerably improved the subjects' absorption of all carotenoids tested such as α-carotene, *β*-carotene, lycopene, and lutein.[69]

Carotenoids are an important group of secondary plant metabolites. These colored compounds are responsible for red, orange, and yellow coloration in many plants, microorganisms, and animals, although the last group is not capable of producing them, and can only obtain them from food. Over 700 different carotenoids have been identified.[70] In most cases carotenoids possess a C-40 skeleton with system conjugated double bonds [71], with other groups like epoxy-, hydroxyl-, and keto-types also present.[72]

The health benefits of carotenoids same with phytochemicals, tannins [73] and polyphenols [74], have been well documented by researchers. Lycopene, a carotenoid from tomato, has antioxidant activity. It is known to induce cell to cell communication, modulates hormones and improves immune system.[75] Also, Kritchevsky et al. suggested that carotenoids can be effective inhibitor of heart disease and risk-reducer to some types of cancer.[76] Xanthophylls, lutein, and zeaxanthin have been associated with improving eye health.[77] Aside from their health benefits, carotenoids play important role as food ingredients due to their provitamin activity. Moreover, recent studies have shown that carotenoids have potential antibacterial activity.[78]

Phenolic compounds are commonly found in plants, including algae, and have been reported to have a wide range of biological activities including antioxidant properties.[79] This good antioxidant activity of *Spirogyra* might be attributed to the presence of phytochemicals such as flavonoids.[80] This indicates that the species of *Spirogyra spp.* are very rich in the contents of a variety of natural products.

The present study is aimed at investigations of the Phytochemicals and Antibacterial properties of the Petroleum ether, Chloroform, Acetone, and Methanolic extracts of fresh water *Spirogyra spp.* against four bacterial strains in order to validate it as an antibacterial remedy.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals

Analytical grade of petroleum ether, chloroform, acetone and methanol were used as solvent for gradient extractions and column elution. Silica gel (60-120 mesh size) for column chromatographic packing. sulfuric acid, hydrochloric acid, ammonia solution, concentrated sulfuric acid, acetic acid, ferric chloride, mercuric chloride, potassium iodide, sodium iodide, bismuth nitrate, olive oil, DMSO, saline solutions, Mueller Hinton agar, gentamicine, iodine.

3.1.2. Apparatus and equipments

Airtight conical flask, orbital shaker, Whatman No 3 filter paper, airtight bottles, oven, refrigerator, boiling tube, water bath, test tube, petri dishes, sterile cork borer, rotary evaporator (Heidolph, UK), column chromatography, UV lamp 254 nm and 365 nm (Uvitec chamber) and iodine chamber used to detect the purity of the compound. ^{1}H-NMR, ^{13}C-NMR and DEPT-135 (Bruker Advance 400 MHz) spectroscopy was done using $CDCl_3$ solvent and an IR spectrum was done using (Perkin-Elmer BX) (KBr) infrared spectrometer at Department of Chemistry, Addis Ababa University.

3.2. Methods

3.2.1. Sample collection

Fresh *Spirogyra spp.* sample was collected from Kito River around Jimma Airport in February, 2014. The collected *Spirogyra* genus was identified in the Department of Biology, Jimma University.

3.2.2. Preparation of algal extracts

The fresh *Spirogyra spp.* was washed under running tap water to remove associated debris, and dried under shade. The dried *Spirogyra spp.* was grounded into fine powder using electric grinder. The resulting powder was extracted as described below.

3.2.3. Extraction

Sequential extraction by petroleum ether, chloroform, acetone and methanol

350 g powder of *Spirogyra spp.* was initially soaked in 900 ml of petroleum ether in airtight conical flask for two days on an orbital shaker and then it was filtered. The filtrates were collected into airtight bottles. Similar process was repeated with fresh petroleum ether and the filtrates were pooled together. Finally, petroleum ether was removed in Rota vapor and the resulting extracts were stored in refrigerator until use for further study. The residue of the algal powder was dried and used further for chloroform, acetone and methanol extractions as similar to the procedures that carried out for the petroleum ether extraction.

3.2.4. Preparation of disk for antibacterial activity

The disk of about 6 mm diameter was prepared from Whatmann No. 3 filter paper using paper puncher. Active cultures of bacterial pathogens were prepared by transferring a loop-full of bacterial colonies from nutrient agar slants were transferred in to test tubes containing Nutrient broth. Then the test tubes were incubated without agitation for 24 hrs at 37°C. Then 0.5 McFarland turbidity standard solution was prepared (Appendix 9). After that, culture turbidity was adjusted to a 0.5 McFarland turbidity standard (1.2×10^8 CFU/ml).

3.2.5. Antibacterial test of algae extracts

Bacteria strains *Staphylococcus aureus (OSM 7346), Escherichia coli (ATCC 25922), Pseudomonas aeruginosa (DSMZ 1117) and Salmonella typhimurium (ATCC 13311)* were obtained from Biology department Jimma University. Antibacterial activity of plant extracts was tested by agar disc diffusion method.[81] To test the antibacterial activities of the bacterial strains, Muller Hinton Agar (MHA) medium was prepared and sterilized and poured into the pre-sterilized Petri dishes in triplet and kept for solidification and about 0.1 ml of each bacterial strain was spread with sterile swabs onto the Petri dishes. After Whatman No.3 filter paper sterilized disks (6 mm in diameter) was placed onto the surface of inoculated Petri dishes and about 100 mg/ml of (petroleum ether, chloroform, acetone and methanol) extracts were placed separately on the inoculated Petri dishes. DMSO (10 µg/µl) will be used as negative control against all the tested bacterial pathogens, while, gentamycin (10 µg/disc) was used as positive control for *Staphylococcus aureus, Escherichia coli, Pseudomonas*

aeruginosa and Salmonella typhimurium. All the Petri dishes were then allowed for 10 min at room temperature for the diffusion of the extract into the agar and finally, incubated at 37°C for 24 hrs for bacterial growth. After incubation, the antibacterial activity was indicated by a clear zone of inhibition and measured with the help of ruler.

3.2.6. Phytochemical analysis

The extracts were subjected to phytochemical tests for presence of following biomolecules by using the standard qualitative procedures as described in literature.[82]

Test for Alkaloids

Mayer's test

Mayer's reagent was prepared (Appendix 9). 1 ml of 1% HCl was added to the 3 ml of extract in a test tube and was treated with few drops of Meyer's reagent. A creamy white precipitate indicated the presence of alkaloids.

Dragendorff's test

Dragendorff's reagent was prepared (Appendix 9). To a few ml of filtrate, 1-2 ml of Dragendorff's reagent will be added. A prominent yellow precipitate indicated the test as positive.

Test for saponins

5 ml of extract was shaken vigorously to obtain a stable persistent froth. The frothing was then mixed with 3 drops of olive oil and observed for the formation of emulsion, which indicated the presence of saponins.

Test for flavonoids

A few drops of 1% NH_3 solution was added to the 2 ml of extract in a test tube. A yellow coloration was observed indicating the presence of flavonoids.

Test for tannins

To 0.5 ml of extract solution, 1 ml of distilled water and 1-2 drops of ferric chloride solution were added and observed for brownish green or a blue black coloration.

Test for terpenoids

5 ml of extract was mixed with 2 ml of $CHCl_3$ in a test tube. 3 ml of concentrated H_2SO_4 was carefully added along the wall of the test tube to form a layer. An interface with a reddish brown coloration was confirmed the presence of terpenoids.

Test for Anthraquinones

Extract was mixed well with benzene, and then half of its own volume of 10% ammonia solution was added. Presence of a pink, red or violet coloration in the ammonial phase indicated the anthraquinones.

3.2.7. Isolation and characterization from chloroform extract of *spirogyra spp.*

The chloroform extract that showed relatively superior antibacterial activity was subjected to column chromatography on silica gel using a mixture of solvent systems. Silica gel (60-120mm mesh size) was used as a stationary phase in column. To prepare the column was clamped vertically. Slurry of silica gel was prepared in solvent used for separation. The silica gel was dried at 100^0C for 2h to activate it and a glass column was packed with the activated 100g silica gel slurry dissolved in chloroform. The slurry was added to the column gradually to avoid cracks. This process was continued till a uniform column of desired length was obtained. 4 g of the crude extract was shaken with 4 g of silica gel and minimum amount of chloroform to obtain homogenous mixture. Then the solvent was allowed to evaporate and the dry sample adsorbed to the silica gel was applied to the column that was already packed with silica gel. This dried sample was poured into the column and different fractions were eluted with different solvent ratio of chloroform: ethyl acetate (in the ratio 100:0, 95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 65:35, 60:40, 55:45, 50:50, 45:55, 40:60, 35:65, 30:70, 25:75, 20:80, 15:85, 10:90, 5:90, 0:100).

4. RESULT AND DISCUSSION

4.1. Percentage yield of crude extracts

Air-dried *Spirogyra spp.* was extracted with gradient solvent systems by petroleum ether, chloroform, acetone and methanol. The amounts of the crude extracts and their percent yields were given in table 1.

Table 1: Percentage yield of crude extracts obtained from 350 g of powdered *Spirogyra spp.*

Solvents	Mass of crude extract (g)	% yield
Petroleum ether	4.227	1.208
Chloroform	10.405	2.973
Acetone	5.748	1.642
Methanol	5.437	1.55

4.2. Antibacterial activities of *Spirogyra spp.*

The Petroleum ether, Chloroform, Acetone, and Methanol extracts of *Spirogyra* spp. were tested for antibacterial activity against four human bacterial pathogens *Salmonella thyphimurium, Pseudomonas aeruginosa, Staphylococcus aureus and Escherichia coli.* Results are presented in the table 2 and appendix 10. The degree of activity was varied with reference to different solvent extracts of *spirogyra spp.* From the Table, the chloroform extracts was found to show the maximum zone of inhibition against *Salmonella thyphimurium* and *Pseudomonas aeruginosa.* The Acetone extract showed the maximum zone of inhibition against *Pseudomonas aeruginosa* and *Staphylococcus aureus*, followed by *Escherichia coli*. The Petroleum ether extract showed the maximum zone of inhibition against *Salmonella thyphimurium*, followed by *Pseudomonas aeruginosa*. The Methanol extract showed the maximum zone of inhibition against *Pseudomonas aeruginosa* followed by *E. coli*.

Table 2: Antibacterial activity of *Spirogyra spp. e*xtracts against different bacterial strains

Solvent extract and controller	**Inhibition zone (mm) against bacterial strains**			
	Escherichia coli	***Salmonella thyphimurium***	***Staphylococcus aureus***	***Pseudomonas aeruginosa***
Petroleum ether	10	15	11	14
Chloroform	14	16	10	15
Acetone	12	10	15	14
Methanol	15	11	10	16
Gentamicine	25	20	23	26
DMSO	NI	NI	NI	NI

NI = Not inhibitory

In the present work of *Spirogyra spp.,* when extracted with different solvents exhibited various range of activity against all bacterial strains tested. The results of the present study agrees with a few earlier reports that the petroleum ether and methanol extracts of *Spirogyra spp.* proved to be comparatively related in exhibiting antibacterial activity against *Pseudomonas and Escherichia coli*.[83] Similar results have also been obtained about the bioactivity of the species of *Spirogyra* collected from the estuarine environment of Miani Hor.[84] Previously, different species of *Spirogyra* were found to show promising results in their tests of antibacterial activity.[85]

4.3. Phytochemical screening of *Spirogyra spp.*

Phytochemical screening of *Spirogyra spp.* extracts showed the presence of most important Phytoconstituents. The medicinal value of *Spirogyra spp.* can be correlated due to the presence of various bioactive chemical constituents (Table 3). The petroleum ether extracts confirms the presence of terpenoids and negative to rest of the phytoconstituents. The chloroform extract gave positive results for terpenoids, tannis and flavonoids and negative to the rest of the Phytoconstituents. Acetone extract showed the presence of Terpenoids, tannis, sapponis and anthraquinone and negative to alkaloids and flanonoids. Similarly methanol extract of *Spirogyra spp*.

gave positive result to Saponins and tannis and negative to the rest. From the above test the chloroform extract was found to possess the most important phytoconstituents like terpenes, tannins and saponins. The previous reports on phytochemical compositions of *spirogyra spp.* also revealed the presence of phenolics, tannins, glycosides, and saponins.[86]

Table 3: Phytoconstituents of *Spirogyra spp.* gradient extracts of different solvents.

Phytochemical constituents	**Test result**			
	P. ether	**Chloroform**	**Acetone**	**Methanol**
Alkaloids	-	-	-	-
Terpenes	+	+	+	-
Tannins	-	+	+	+
Saponins	-	+	+	+
Flavonoids	-	-	-	+
Antraquinones	-	-	+	-

+ = Presence; - = Absence

4.4. Isolation of compounds from chloroform extracts of *Spirogyra spp.*

The crude chloroform extract with relatively better activity against the test strains was selected for chromatographic separation. The solvent system for isolation of compounds was selected by carrying out the TLC analyses of the crude extract in various combinations of solvents with increasing polarity. Chloroform and ethyl acetate (80:20) mixture were found to show good separation of components on the TLC plate. 4 g of *Spirogyra spp.* extract of chloroform was subjected to column chromatography in which 100 g of Silica gel was packed with chloroform. Successive elution with increasing polarity with chloroform and ethyl acetate resulted in 162 fractions. The isolated fractions were checked by TLC. The spots developed on TLC were visualized under UV light at 254 nm and 365 nm and then by exposure to iodine vapor. Fractions which have similar spot were collected separately in volumetric flask. Based on TLC analysis fractions with similar spots were combined. Totally 23 combined fractions were collected and labeled by letters from A-W. Fractions 1-6 (A), 7-9 (B), 10-15 (C), 16-19 (D), 20-26 (E), 27-30 (F), 31-38 (G), 39-40 (H), 41-43 (I), 44-53 (J), 54-57 (K), 58-60 (L), 61-71(M), 72-79 (N), 80-93 (O), 94-110 (P), 111-120 (Q), 121-130 (R), 131-142 (S), 143-151 (T), 152-165 (U), 156-160 (V) and

161-162 (W) were combined according to TLC profile. From those fractions two combined fractions B and K [which was obtained from chloroform-ethyl acetate (100:0 and 95:05 respectively] have one spot on TLC when analysed with different solvent system. However, the rest have more than one spot. Further TLC analysis was done and fractions A, E and J were selected for further purification in small column.

Fractions A (1-6) was chromatographed using chloroform-ethyl acetate solvent system and 40 fractions were collected by increasing polarity. Fraction A12-A25 gave a yellowish crystal (12 mg from chloroform-ethyl acetate (95:05)).

Fractions E (20-26) was chromatographed using chloroform-ethyl acetate solvent system and resulted in 20 fractions. The second fraction [which was obtained by elution with chloroform-ethyl acetate (90:10)] gave a pure compound. Fractions J (44-53) was chromatographed with increasing polarity of chloroform-ethyl acetate solvent systems gave 25 fractions. This successive elution resulted in isolation of J3-5 and J14-20 and J21-25 (with chloroform-ethyl acetate (100:0), (90:10) and (80:20) respectively). Then four fractions that have one spot on TLC profile and relatively high yield were selected and coded as SP-1 to SP-4.

4.5. Structural elucidation of isolated compound

The structures of the compounds SP-1 and SP-2 that were isolated from chloroform extract of *Spirogyra spp.* were proposed based on data obtained from spectroscopic (IR, ^{1}H NMR, ^{13}C NMR, and DEPT-135) analyses and comparing with reported data in literature. However the structures of compounds (SP-3 and SP-4) were not characterized due to their lack of full spectral data.

Structural elucidation of compound SP-1

Compound SP-1 was obtained as yellowish oily substance with R_f value of 0.25 in chloroform and ethyl acetate combination (95:5). The IR spectrum (Appendix 1) of SP-1 revealed the presence of hydroxyl (3400 cm-1) and alkene (1661 cm-1) functional groups. The ^{13}C NMR spectrum (Table 4, Appendix 3) of SP-1 showed carbon resonances in the olefinic region (δ 124.95 to 138.51) indicating double bonds. In the ^{13}C-NMR spectrum, the signals due to the double bonds in the cyclic alkene systems were observed at δ 125.60 (C-5) and 137.57 (C-6) as well as δ 124.95 (C-4') and 137.75 (C-5'), and two oxygenated carbon signals were identified at δ 65.12 (C-3) and 65.96 (C-3'). These facts suggested that compound **1** was of a carotenoid (C40) derivative. The ^{1}H-NMR spectrum of SP-1 displayed ten methyl protons at δ 0.9-2.04, two carbinol protons at δ 4.06 and 4.30, and fifteen olefinic protons at δ 5.51-6.72. The ^{1}H NMR data (Table 4, Appendix 2) displayed two methyl groups bound to aliphatic carbons at δ H 0.97 and δH 0.89 and an overcrowded olefinic signals, which appeared between δ 5 and 7, characteristic of polyenes. Based on the information from ^{13}C-NMR, as well as ^{1}H-NMR, DEPT-135 and IR spectral data led to the identification of the structure of compound SP-1 displayed high structural similarity to lutein.[87]

Table 4: ^{13}C-NMR, ^{1}H-NMR and DEPT-135 spectral data of compound SP-1 along with the reported data of lutein

C No.	^{13}C-NMR of SP-1 (δ)	^{13}C-NMR of Lutein (δ)	^{1}H-NMR of SP-1	^{1}H-NMR of Lutein (δ)	DEPT-135 SP-1 (δ)	Nature of the carbon
1	37.12	37.13				C
2	48.44	48.45	1.75, 1.64	1.78, 1.50	48.44	CH_2
3	65.10	65.10	4.03	4.03	65.10	CH
4	42.55	42.56	2.32, 2.44	2.05, 2.42	42.55	CH_2
5	125.60	126.17				C
6	138.00	138.00				C
7	128.73	128.73	6.14	6.12	128.72	CH
8	130.82	130.81	6.66	6.65	130.81	CH
9	135.70	135.07				C
10	130.05	130.04	6.14	6.07	130.09	CH
11	124.48	124.49	6.66	6.73	124.48	CH
12	137.56	137.57	6.28	6.28	137.56	CH
13	136.50	136.42				C
14	132.57	132.58	6.26	6.23	132.57	CH
15	130.06	130.09	6.64	6.63	130.09	CH
16	30.26	30.26	1.02	1.08	30.26	CH_3
17	28.73	28.73	1.09	1.09	28.73	CH_3
18	21.61	21.62	1.75	1.78	21.61	CH_3
19	29.69	29.70	1.93	1.97	29.69	CH_3
20	12.81	12.81	1.98	1.97	12.81	CH_3
1'	34.03	34.04				C
2'	44.63	44.64	1.27, 1.98	1.37, 1.85	44.63	CH_2
3'	65.93	65.93	4.25	4.25	65.93	CH
4'	128.54	128.81	5.46	5.50	128.72	CH
5'	137.77	137.77				C
6'	54.98	54.97	2.44	2.47	54.98	CH
7'	131.31	131.30	5.48	5.47	131.31	CH
8'	130.10	130.09	6.66	6.65	130.09	CH
9'	135.70	135.70				C
10'	125.59	125.60	6.14	6.05	125.59	CH
11'	124.93	124.94	6.66	6.74	124.94	CH
12'	138.50	138.50	6.28	6.28	138.50	CH
13'	136.70	136.49				C
14'	137.77	137.73	6.26	6.23	137.73	CH
15'	130.82	130.81	6.64	6.63	130.81	CH
16'	29.50	29.50	0.87	0.85	29.50	CH_3
17'	24.29	24.29	1.02	1.03	24.28	CH_3
18'	22.85	22.86	1.64	1.64	22.85	CH_3
19'	12.77	12.76	1.92	1.90	12.75	CH_3
20'	13.10	13.11	1.99	1.95	13.11	CH_3

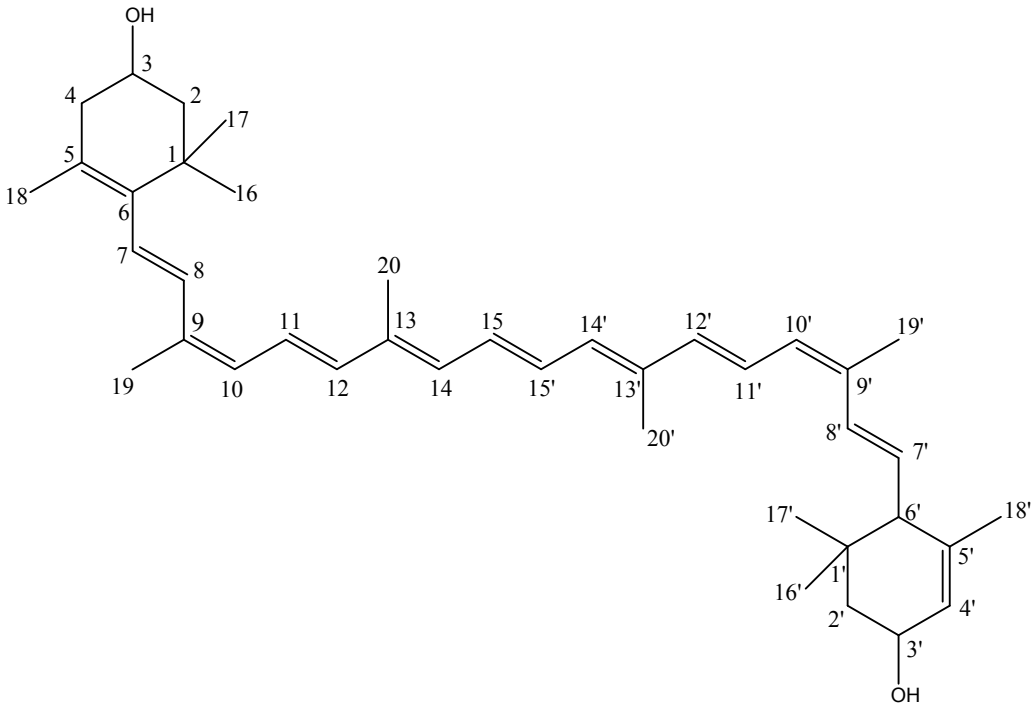
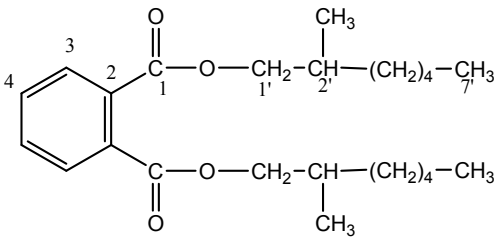


Figure 4: Proposed structure of compound SP-1(Lutein)

Partial characterization of compound SP-2

The IR spectrum clearly showed the presence of ester moiety (1731 and 1272 cm^{-1}), and presence of hydroxyl (3427 cm^{-1}) (Appendix 5). The ^{1}H NMR of the compound showed the aromatic protons H-3 and H-4 appeared at δ 7.71 and 7.55. The methylene protons at H-1 attached to the ester appeared at δ 4.23. The other methylene protons appeared at δ 1.27 (Appendix 6). The ^{13}C NMR spectrum also confirmed the structure. The ester carbonyl appeared at δ 167.78 while the methylene attached to ester oxygen appeared δ 68.19. The terminal methyl appeared at δ 14.03 while the methyl attached to another carbon appeared at δ10.95. The three aromatic carbons C-2, C-3 and C-4 appeared at δ 130.89, 128.81 and 132.46, respectively. The other carbons are also accounted for 167.78, 68.19, 38.76, 23.77, 28.94, 30.38, 22.99, 14.05, 10.98 (Appendix 7). The above spectral data related with the structure of the compound bis(2-methylheptyl)phthalate.[88]



4.6. Antibacterial activities of *the isolated compounds*

The isolated compounds of *Spirogyra* spp. Coded as SP-1, SP-2, SP-3 and SP-4 were tested for antibacterial activity against four human bacterial pathogens *Salmonella thyphimurium, Pseudomonas aeruginosa, Staphylococcus aureus and Escherichia coli.* Results were presented in the Table 5 and appendix 11. The degree of activity was higher than crude extracts. From the Table, SP-2 isolate was found to show the maximum zone of inhibition against *Salmonella thyphimurium* and *Pseudomonas aeruginosa.* SP-1 isolate showed the maximum zone of inhibition against *Pseudomonas aeruginosa* and *Staphylococcus aureus*, followed by *Escherichia coli*. Similarly, SP-4 isolate showed the maximum zone of inhibition against *Salmonella thyphimurium*, followed by *Pseudomonas aeruginosa*. However, SP-3 isolates were not inhibiting all bacteria strains.

Table 5: Antibacterial activity of isolated compounds from *Spirogyra spp.* chloroform extract

Test compounds	**Inhibition zone (mm) against bacterial strains**			
	Escherichia coli	***Salmonella thyphimurium***	***Staphylococcus aureus***	***Pseudomonas aeruginosa***
SP-1	17	18	20	20
SP-2	20	21	18	28
SP-3	NI	NI	NI	NI
SP-4	18	19	16	27
Gentamicine	27	23	25	29
DMSO	NI	NI	NI	NI

NI = Not inhibitory

According to previous reports in literature the isolated and characterized compound lutein is an antioxidant which is believed to be an essential nutrient for normal vision.[89] Studies have also indicated that Lutein improves heart health, protects our skin against UV damage, reduces diabetes induced oxidative stress, and possesses anti-inflammatory and anti-cancer properties .[90] Lutein offers eye protection by lowering the risk of age related vision loss, which causes gradual loss of central vision.[91] In the present investigation, Lutein isolated from green algae *Spirogyra spp.* was investigated for its antibacterial activity against pathogenic bacteria.

5. CONCLUSION AND RECOMMENDATION

Our results shows, green algae *spirogyra spp.* extract possess a significant antibacterial activity due to the presence of bioactive compounds. These results also suggest that the extract could serve as potential source of bioactive compounds. The petroleum ether, chloroform, acetone, and methanol extracts of *Spirogyra* spp. were tested for antibacterial activity against four human bacterial pathogens *Salmonella thyphimurium, Pseudomonas aeruginosa, Staphylococcus aureus and Escherichia coli.* The extracts were inhibiting the growth of bacteria. The chloroform extract was most antibacterial active. Then, from chloroform extract four compounds SP-1, SP-2, SP-3 and SP-4) were isolated by column chromatography. SP-1, SP-2 and SP-4 fractions of *Spyrogyra spp.* also results in the microbial growth inhibition. From those isolated compound SP-1 was fully characterized and SP-2 was partially characterized. Information obtained from ^{13}C-NMR, as well as ^{1}H-NMR and DEPT-135 spectral data led to the identification of the structure of compound SP-1 displayed high structural similarity to lutein. Phytochemical screening of *Spirogyra spp.* extracts showed the presence of most important Phytoconstituents like terpenoids, tannins, flavonoids, saponins and anthraquinone. The present study indicates that green algae *Spirogyra spp.* collected from Jimma town are potential sources of bioactive compounds and can be used as source for antibacterial agent. Further research is needed in which the extract could possibly be exploited for pharmaceutical and nutritional use.

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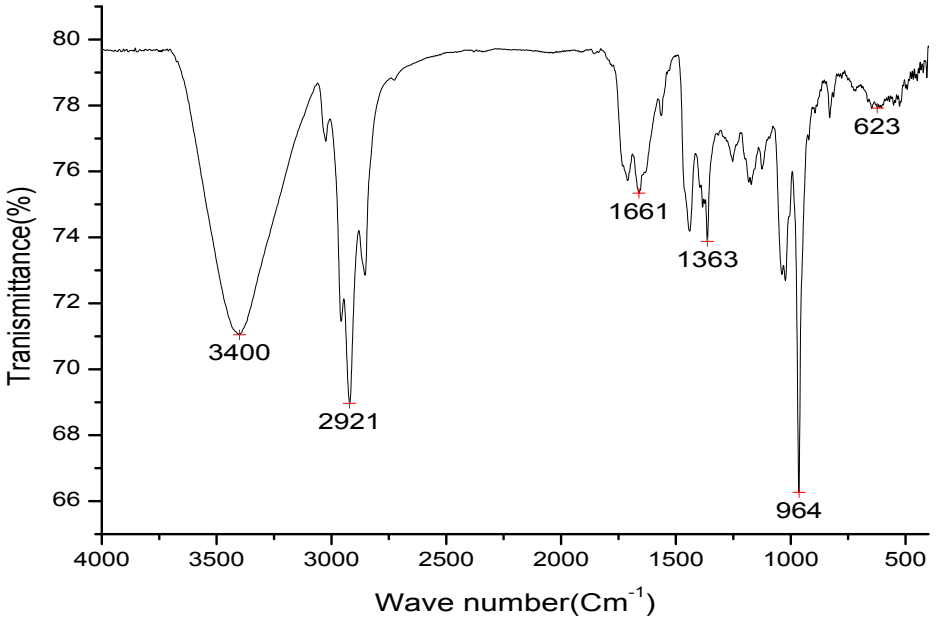
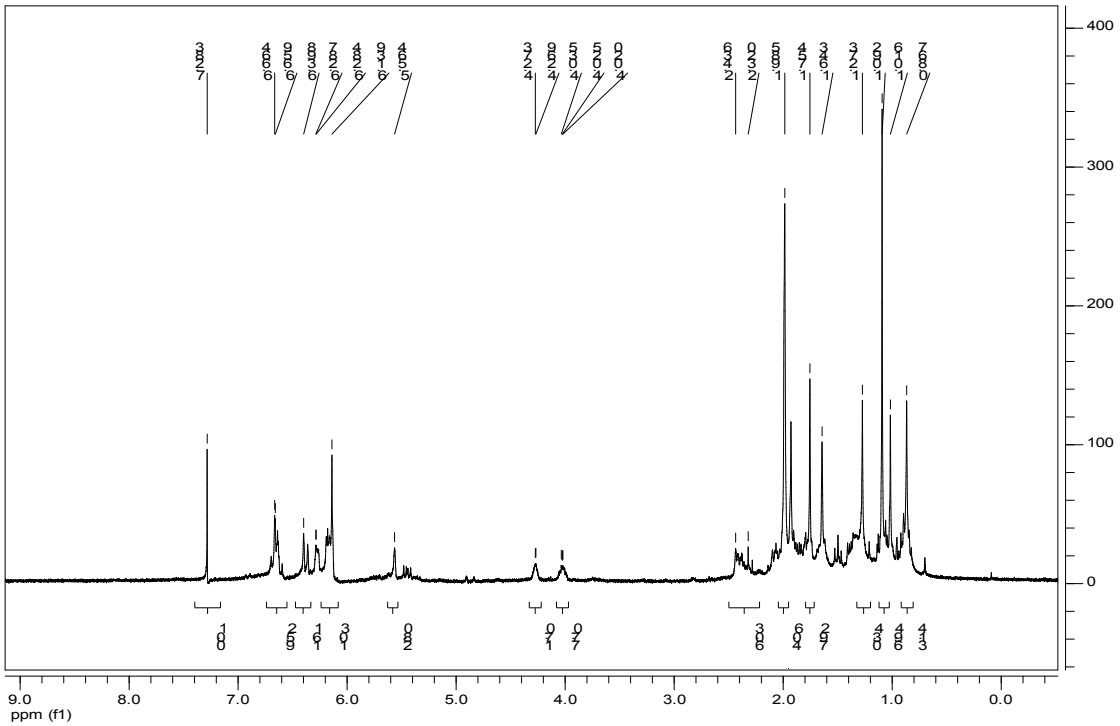
Appendixes

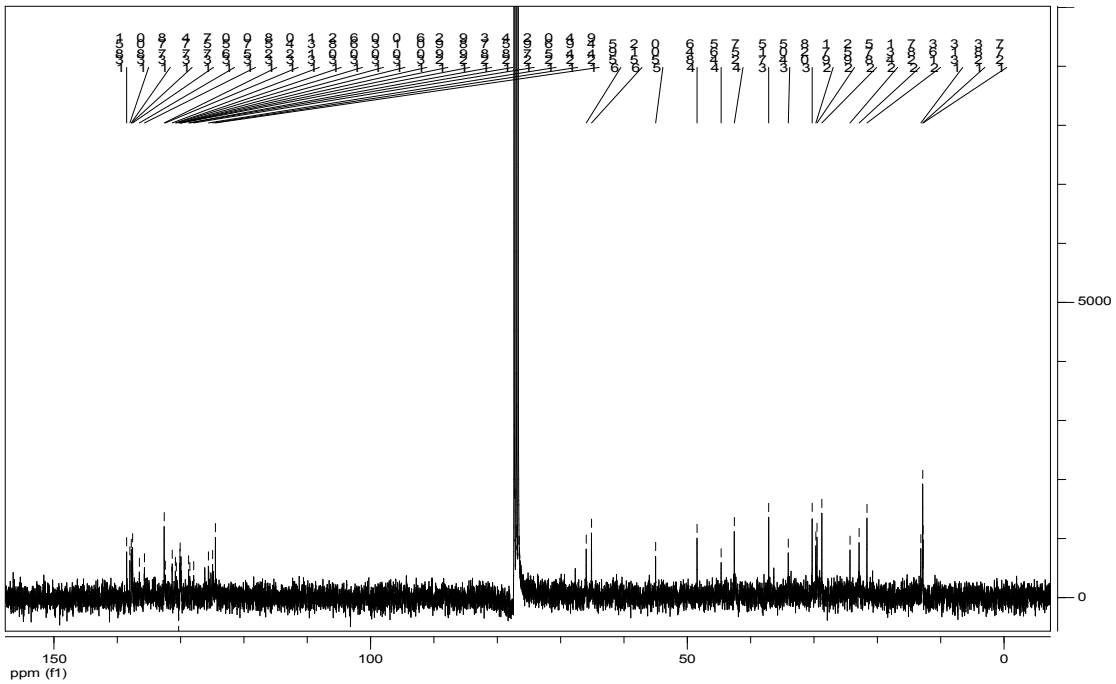
Appendix 1: IR spectrum of compound SP-1



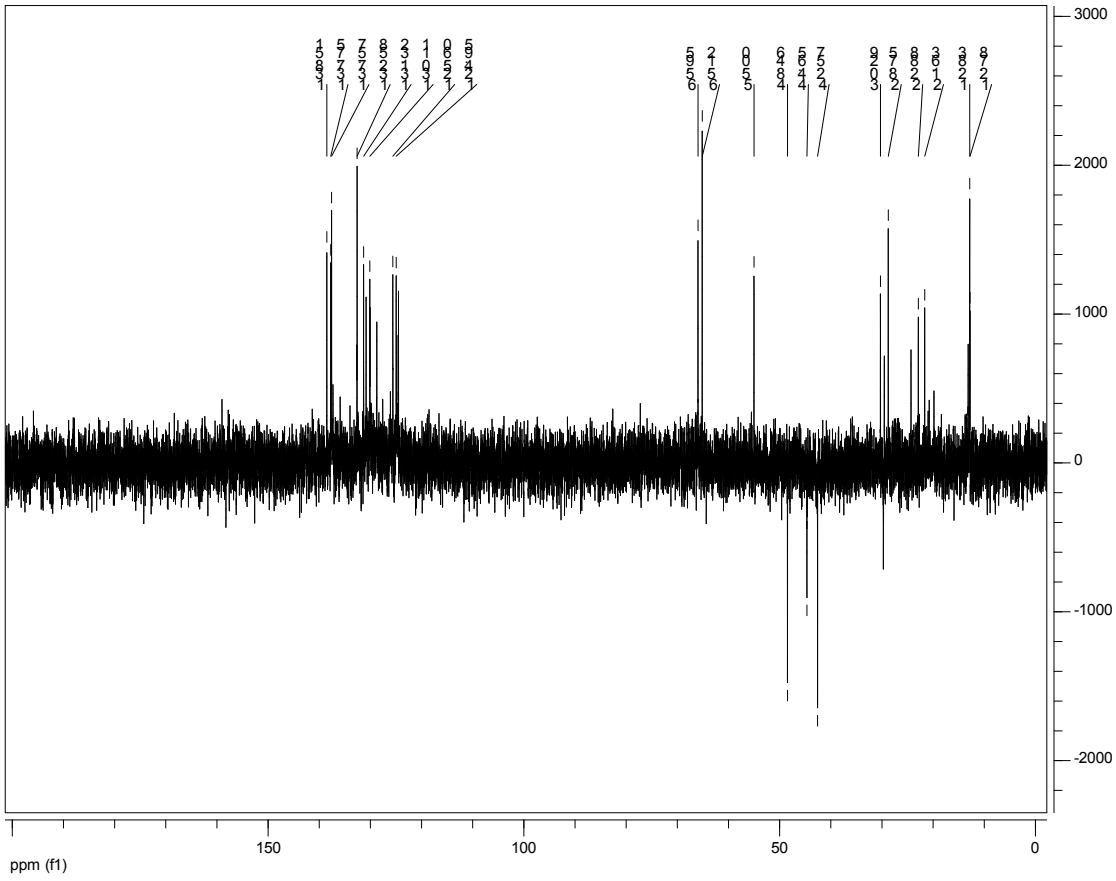
Appendix 2: ^{1}H-NMR spectrum of compound SP-1 in $CDCl_3$



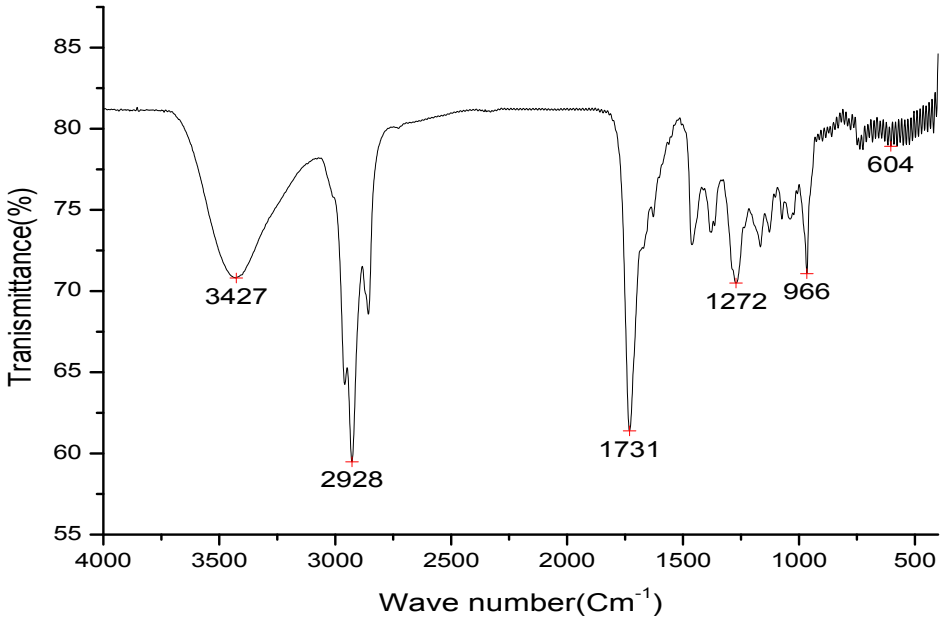
Appendix 3: ^{13}C-NMR spectrum of compound SP-1 in $CDCl_3$



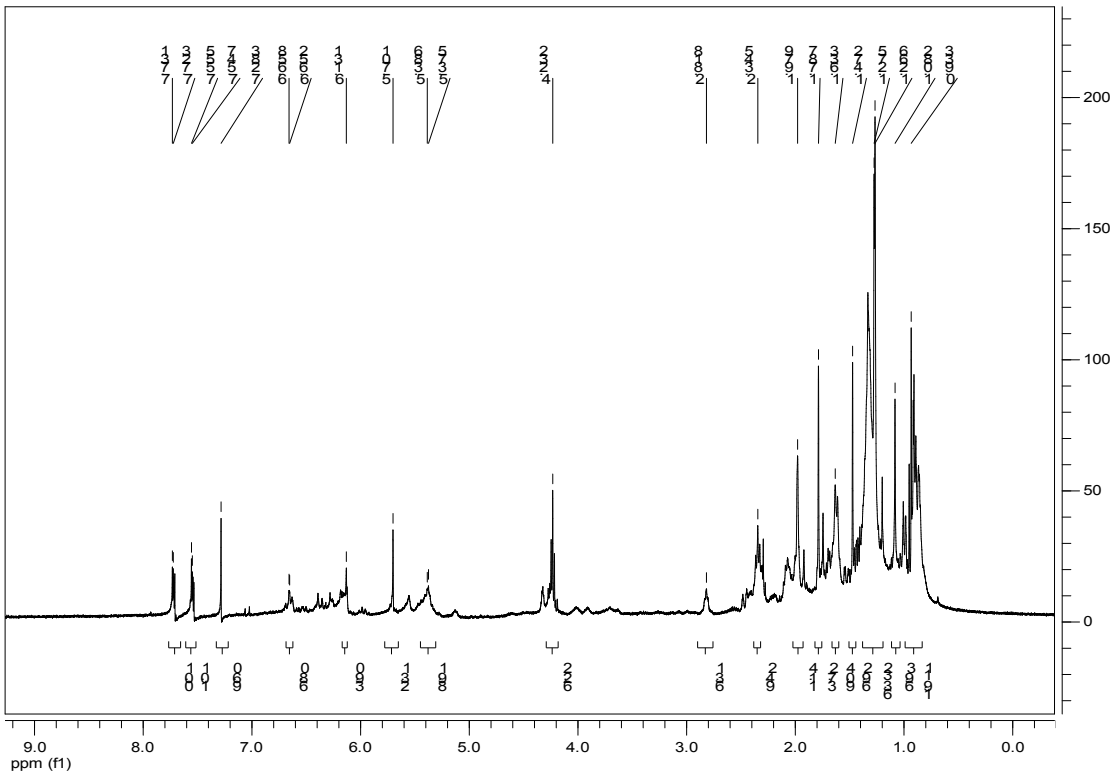
Appendix 4: DEPT-135 spectrum of compound SP-1 in $CDCl_3$



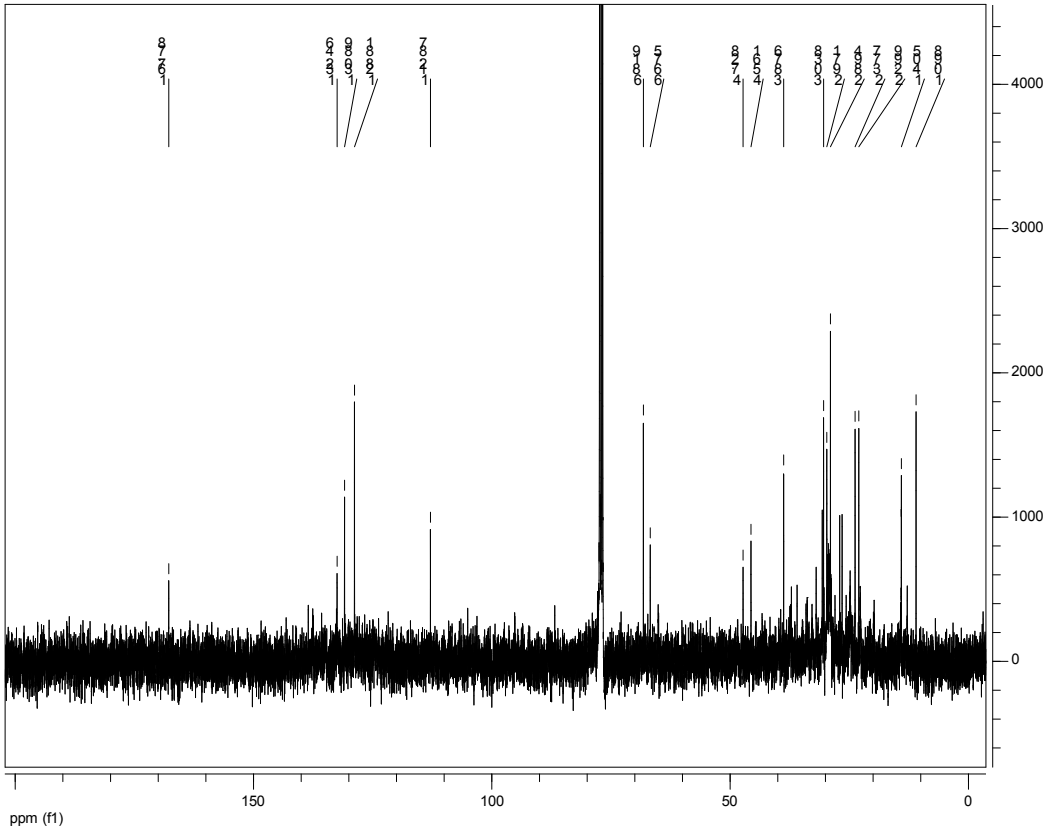
Appendix 5: IR spectrum of compound SP-2



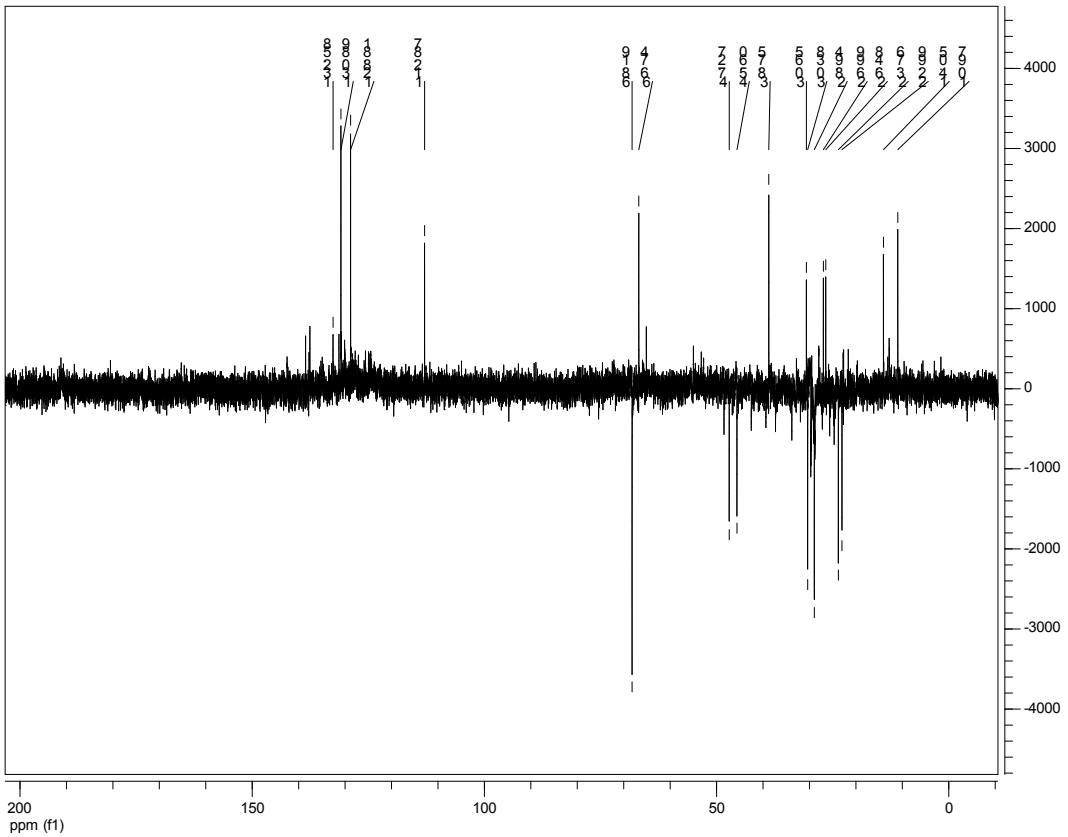
Appendix 6: ^{1}H-NMR spectrum of compound SP-2 in $CDCl_3$



Appendix 7: ^{13}C-NMR spectrum of compound SP-2 in $CDCl_3$



Appendix 8: DEPT-135 spectrum of compound SP-2 in $CDCl_3$



Appendix 9: Preparation of Mayer's reagent, Dragendorff's reagent and 0.5 McFarland standard solutions

Mayer's reagent

Mercuric chloride (1.35g) was dissolved in 60ml of water and potassium iodide (5.0g) was dissolved in 10ml of water. The two solutions was mixed and made up to 100ml with water.

Dragendorff's reagent

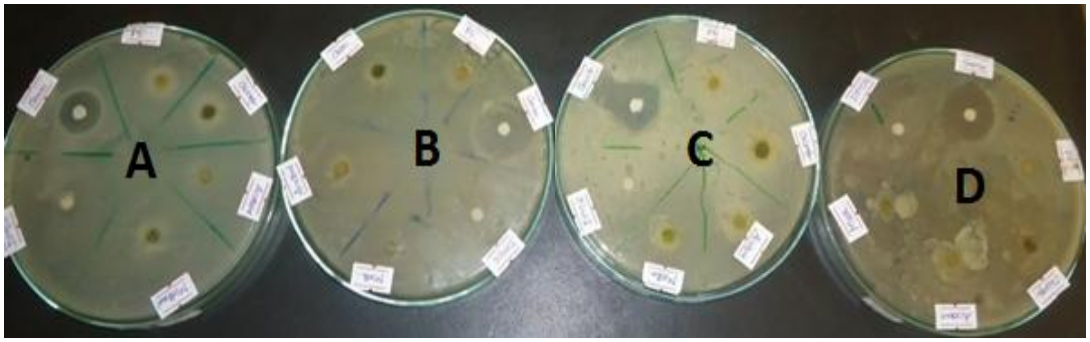
Dragendorff reagent: Solution (a): 0.85g basic bismuth nitrate in 10ml glacial acetic acid and 40 ml water was dissolved under heating. Solution (b): 8 g potassium iodide in 30 ml water was dissolved. Stock solution: (a) + (b) are mixed 1:1 Spray Reagent: 1 ml stock solution was mixed with 2 ml glacial acetic acid and10 ml water. Used for detection of alkaloids.

0.5 McFarland standard solutions

Turbidity standard 0.5 McFarland was prepared by mixing two solutions; solution "A" and solution "B". Solution "A" is 1 % v/v solution of sulphuric acid (H_2SO_4) and solution "B" is 1 % w/v solution of barium chloride ($BaCl_2$).

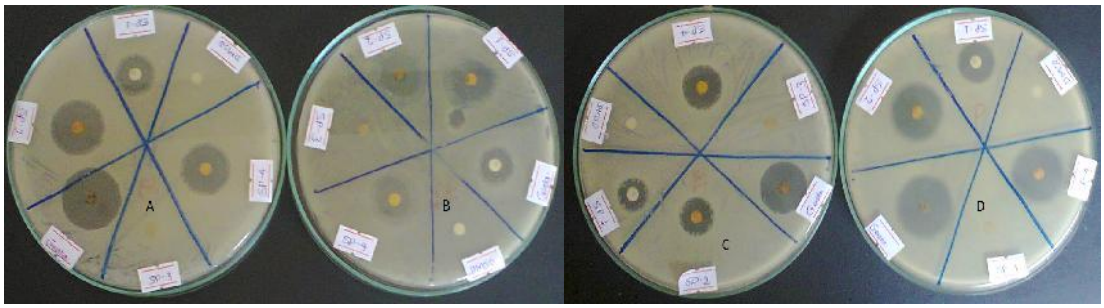
To get 0.5 McFarland standard, concentration equivalents to cell density of about 10^7-10^8 $CFUg^{-1}$, the amount of 0.5 ml $BaCl_2$ of 1 % solution "A" was mixed with 99.5 ml H_2SO_4 of 1 % solution "B". A small volume of the turbid solution was transferred to a screw-cap bottle of the same types as used for preparing test and control inoculums. Culture containing test tube with approximately equal concentration or density with 0.5 McFarland standards is used for inoculation of media. The standard was shaken immediately before used; and stored in a well sealed container in a dark place at room temperature (20 - 28 ^oC) when not used.

Appendix 10: Antibacterial activity of gradiant petroleum ether, chloroform, aceton and methanol crude extracts (100 mg/ml)



A. *Salmonella thyphimurium* B. *Pseudomonas aeruginosa* C. *Staphylococcus aureus* D. *Escherichia coli*

Appendix 11: Antibacterial activity of SP-1, SP-2, SP-3 and SP-4 isolated compounds (100 mg/ml)



A. *Salmonella thyphimurium* B. *Staphylococcus aureus* C. *Escherichia coli* D. *Pseudomonas aeruginosa*