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Phytochemicals, total lipids and molecular characterization of West African strain of *Oscillatoria* sp. (Cyanobacterium) isolated from *Ceratophyllum demersum* L. (Hornwort)

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Abstract
Cyanobacteria are potential sources of biologically active compounds with antiviral, antibacterial, antifungal, and anticancer activities. Bioactive compounds were obtained from the batch culture using Wright's Cryptophyte (WC) medium for *Oscillatoria* sp. isolated from *Ceratophyllum demersum* L. The DNA of the pure culture was extracted using DNeasy® Plant Mini Kit following the protocols, conserved DNA regions of chloroplast genome corresponding to SSU/18S rDNA and Rbcl were amplified and Polymerase Chain Reaction (PCR) product was sequenced. Lipids analysis and the active compounds of *Oscillatoria* strain were evaluated using Agilent 7890A Gas Chromatography (GC) and Agilent 5975C inert Mass Spectrometer detector. GC-MS analysis of hexane extract of *Oscillatoria* strain showed the presence of bicyclic sesquiterpene (Caryophyllene), α–Caryophyllene (monocyclic sesquiterpene) and terpenoids (Caryophyllene oxide). The result implied a closely related but unique strain which is probably endemic to West Africa and Nigeria as other strains are from other parts of the world, the potentials of this *Oscillatoria* strain in pharmaceutical industries is also worthy of note.

Keywords: Cyanobacteria, hydrocarbon, macrophytes, terpenoids, GC-MS

1. Introduction

Research on the cyanobacteria continues because of its potential to serve as source for drugs, renewable fuel, feeds, chemical feedstock, lubricant and bio-fertilizers due to reasons such as their ability to grow faster in different environments; have high yields per area, use up carbon (iv) oxide and release oxygen, an excellent source of vitamins and proteins. Compared to other biomass source, they do not compete with agriculture due to their ability to grow in areas not used for normal agricultural purposes. Cyanobacteria represent a morphologically diverse and widely distributed group of photosynthetic prokaryotes with significant roles in aquatic and terrestrial ecosystems. These bacteria have gained a lot of attention in recent years, because of their possible applications in the bioconversion of structurally different substrates into the desired usable products [1]. The key advantage of cyanobacteria over other microbes such as the bacterium *Escherichia coli* and the yeast *Saccharomyces cerevisiae* that are of common industrial use, is their ability to use light energy to reduce CO_2 to products of interest [1]. Cyanophyta are morphologically, physiologically and metabolically very diverse group, which makes them as a promising group of organisms for research on drugs discovery [2]. They are also known to contain bioactive compound which are active against fungi, bacteria and viruses [3, 4].The bioactive compounds are generally extracted using different methods such as: sonification, heating under reflux, Soxhlet extraction (hot or cold method) and others [5] from plant samples. In addition, extracts could also be prepared from percolation and maceration of fresh green plants or dried powdered plant material in water or organic solvent [6]. The extracts are used in perfumery, aromatherapy, medicine, incense, and flavoring food. The bioactive compounds can be extracted from plants using different solvents either polar or non-polar such as ethanol, hexane, methanol, acetone, or water. According to [7], methanoic extract of *Oscillatoria* sp. has some potential bioactive compounds which inhibit the growth of pathenogenic bacteria. Also [2], reported that the bioactive compounds from both methanoic and acetone extract of *Oscillatoria agardii* and *Anabaena* inhibited the growth of some bacteria. *Ceratophyllum demersum* L., a submerged aquatic plant commonly occurred with luxuriant growth mostly in polluted water body. Efforts were made to culture, isolate and extract crude sample of *Oscillatoria* sp. from pure culture for the bioactive components using GC-MS.

2. Materials and Methods

2.1 Description of the Study Site

Akute-Odo, part of Ogun River (Plate 1) harbors many aquatic macrophytes such as *Pistia stratiotes L., Ipomoea aquatic* Forssk*, Ceratophyllum demersum* L. and *Eichorrnia crassipies* (Mart.) Solms which at certain time of the year completely cover a large part of the water surface thereby impeding transportation. The river serves as a source of water processed by Lagos State Water Corporation for domestic and industrial consumption. The river also serves as major source of water to surrounding villages like Akute, Ajuwon, Alagbole, Ilu- Iga'un and means of transportation and other activities (Plate 1b). The river took its source from Igaran Hills at an elevation of about 530m above the sea level and flows directly Southwards over about 480km before it discharges into the Lagos Lagoon [8]. The main occupation of the people living around the river is farming, fishing and logging. Five sampling stations (Table 1) were created in order of accessibility.

Table 1: Geographic positioning of the sample stations at Akute-Odo part of Ogun River.

Stations	Names of accessible stations	Geographical position reading of the stations	
		Latitude	Longitude
1	Dam	06º40'672"N	003º22'730"E
2	Water Cooperation	06º40'026"N	003º22'521"E
3	Power line (Agogo)	06º41'362"N	003º23'004"E
4	Ilu- Iga'un	06º42'064"N	003º23'031"E
5	Odo Otun	06º41'946"N	003º22'720"E

Plate 1: Akute-Odo part of Ogun River and its Environs (A). Activities along the River Bank (B).

2.2 Collection of Samples

At each station, a whole plant of *Ceratophyllum demersum* L. was collected in a bowl. Agitation method, [9] was used to dislodge attached algae from the host plant, *Ceratophyllum demersum* using 60cl purified water. Samples were transferred to the laboratory without preservation.

2.3 Biological sample analysis

To the 100ml conical flasks for each station, 5ml of Wright's Cryptophyte media (WC) was added and covered with absorbent cotton wool to filter air and prevent contamination. The conical flasks were arranged close to the window for light intensity to facilitate growth process. The conical flasks containing the cultures were shaken every day to avoid clumping during growth. During the culturing period and subsequent observation of the samples from different fields under the microscope, species of *Oscillatoria* sp. (Plate 2) was selected and mono culture *w*as carefully isolated and the growth was monitored for 3 weeks, after which 2g from sufficient biomass obtained was extracted using n-hexane for Soxhlet apparatus (hot method) and then taken for Gas Chromatography and Mass Spectrometry (GC-MS).

Plate 2: Pure culture of *Oscillatoria* sp**.**

2.4 DNA extraction, PCR amplification and Sequence

Algal cultures preliminarily identified as *Oscillatoria* sp. were centrifuged for 15 min. in 15 ml centrifuge tubes. The pellet was then retrieved and immersed in liquid nitrogen for 2 min. Frozen pellets were then ground, and biomass was transferred to a 1.5ml centrifuge tube. Total nucleic acids were extracted with a DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany). Extracted DNA was amplified via PCR targeting portions of

SSU rDNA (~1550 nucleotide) and rbcL (~1250 nucleotide). The volume of each PCR was 50µL: 1.0 µL purified DNA; 5 µL 10× buffer; 2.5mM $MgCl_2$ (5µL of 25mM stock); 0.2 mM each dATP, dCTP, dGTP, dTTP (1 µL each of 10 mM stocks); 0.1 µM each primer (0.25 µL each of 20 µM stocks); 0.2 units Taq polymerase; and ddH_2O to a final volume of 50 µL. PCR conditions for SSU followed [10]. The crude PCR product was purified using ExoSAP-IT (USB Corporation, Cleveland, Ohio) following manufacturer's specifications. The pure PCR product was run in a FlashGel System (Lonza, Rockland, ME) to confirm nucleotide lengths of amplified products. Forward and reverse strands were cycle sequenced with BigDye (Applied Biosystems, Foster City, CA, USA) using a combination of nested primers (Table 4). Sequences were resolved with an ABI 3700 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence chromatograms were edited and assembled into contigs with Sequencher ver. 5.1 (Gene Codes Corporation, Ann Arbor, MI, USA). Assembled sequences for each target gene were compared to other taxa to confirm identification using NIH's BLAST system (http://blast.ncbi.nlm.nih.gov/Blast.cgi) as described in [11].

2.5 Analysis of Total Extracted Lipids

The protocols of [12,13] was followed as described by [11], total extracted lipids quantification, Two hundred milliliters of culture liquid was sub-sampled and filtered onto a 47 mm glass fiber filter, pore size ~0.8nm, and placed into a labeled plastic Petri dish and stored in a -80 ^{0}C freezer. Filters were removed from the freezer and placed in a plastic desiccator. The desiccator was then placed in a drying oven at 60 ^{0}C for 24 h. Upon removal from the drying oven the desiccator was connected to a vacuum pump for 1 h to remove water vapour. The filters were immediately removed from the desiccator and weighed with a Mettler AG245 analytical scale. Filters were then placed in individual Soxhlet extraction apparatus with 80 ml of a 2:1 methanol and chloroform solution. Extraction could proceed for 8–20h. At the end of the extraction period, the extract was transferred to a Kuderna-Danish Concentrator. The extract was condensed to 1ml and the extraction apparatus was rinsed with an additional 1ml of 2:1 methanol and chloroform. This 2ml of extract was transferred to a dry weigh ampoule and the solution was placed under a nitrogen stream until dry. The ampoule was then weighed on a Mettler AG245 analytical scale. Based upon these values mg/L of lipid was calculated for culture flask.

2.6 Gas Chromatography Mass Spectrometry (GC-MS) Analysis

The GC-MS uses the principle of separation technique, sample was analyzed in Agilent 7890A Gas Chromatography (GC) system equipped with a HP-5 column (30ml x 250µm ID, 0.25µm film thickness, Agilent 122-0132) and Agilent 5975C inert Mass Spectrometer Detector with triple-axis detection using Helium as carrier gas. The flow rate of 1.50ml/min; injector and column oven temperature 280 ^{0}C and 80 ^{0}C; injector mode split ratio of 20:1. GC-MS consists of an injection port at one end of a metal column packed with the analyte solution and a detector at the other end of the column. It is made up of the mobile phase which is the Helium gas and a stationary phase which serves as the column. The sample is injected into the injector with a liner under, the syringe picked the sample and the mobile phase, and the Helium gas propelled the sample from the liner down the column where separation into different components occurs. The injection port was maintained at a temperature 80ºC for 4 mins, it increased 10 ºC per min to 24 ºC for 20 min. The run time was 40 minutes. The column used was a metal tube (HP5MS) often packed with a sand-like material to promote maximum separation. As the sample moved through the column, the different molecular characteristics determined how each substance in the sample interacted with the column surface. The column allowed the various substances to partition themselves and the various components in the sample separated before eluting from the column. The length of the column was 30m, internal diameter of 0.320mm and thickness of 0.25µm. The amount of time at which each particular component elute from the compound was retained in the GC column known as the retention time which helped in differentiating between the components such that if two samples do not have equal retention times, those samples are not the same substance.

2.7 Identification of Compounds

Interpretation on mass spectrum GC-MS was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns and Fiehn Mass Spectra Libraries. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The Name, Molecular weight and Structure of the components of the test materials were ascertained as done and described by [11].

3. Results and Discussion

3.1 Physico-Chemical Parameters

The results of the physico-chemical parameters (Table 2) for each station showed different variations. The pH value ranges from 6.65 - 6.81 from stations 1-5, an indication that the water is slightly acidic. Salinity values ranged between 0.08‰ at station 1 to 0.11mg/L (station 5) an indication of freshwater environment. The highest Chemical Oxygen Demand value 25mg/L was recorded at station 2 while 12mg/L recorded as lowest value was observed at station 5. The Total dissolved solids value ranged from 102.1 mg/L to 106.0 mg/L from station 1 to 5 while the Dissolved Oxygen (DO) value which is an important water quality parameter with special significance for aquatic organisms in natural waters [14] fluctuated from 3.40 mg/L (station 2) to 3.55 mg/L (station 5). The BOD_5 values ranged between 4.00 mg/L to 6.00 mg/L (station 2), the minimum value (4mg/L) was recorded at stations 1 and 5 an indication that the river water could support aquatic life. Nitrate-nitrogen values ranged between 6.20 mg/L (station 2) to 14.62 mg/L at station 1 while Phosphate-phosphorus values fluctuated from 1.10 mg/L to 4.44 mg/L (station 3), Lowest Sulphates value (13.7mg/L) was recorded at station 2 while the highest value (22.8 mg/L) was recorded at station 3 (Table 2). Highest and lowest chlorophyll 'a' values (10.1µg/L and 5.6 µg/L) was recorded at station 3 and 5 respectively. Zinc recorded 0.058mg/L at station 1 and 0.09 mg/L at station 5 (Table 2).

Table 2: Physico-chemical parameters analysis of the water samples at Stations 1-5

Parameters	Station 1	Station 2	Station 3	Station 4	Station 5
pH at 25 °C	6.68	6.65	6.7	6.81	6.79
Conductivity (µS/cm)	193.2	194.9	196.1	197.1	199.9

TSS (mg/L)	23	30	29	32	21
TDS (mg/L)	102.1	103	104	104.3	106
Salinity (ppt at 25 °C)	0.08	0.09	0.09	0.1	0.11
DO (mg/L)	3.46	3.4	3.52	3.47	3.55
BOD_5 (mg/L)	4.00	6.00	5.00	5.00	4.00
COD (mg/L)	18	25	16	18	12
Nitrate (mg/L)	14.62	6.2	11.08	15.51	6.65
Sulphate (mg/L)	14.1	13.7	22.8	21.1	20.4
Phosphate (mg/L)	2.22	3.38	4.44	3.6	1.1
Silica (mg/L)	12.3	14.8	10.8	12.7	11.3
Zinc (mg/L)	0.058	0.074	0.081	0.082	0.09
Iron (mg/L)	0.21	0.245	0.197	0.202	0.147
Copper (mg/L)	0.0042	0.0047	0.0054	0.005	0.0036
Lead (mg/L)	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Oil & Grease (mg/L)	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
Chlorophyll a (µg/L)	8.4	6.6	10.1	6.1	5.6

3.2 DNA Extraction, PCR Amplification and Sequence

The pure PCR product run in a FlashGel System (Lonza, Rockland, ME) confirmed nt lengths of amplified products. A partial SSU rRNA fragment 1446nt in length and a partial RBCL fragment 1033nt in length were recovered. BLAST results for the partial SSU rRNA fragment identified a 97% similarity to an existing *Oscillatoria acuminatum* sequence (KM019978.1). BLAST results for the partial RBCL fragment identified a 98% similarity to an existing *Oscillatoria sancta* sequence (FN813331.1). From the analysis, the total extracted lipid was 10.88% with 60.2 biomass of the strain.

3.3 Total Lipid Extracted

The total lipid extracted was 10.88% as presented in Table 3.

Table 3: Total Lipid extracts of the *Oscillatoria* sp.

	Vial	Vial +Lipid	Lipid	Filter	Filter +Biomass	Biomass	Lipid%
Oscillatoria sp.	13118.93	13125.48	6.55	118.58	178.78	60.2	10.88039867

3.4 Gas Chromatography Mass Spectrum

Sixteen compounds were identified in this strain of O*scillatoria* by GC-MS analysis. The bioactive compounds observed were generally terpenes and terpenoids; hydrocarbons and oxygenated compounds (Table 4) respectively. The active compounds with their retention time (RT), molecular formula, nature of the compound, composition percentage and quality in the hexane extract are presented in Table 3 and Figure 1while the chemical structures were shown in Figures 2 (a-f); 3(a-f) and 4. Some of the hydrocarbon compounds recorded were Copaene, Caryophyllene, and α-Cubebene while Naphthalene, 1, 2, 3, 4, 4a, 5, 6, 8a-octahydro-4a, 8-dimethyl-2-(1-methylethenyl); Naphthalene,1,2,3,5,6,8a-hexahydro-4, 7-dimethyl-1-(1-methylethyl) and 1H Cycloprop [e] azulen-7-ol, decahydro-1, 1, 7-trimethyl-4-methylene which are oxygenated compounds.

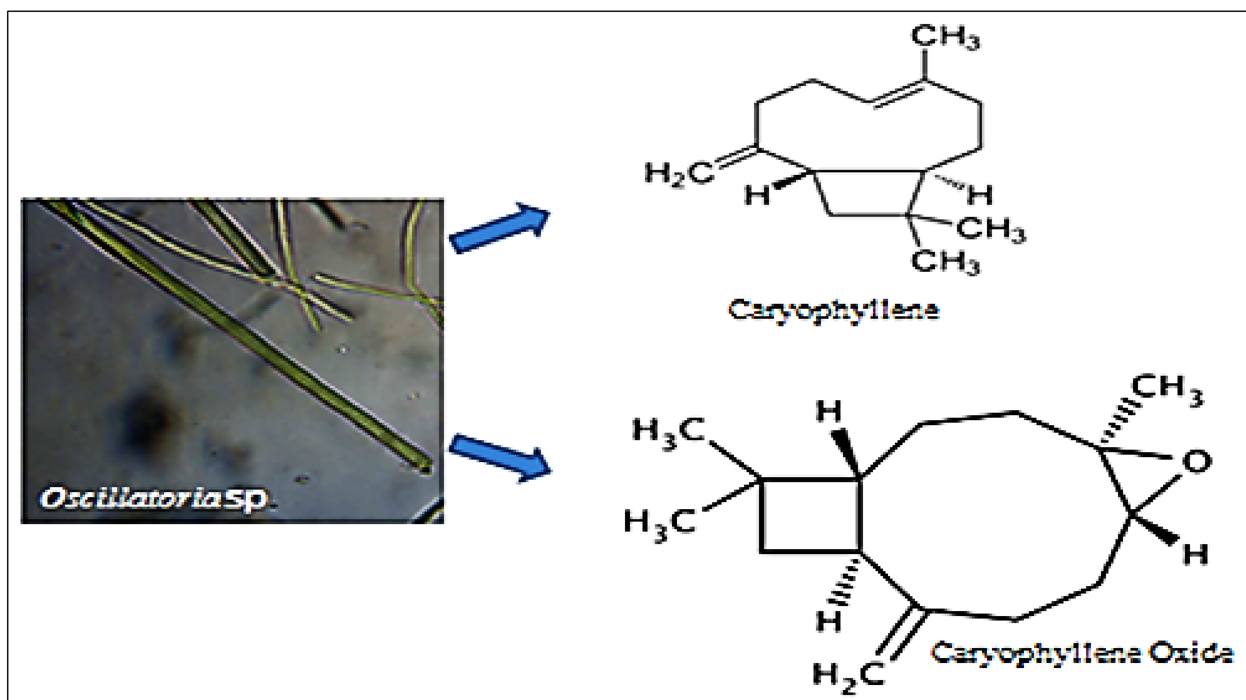
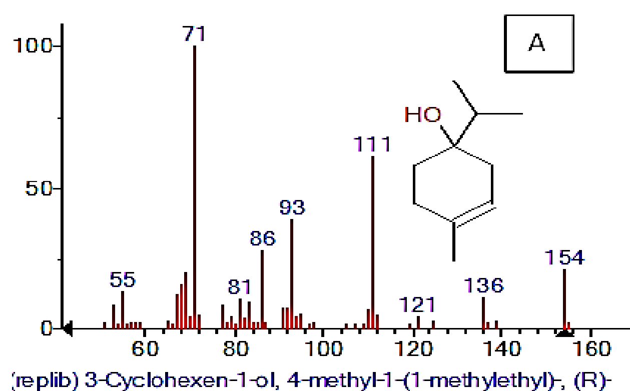
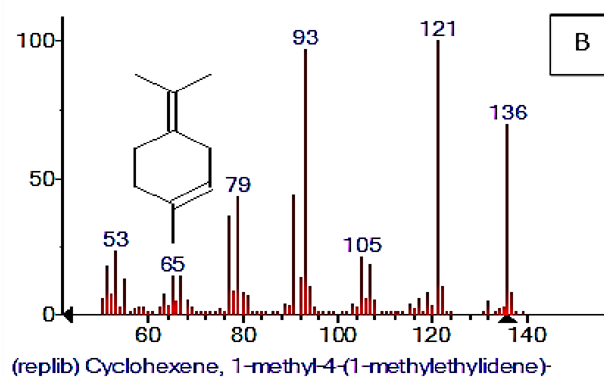


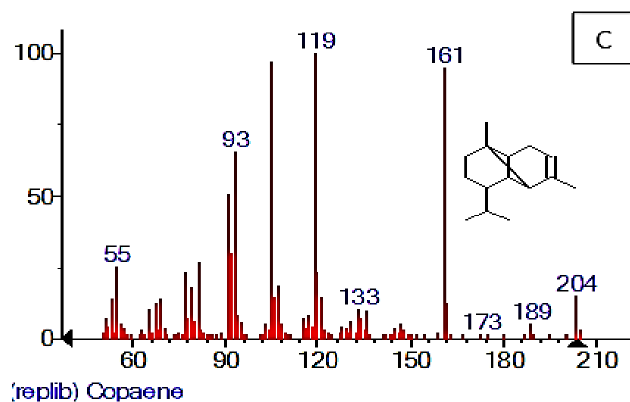
Fig 1: Graphic explanation of caryophyllene and its oxidized form (Caryophyllene oxide) from GC-MS analysis of *Oscillatoria* sp.

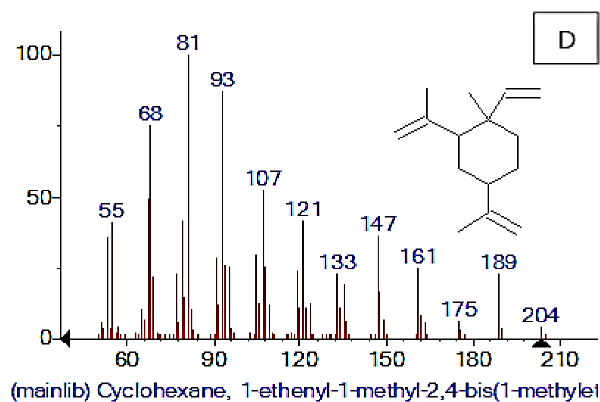
Table 4: Crude extract analysis results of *Oscillatoria* sp. using GC-MS

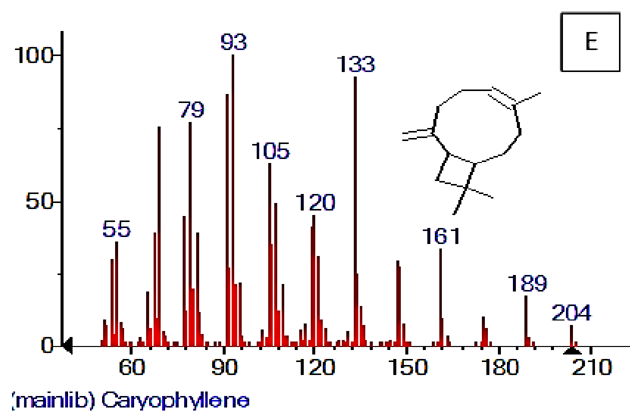
S/N	Compounds Names	Molecular Formular	Percentage (%)	Retention time (min)	Quality
1	3-Cyclohexen-1-ol,4-methyl-1-(-1-methylethyl)	$C_{10}H_{18}O$	5.78	6.426	70
2	Cyclohexene,1-methyl-4-(-1-methylethylidene)	$C_{10}H_{16}$	2.01	6.672	87
3	Copaene	$C_{15}H_{24}$	1.36	9.905	98
4	Cyclohexane,1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)	$C_{15}H_{24}$	32.3	10.191	64
5	Caryophyllene	$C_{15}H_{24}$	8.58	10.58	99
6	α–Caryophyllene (Humulene)	$C_{15}H_{24}$	7.23	11.072	96
7	Naphthalene,1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-2-(1-methylethenyl)	$C_{15}H_{24}$	2.64	11.53	98
8	Naphthalene,decahydro-4a-methyl-1-methylene-7-(1-methylethylidene)	$C_{15}H_{24}$	2.43	11.65	89
9	Naphthalene,1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)	$C_{15}H_{24}$	1.51	12.022	94
10	1H-Cycloprop[e]azulen-7-ol,decahydro-1,1,7-trimethyl-4-methylene	$C_{15}H_{24}O$	14.37	12.789	93
11	Caryophyllene oxide	$C_{15}H_{24}O$	8.98	12.857	92
12	Cis-Z-α-Bisabolene epoxide	$C_{15}H_{24}O$	3.36	13.166	94
13	1H-Cycloprop[e]azulen-7-ol,decahydro-1,1,7-trimethyl-4-methylene	$C_{15}H_{24}O$	2.78	13.922	78
14	2,6,9,11-Dodecatetraenal, 2,6,10-trimethyl	$C_{15}H_{22}O$	1.48	14.202	87
15	3-(2-Isopropyl-5-methylphenyl)-2-methylpropionic acid	$C_{14}H_{20}O_2$	2.61	14.362	55
16	7R,8R-8-Hydroxy-4-isopropylidene-7 methylbicyclo [5.3.1]undec-1-ene	$C_{15}H_{24}O$	2.6	14.528	95











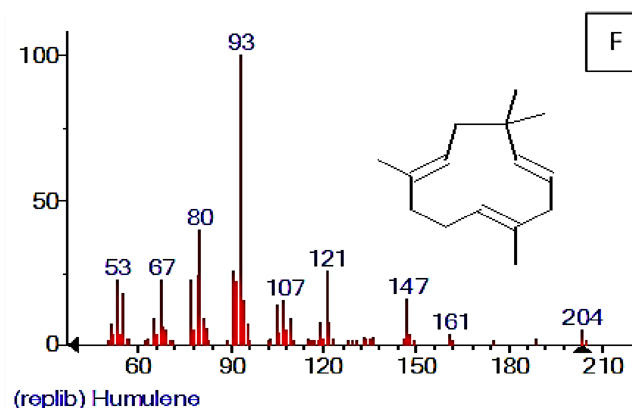


Fig 2A-F: Chemical structure of different compounds isolated from *Oscillatoria* sp.

A

(replib) Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-4a,8-dim

B

(mainlib) Naphthalene, decahydro-4a-methyl-1-methylene-7-

C

(replib) Caryophyllene oxide

D

(mainlib) cis-Z-a-Bisabolene epoxide

E

(mainlib) 1H-Cycloprop[e]azulen-7-ol, decahydro-1,1,7-trime

F

(mainlib) 2,6,9,11-Dodecatetraenal, 2,6,10-trimethyl-

Fig 3A-F: Chemical structure of different terpenoids isolated from *Oscillatoria* sp.

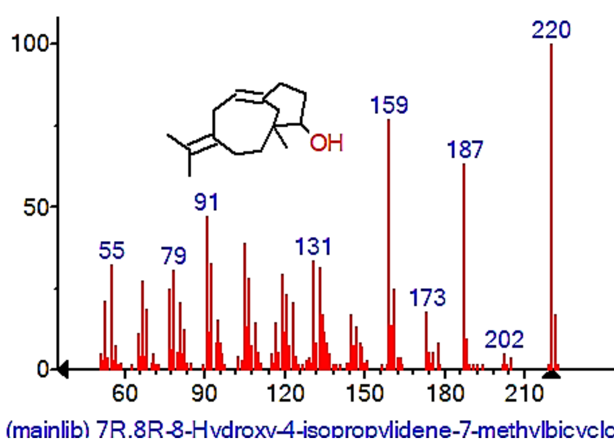


Fig 4: Chemical the structure of 7R, 8R-8-Hydroxy-4-isopropylidene-7 methylbicyclo [5.3.1] undec-1-ene extracted from *Oscillatoria* sp.

4. Discussion

The freshwater species, *Oscillatoria* produced different bioactive compounds, which are known to have potentials in pharmaceuticals. They are generally grouped into terpenes (hydrocarbons) and terpenoids (oxygenated compounds) such as Copaene, Caryophyllene and Naphthalene, decahydro-4a-methyl-1-methylene, 7-(1-methyethenyl), Cis-Z-α-Bisabolene epoxide, 7R, 8R-8-Hydroxy-4-isopropylidene-7-methylbicyclo [5.3.1] undec-1-ene respectively. Oxygenated compounds (terpenoids) are known to have potentials in pharmaceuticals. The GC-MS analysis of the hexane fraction from the work of [15] showed the presence of several compounds belonging to the terpenoids. According to [16], terpenoid compounds commonly found abundant in plants exhibited anti-cancer property by killing cancer cells. The solvent employed for extraction and method used also revealed these different bioactive compounds. Previous studies had reported anti-inflammatory activity of non-polar solvent extract from various plant species. [17] reported that ethyl acetate fraction of *Ochina squarrosa* L. showed high anti-inflammatory activity when compared to diclofenac sodium while [16], in their findings showed that hexane fraction of *Jatropha curcas* root extract had the strongest inhibiting power, anti-inflammatory and cytotoxicity activities. The isolated compounds in this study have been reported for their pharmaceutical potentials. Copaene which was found in abundance in the extract has pharmacological effect for been an anti-infective, non-steroidal, and anti-inflammatory agent. Caryophyllene is an anti-depressant and known to exert cannabimimetic anti-inflammatory effects in mice [18]. Among the oxygenated compounds; Caryophyllene oxide has also shown to possess antibacterial properties [19] while Naphthalene, decahydro-4a-methyl-1-methylene, 7-(1-methyethenyl) and Naphthalene, 1, 2, 3, 4, 4a, 5, 6, 8a-octahydro-4a, 8- dimethyl-2-(1-methylethenyl) are known to possess anti-fungal properties [20]. There is a dearth of literature on extraction of volatile compound from *Oscillatoria* in Nigeria in particular. Therefore, this study has established the endemic nature of *Oscillatoria* strain using molecular techniques and been able to showcase the potentials of using this cyanobacterium (*Oscillatoria).* This study is the pioneer work on the potentials of *Oscillatoria* species in Nigeria and it has explored *Oscillatoria* species in medicine and phytopharmaceutical importance.

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