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Extraction and characterization of the novel compound, 6 – methanol – 1 – methyl – 4 – isopropenyl cyclohexen – 1 – ene from the Petroleum ether (60 –80 Degree Centigrade) of the dried powdered fruit with seeds of the traditional medicinal plant Xylopia aethiopica.

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ABSTRACT: Fresh fruits with seeds of ***Xylopia aethiopica*** were collected from the Gola Forest, dried, grounded using a mortar and pestle and kept in specially sealed container until the time of the extraction. The extraction was done in a Soxhlet Extractor at a temperature of 70 ^{o}C, using solvents of increasing polarity i.e. petroleum ether 60 – 80 ^{o}C, acetone, methanol, ethanol, and water: ethanol (50:50). The Petroleum ether solvent extracts was concentrated and reduced to halve of its volume under reduced pressure using a Buchi Rotary Evaporator at 50 ^{o}C and stored in a refrigerator for 48 hours. 300g of the powdered fruit with seeds gave 255mg of brown oily liquid with sweet odour labelled LK005 which was Soluble in petroleum ether, water, Ethanol, Chloroform Dichloromethane and: diethyl ether. Tested positive for unsaturation contains the elements Carbon, Hydrogen and Oxygen.Elemental analysis was performed by wet chemical methods and confirmed by the Carlo Elba elemental analyzer. ^{1}H spectra were acquired on an Agilent DirectDrive2 500 MHz NMR spectrometer equipped with a One-Probe operating at 500 MHz for ^{1}H NMR and 126 MHz for ^{13}C NMR in $CDCl_3$, deuterated DMSO, $(CD_3)_2CO$, D_2O or toluene-d_8 and recorded at 25 °C. ^{1}H-NMR spectra were recorded with 8 scans. The fragmentation patterns obtained from the MS spectra and ^{1}H spectra data obtained from abroad confirmed the structure of Sample LK005 as 6- methanol-1-methyl -4-isopropenyl cyclohex-1-ene. Sample LK005 identified as an essential oil which support the use of the plant in traditional medicine.

KEY WORDS: Essential oils, unsaturation, fragmentation patterns, pain, proton NMR spectra.

I. INTRODUCTION

This research work was geared towards isolation and characterization of compounds from the petroleum ether extract of the traditional medicinal plant ***Xylopia aethiopica*** used for the treatment of both internal and external pain in Sierra Leone. Plants synthesize chemical compounds that help defend them against attack from a wide variety of predators such as insects, bacteria, virus, fungi and herbivorous mammals (allelopathy) **[1, 2 & 3]**. Some of these compounds, whilst being toxic to plant predators, turned out to have beneficial effects when used to treat human diseases. Traditional Healers in Sierra Leone and elsewhere in the world are not aware of the active compounds in the medicinal plants responsible for the numerous diseases they claim to cure. Thus the treatments they offer appear to be more of placebo than nocebo. Pharmacognostic potentials of the dried powdered fruit with seeds of the traditional medicinal plant **Xylopia aethiopica** used for the treatment of both internal and external pains in Sierra Leone has been investigated and reported **[4, 5]**. The powdered plant organ gave fluorescent derivatives with NaOH solution, ammonia solution, 50% HCl and 50% HNO_3 when viewed under UV/Lamp confirming the presence crude drugs in the plant organ investigated. Phytochemical evaluation of the plant organ was reported to revealfrom moderate to high contents of carbohydrates, alkaloid, flavonoids, proteins sterols/terpenes, tannins and phenolic compounds and saponins in the Ethanolic, methanol and aqueous extract [4]. The plant organ investigated was reported to have large amounts of nutrients and rich in **K** (42283 ± 194.00 ppm), **Ca** (8682 ± 80.00 ppm), **Mg** (4016 ± 1216 ppm), **Al** (2600 ±196.00 ppm) and **Fe** (768.22 ± 14.36 ppm). The other elements present in smaller quantities were included **Ti (**211 ±15.00 ppm), **Rb** (62.24 ± 1.00 ppm), **Sr** (39.87 ± 0.71 ppm) **Zr** (23.74 ± 0.72ppm), **Zn** (22.09 ± 1.96 ppm), **Sc** (22.00 ± 11.00 ppm), **Cu (**9.99 ± 4.03 ppm) and **Mo** (4.92 ± 0.73ppm). The above elements have been reported to play great role in metabolic processes in humans thus preventing various types of mineral deficiency diseases that could be associated with pains and degenerative diseases **[5]**. The isolation and characterization of active compounds in *Xylopia aethiopica* will support the use the plant in traditional Medicine.

Figure 1: Dried fruits with seeds of Xylopia aethiopica

Botanical Name: *Xylopia aethiopica* (Dunal.) A. Rich
Local vernacular names in Sierra Leone [6,7]
Creole: Spais-Tik
Mende: Hewe
Temne: Ma-Tel
Kissi: Siawo
Karim: Sɔ
Xylopia aethiopica is an ever green, aromatic tree belonging to the Annonaceae family and found in most African Countries [**8, 9, 10, 11, 12, 13& 14].** The fruits of the plant are harvested twice a year in Sierra Leone. The dried fruits of *X. aethiopica* (Grains of Selim) have been reported to be used as treatment of bronchitis, dysenteric conditions, or as a mouthwash to treat toothaches, febrile pains, to treat asthma, stomach-aches, spice, headaches, constipation and rheumatism **[15]**.

Extracts from *Xylopia spp*. have been reported to possess antiseptic and analgesic properties, insecticidal activity, treatment of bronchitis and dysenteric conditions using different therapeutic preparations **[16]**. In Congo, it is used against the attacks of asthma, stomach aches and rheumatism, as a tonic in the Ivory Coast for women who have newly given birth, fertility and for ease of childbirth **[12, 15, 17, 18, 19, 20, 21 & 22]**. Aqueous Ethanol Extract of the Fruit of Xylopia Aethiopica (Annonaceae) has been reported to exhibit Anti-Anaphylactic and Anti-Inflammatory Actions in Mice **[21].** The non – traditional medicinal use of *Xylopia aethiopica* includes the use of the bark of the plant to make doors and partitions. Its termite resistant properties enable the wood to be used in hut construction: posts, scantlings, roof-ridges and joists. The wood is also used for boat construction: masts, oars, paddles and spars. In Togo, and Gabon and Cameroon, the wood was traditionally used to make bows and crossbows for hunters warriors **[11, 14].**Kaurane-type diterpenoids known as xylopioxyde (16, 17-epoxy-15-oxo-*ent*-kauran-19-oic acid) and Xylopic acid have been isolated from the fruits of ***Xylopia aethiopica*[21, 22, 23, 23, 24& 25]**.Insect anti feedant, immunomodulatory activities as well as antimicrobial, anti-parasitic, antitumoral **[26, 27& 28]**of theplant has also been reported.

II. MATERIALS AND METHODS

Collection of Plant Materials : Fresh plant materials of ***Xylopia aethiopica*** was collected in January, 2020 from the Gola Forest in the Eastern Province of Sierra Leone and identified with assistance of Mr. M. A. Feika, former Laboratory technician of the Botany Department, Fourah Bay College, University of Sierra Leonenow Project Supervisor attached to the Gola Forest, Kenema

Preparation of dried plant materials : The Fresh plant materials were reduced in size by crushing it into smaller pieces using a cutlass and dried under shade. The dried plant material was grounded using a mortar and pestle and kept in specially sealed container until the time of the extraction.

General Methods : Extraction was done in a Soxhlet Extractor at a temperature of 70 ^{o}C, using solvents of increasing polarity i.e. petroleum ether 60 – 80 ^{o}C, acetone, methanol, ethanol, and water: ethanol (50:50). Each time before extracting with next solvent, the powdered material (in the thimble) was air dried below 50 ^{o}C and then subjected to further extraction. The solvent extracts was concentrated and reduced to halve of its volume under reduced pressure using a Buchi Rotary Evaporator at 50 ^{o}C and stored in a refrigerator for 48 hours. The percentage extractive yield of each of the solvent extracts was calculated by using the formula below:

$$\% \text{ Extractive Yield (WW)} = \frac{\text{Weight of dried solvent extract}}{\text{Weight of dried powdered plant material}} \times 100$$

Equation1.0.: Formula to determine the percentage extractive yield of powdered plant extract

2.4. LC-MS and NMR spectrophotometry for Samples LK005 and LK006 (USA &UK)

Elemental analysis was performed by wet chemical methods and confirmed by the Carlo Elba elemental analyzer. ^{1}H spectra were acquired on an Agilent DirectDrive2 500 MHz NMR spectrometer equipped with a One-Probe operating at 500 MHz for ^{1}H NMR and 126 MHz for ^{13}C NMR in $CDCl_3$, deuterated DMSO, $(CD_3)_2CO$, D_2O or toluene-d_8 and recorded at 25 °C. ^{1}H-NMR spectra were recorded with 8 scans, a relaxation delay of 1s, and a pulse angle of 45° and referenced to the various NMR solvents as necessary. ^{13}C-NMR spectra were collected with 254 scans, a relaxation delay of 0.1 s, and a pulse angle 45. High-resolution mass spectroscopy was performed with APCI mass spectra recorded on Finnegan LCQ Deca (Thermo Quest) technologies with LC/MS/MS (quadruple/time-of-flight) and Waters Xevo G2-XS UPLC/MS/MS inert XL MSD with SIS Direct Insertion Probe. Melting points for all products were measured with a Thomas HOOVER capillary Uni-melt melting point apparatus and are uncorrected.

A set of procedures for routine characterization of samples that require a high level of confidence to assign purity by reverse phase Ultra high performance liquid chromatography (RP-UHPLC) under acidic mobile phase conditions were carried out. The Sample was labelled as MSQ3AB_15NOV2019SLK_00A – 10 with file name EV-SLK_ and MS file number IM-METCR-AB101-PosNeg, inlet file Number METCR-AB101 using the Open-Lynx equipment.The specification was typically comprised a test for the determination of the compound's identity and a test for the determination of the compound's purity with a high degree of confidence. Elemental analysis, UHPLC-MS, coupled with other ancillary detectors, were the predominant method of analysis used.
Common Apparatus and Reagents used were:

- 0.1% Formic acid in water – Mobile phase "A"
- 0.1% Formic acid in acetonitrile – Mobile phase "B"
- Waters ACQUITY UPLC CSH C18 Column, 130Å, 1.7 µm, 2.1 mm X 100 mm column

UHPLC system that is capable of gradient elution with UV or diode array detection with other detectors as required (e.g. MS, ELS) were used in the instrumental analysis.
No test sample was used to confirm operational performance of the system during daily setup of the system as the samples were all-natural products. For the LC Conditions**;** a flow rate= 0.6 mL/min;Column temperature = 40 ºC in 5.82 minutes. UV detection was typically performed at a selected wavelength or over a scan range. MS detection was typically performed over a mass range to include target masses and other ions of interest. Additional detectors such as ELS can also be included to meet specific project requirements. Acquired data was processed automatically using Open-Lynx Software, the data is then distributed electronically and read using the Open-Lynx data browser applications.

III. RESULTS AND DISCUSSIONS

Table 1.0.: Mass of solvent extracts of the plant organof*Xylopia aethiopica* by Soxhlet extraction

Item	Name of Plant	Plant organ used	Solvent used	Mass of powdered plant material (g)	Mass of solvent extract (g)
1	*Xylopia aethiopica*	Fruit with seeds	Petroleum ether	300	18.12
			Acetone	300	21.78
			Methanol	300	25.62
			Ethanol	300	29.31
			Ethanol: Water (50:50)	300	31.77

To extract compounds from ***Xylopia aethiopica,*** the Petroleum ether extract was concentrated to half of its volume, allowed to cool down and stored in a refrigerator for 48 hours. A brown liquid from the mixture was poured into another container purified, weighed,labelled **LK005**and sent abroad for Instrumental analysis.

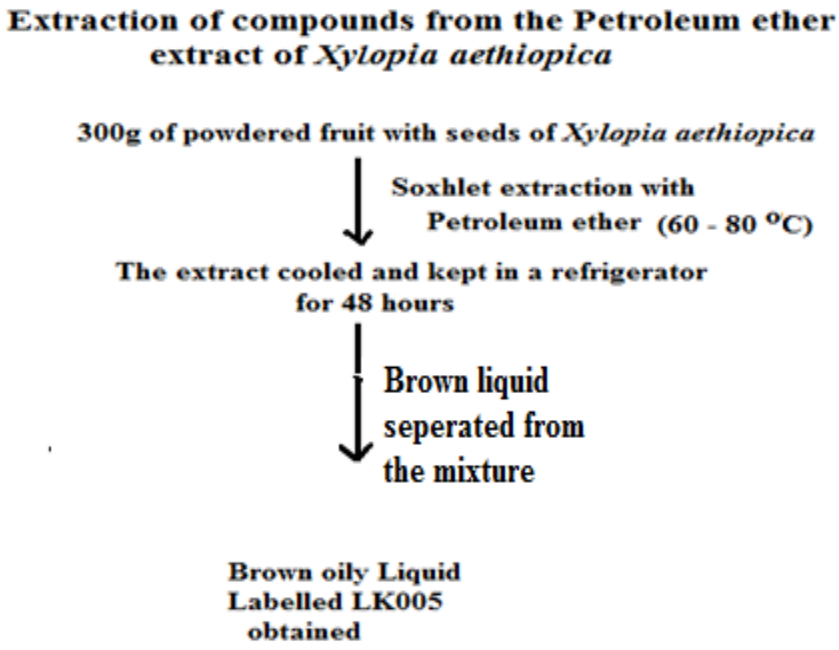


Figure 2.0.: Flow Chart for the extraction of compounds from Xylopia aethiopica

Extraction of compounds from powdered fruit with seeds of *Xylopia aethiopica* plants by Soxhlet extraction.

Mass of powdered leaves = 300g

Mass of Compound isolated = 0.255g

$$\text{Percentage by mass of sample LK005} = \frac{0.255\text{g} \times 100}{300\text{g}} = 0.085\%$$

Results of wet chemical analysis Sample LK005
Mass = 200mg
Nature = Brown oily liquid (Essential oil)
Solubility: Soluble in petroleum ether, water, Ethanol, Chloroform Dichloromethane and diethyl ether

Table 2.0.: Results of Wet Chemical Analysis on Sample LK005

Test	**Observation**	**Inference**
a. Acid Test – Solutions of sample LK003 was tested with Litmus paper	Red litmus paper turned blue	Sample LK005 is basic
Sample + Ethanoic acid	Smell of ester observed	Contains OH group
Solution of Sample LK005 + $NaHCO_3$	No reaction observed	Sample LK005 is not acidic
b. Phenol Test	No reaction observed	Sample LK005 does not contain Phenolic compound
a. Test for unsaturation	The colour of 0.1M $KMnO_4$ solution changes from purple to colourless	Sample LK005 is unsaturated
b. Test for aromaticity	smoky flame	Sample LK005 is aromatic
c. Carbohydrate		
Portion of Sample LK005 was strongly heated with in a boiling tube until no further change occurred.	Sample LK005 turned black with a colourless gas and droplets of colourless at the mouth of the test	Probably carbohydrate present Presence of Carbon dioxide

d. Gas + Lime water e. Liquid + $CuSO_4$	tube. Turns lime water milky. Colour changes from white to blue	Presence of water Hence Sample LK005 contains Carbon, Hydrogen and Oxygen
f. The Middleton's test		
5mg of Sample LK005 was mixed with 1g of Middleton's mixture in small test tube and heated for two minutes in a hot Bunsen flame. The red-hot test tube was plunged into 20ml of water in a beaker. whole mixture was boiled to dissolve the sodium salts formed, filtered and the filtrate divided into three portions		
g. Test for cyanide ions	No Specks of Prussian blue precipitated seen on the filter paper	Sample LK005 Nitrogen atoms absent.
ii Test for sulphide ions	No visible reaction seen	Sulphide ions are absent.
h. Test for halides ions	No visible reaction seen	Halides ions are absent

Hence Sample LK005 is Brown liquid with sweet odour, slightly soluble in water, Ethanol and Chloroform. Tested positive for unsaturation contains the elements Carbon, Hydrogen and Oxygen.

Instrumental Methods of Analysis of Sample LK005

Table 3.0.: Results of Elemental composition on Sample LK005

Symbol	Element	Atomic weight	Atoms	Mass percent
C	Carbon	12.0107	11	77.8655 %
H	Hydrogen	1.00794	18	11.7621 %
O	Oxygen	15.9994	1	10.3724 %

Elemental composition of $C_{11}H_{18}O$:
Expected Molecular Formula = $C_{11}H_{18}O$
Molar mass = 166.3 $gmol^{-1}$
Expected Structure

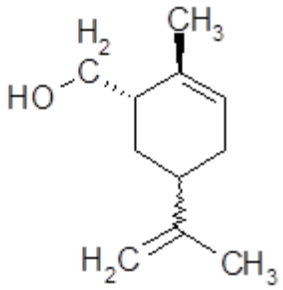


Figure 3.0.: Proposed structure of Sample LK005

Results of Proton NMR Spectroscopy (USA) :

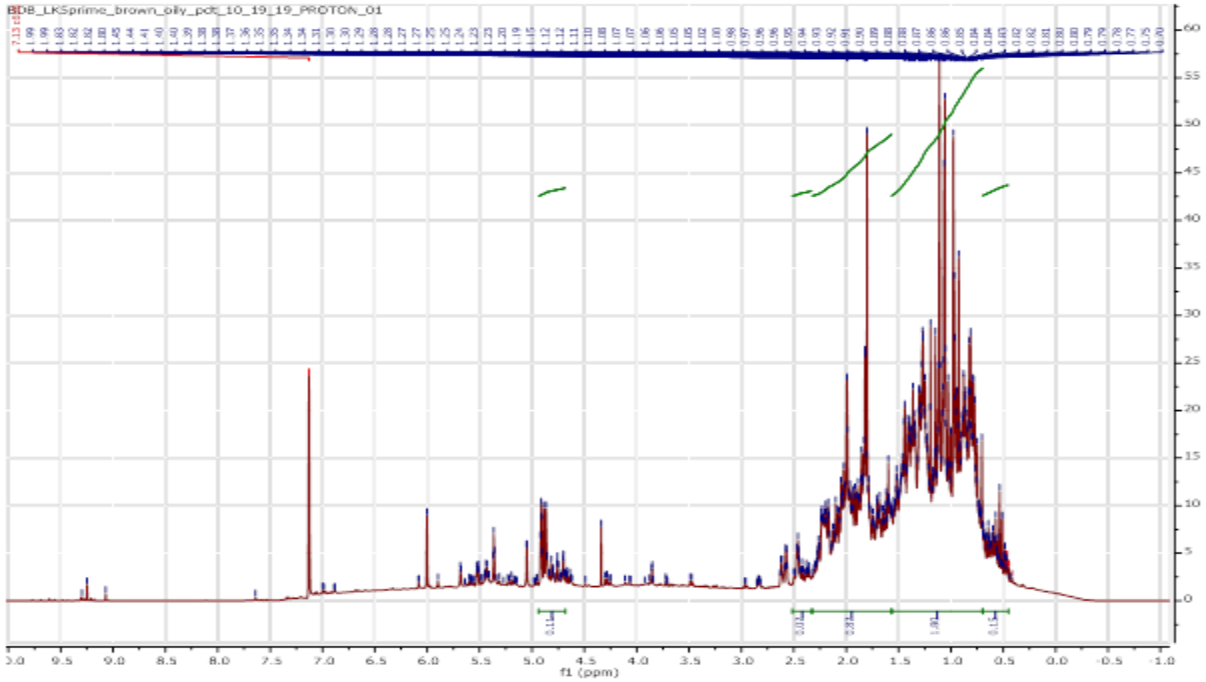
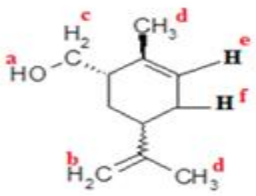


Figure 3.1.: Proton NMR of Sample LK005

The ^{1}H NMR Spectrum for LK005 is worthy of some comments according to Figure 3.1.
The interpretations of δ – values (ppm) for ^{a} H – O, ^{b} H_2C=, ^{c} H_2 –C, $C^{d}H_3$ –C-- and ^{e} H –C== shifts drawn above are shown below;



Solvent peak = 7.13 ppm
^{a}H – O = 6.00 ppm
$^{b}H_2C$= = 1 - 1.5 ppm
$^{c}H_2$ –C = 4.5 - 5 ppm J - values = 0.01
$C^{d}H_3$ –C-- = 0.5 - 1.00 J - values 0.15 - 1.80
^{e}H –C= = 2.0 - 2.5 ppm J - values 0.87, 0,07

Figure 3.2.: Different proton environments around the proposed structure of sample LK005

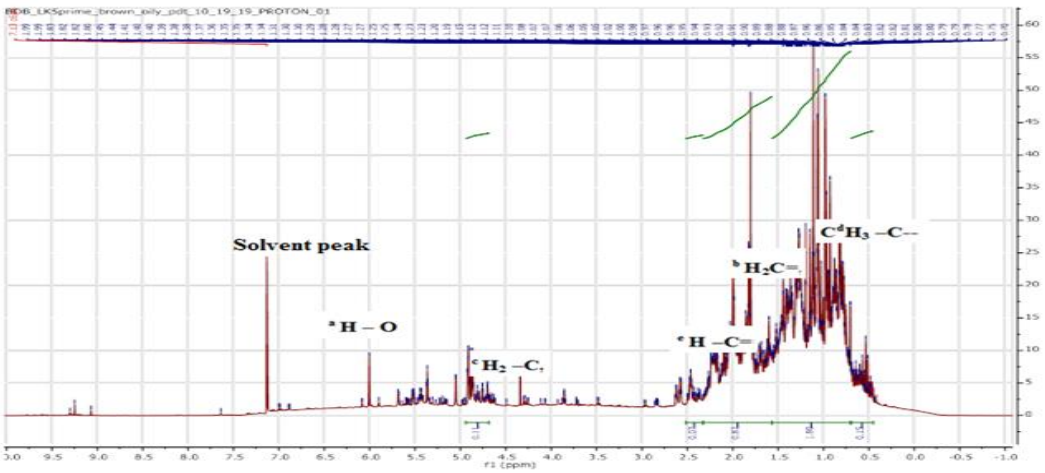


Figure 3.3.: Location of proton environments on the NMR spectrum of Sample LK005

Results of LCMS/Mass Spectroscopy of Sample LK005

Table 3.1.: ID and Description of mass spectrum of Sample LK005

Sample	Vial	ID	File	Date	Time	Description
6	1:14:00 AM	A6	MSQ3AB_15NOV2019SLK_005	15-Nov-19	3:46:40 PM	EV-SLK_005

Figure 3. 4: Results of LCMS-Mass Spectroscopy of Sample LK005

Table 3.2.: Number of Peaks used in obtaining Fragments of Sample LK005

Peak Number	Vial	Function	Trace	BPI	Area Abs.	Area %BP	Width	Height
1	1:14	1:MS ES+	MS ES+ :TIC	1.80E+06	6.00E+05	33.98	0.07	18981312
2	1:14	1:MS ES+	DAD: 215	1.56E+06	7.00E+03	16.52	0.052	341345.906
5	1:14	1:MS ES+	DAD: 215	9.49E+05	8.00E+03	18.82	0.048	390768.688
6	1:14	1:MS ES+	MS ES+ :TIC	2.99E+06	5.00E+05	30.86	0.043	20983352
7	1:14	1:MS ES+	DAD: 215	2.26E+06	6.00E+03	13.68	0.048	317418.469
8	1:14	1:MS ES+	MS ES+ :TIC	4.54E+06	1.00E+06	62.83	0.05	33186338
9	1:14	1:MS ES+	MS ES+ :TIC	4.34E+06	9.00E+05	49.56	0.057	27259180
10	1:14	1:MS ES+	MS ES+ :TIC	9.34E+06	2.00E+06	97.39	0.077	55629048
11	1:14	1:MS ES+	DAD: 215	3.79E+06	4.00E+04	100	0.148	938475
12	1:14	1:MS ES+	MS ES+ :TIC	9.01E+06	6.00E+05	32.66	0.04	19748748
13	1:14	1:MS ES+	MS ES+ :TIC	1.04E+07	1.00E+06	81.44	0.053	38801744
14	1:14	1:MS ES+	MS ES+ :TIC	9.92E+06	2.00E+06	100	0.09	32508732
15	1:14	1:MS ES+	DAD: 215	1.11E+05	3.00E+04	63.27	0.087	756921.625
16	1:14	1:MS ES+	DAD: 215	4.09E+03	6.00E+03	14.88	0.092	145326.406
17	1:14	1:MS ES+	DAD: 215	3.58E+03	5.00E+03	12.01	0.092	159670.141
18	1:14	1:MS ES+	DAD: 215	1.50E+03	2.00E+04	57.79	0.065	705532
19	1:14	1:MS ES+	DAD: 215	1.44E+03	5.00E+03	12.79	0.042	265183.938

The following results are obtained from nineteen peaks of Mass Spectroscopy with respect to the fragments that could be possibly obtained from Sample LK005 and by McLafferty Rearrangement with ID. NO of the MSQ3AB_15NOV2019SLK_005 are interpreted as shown below

Usual fragmentation patterns and by Maclerfty Rule corresponding to molecular ions in the various peak spectrums

– OH = 17, - 2OH = 34, --3OH = 51, – CH_3 = 15, – $2CH_3$ = 30, – $3CH_3$ = 45, – 4 CH_3 = 60, - CCH_3 = 27, $2CCH_3$ = 54, $3CCH_3$ = 81, $4CCH_3$ = 108. CCH_2 = 26, $2CCH_2$ = 52. $3CCH_2$ = 78, $4CCH_2$ = 104, $M^+ + CH_3CCH_2$ = 41, $M^+ + 2CH_3CCH_2$ = 82, $M^+ + 3CH_3CCH_2$ = 123,
$M^+ + 4CH_3CCH_2$ = 164, $M^+ + CH_2OH$ = 31, $M^+ + 2CH_2OH$ = 62, $M^+ + 3CH_2OH$ = 93
$M^+ + 4CH_2OH$ = 124, $M^+ + C_6H_7CH_3$ = 94, $M^+ + 2C_6H_7CH_3$ = 188, $M^+ + 3C_6H_7CH_3$ = 282, $M^+ + 4C_6H_7CH_3$ = 376

Ion	Expected Molecular mass	Peak position	Actual Molecular Mass	Intensity
M^+	165.27	735	165.03	28658
$M^+ + OH$	182.27	744	182.17	5550.48
$M^+ + 2OH$	199.27	1283	199.4	18634.2
$M^+ +$ **3OH**	216.27	756	216.21	2213.64
$M^+ + CH_3$	180.27	424	180.06	14694.4
$M^+ + 2CH_3$	195.27	1279	195.12	368264
$M^+ + 3CH_3$	210.27	755	210.18	4570.20
$M^+ + 4CH_3$	225.27	1296	225.23	2791.44
$M^+ + CCH_3$	192.27	1225	192.42	9439.60
$M^+ + 2CCH_3$	219.27	1292	219.7	2993.99
$M^+ + 3CCH_3$	246.27	1659	246.63	3609.37
$M^+ + 4CCH_3$	273.27	1906	273.11	664.835
$M^+ + CCH_2$	191.27	2241	191.64	472.868
$M^+ + 2CCH_2$	217,27	429	217.08	179676.0
$M^+ + 3CCH_2$	243.27	1076	243.12	135840.0
$M^+ + 4CCH_2$	269.27	1091	269,93	9726.14
$M^+ + CH_3CCH_2$	206.27	427	206.99	15616.60
$M^+ + 2CH_3CCH_2$	247.27	438	247.97	14335.00
$M^+ + 3CH_3CCH_2$	288.27	1095	288.02	12322.0
$M^+ + 3CH_3CCH_2$	329.27	1118	329.50	1429.04
$M^+ + CH_2OH$	196.27	749	196.22	1752.66
$M^+ + 2CH_2OH$	227.27	432	227.01	678098.00
$M^+ + 3CH_2OH$	258.27	442	258.10	227170.0
$M^+ + 4CH_2OH$	289.27	1680	289.17	1577.00
$M^+ + C_6H_7CH_3$	259.27	443	259.10	14583.90
$M^+ + 2C_6H_7CH_3$	353.27	825	353.70	2850.12
$M^+ + 3C_6H_7CH_3$	447.27	1504	447.60	1480.29
$M^+ + 4C_6H_7CH_3$	541.27	1539	541.34	2393.18

The fragmentation patterns as outlined in the table above support the fragmentation patterns in the proposed structure of Sample LK005. This confirms the structure of Sample LK005.

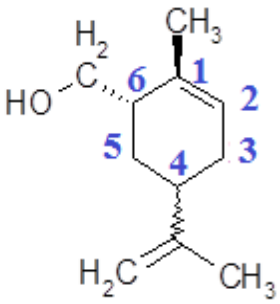


Figure 3.5.: Confirmed Structure of Sample LK005 and named as 6- methanol-1-methyl -4-isopropenyl cyclohex-1-ene

IV. CONCLUSION AND RECOMMENDATIONS

Conlcusion : The brown essential oil labelled Sample LK005 obtained from the petroleum ether extract has been identified and named as **6- methanol-1-methyl -4-isopropenyl cyclohex-1-ene.** Essential oils have been reported to be used as medicinal products, in the food industry as flavour and in the cosmetic industry as fragrances [**29]**.They have all been reported to exert broad spectrum antimicrobial activity [**30, 31]**. Xylopia aethiopica been a valuable medicinal plant widely [**32, 12, 33&19]**is used in traditional medicine for managing various ailments including skin infections, candidiasis, dyspepsia, cough and fever. The composition of the essential oils from the leaves, stem and root barks, and fresh and dried fruits of the plant are reported to have antioxidant properties and the principal constituents as mono- and sesqui-terpene hydrocarbons [**34]**. Several reports on the antimicrobial property against a wide range of Gram positive and Gram negative bacteria, and Candida albicans [**35, 36, 37, 38& 39]**of essential oilsare available in the literature. Mosquito repellent activity of the fruit essential oils has also been reported**[40].** Hence the work done in isolating Sample LK005 from *Xylopia aethiopica*, named as 6- methanol-1-methyl -4-isopropenyl cyclohex-1-ene being an essential oil is responsible for the medicinal property of *Xylopia aethiopica*which is in agreement with the previous works reported.

RECOMMENDATIONS : The brown essential oil identified as **6- methanol-1-methyl -4-isopropenyl cyclohex-1-ene,** extracted from the petroleum ether extract ofthe dried powdered fruit with seeds of Xylopia aethiopica plant is responsible for the medicinal use of the plant. We there recommend that a further work be done in synthesizing the compound and using the compound to carry out broad spectrum antimicrobial sensitivity testing. Mosquito repellent activity of the compound should be also carried out in order to produce mosquito repellant products.

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CONFLICT OF INTEREST : The authors have declared that no competing interests exist.

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