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Extraction, characterization and confirmation of Xylopic acid from the Petroleum ether 60 –80^{O}C extract of the dried powdered fruit with seeds of the traditional medicinal plant Xylopia aethiopica from Sierra Leone.

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Abstract: Xylopic acid identified as 15-(Acetyloxy) Kaur-16-en-18-oic acid has been extracted from the petroleum ether extract ofdried powdered fruit with seeds from the traditional medicinal plant Xylopia aethiopica. The petroleum ether extract after Soxhlet extraction was concentrated to one third of its volume and kept in a refrigerator for 48 hours. The oily liquid was separated from the mixture and the crystals remaining on the walls of the flask collected, weighed and labelled as LK006.
The Sample LK006 was light yellow crystals, slightly soluble in water, Ethanol and Chloroform. It tested positive for unsaturation contains the elements Carbon, Hydrogen and Oxygen during wet chemical methods of analysis.The melting point of the crystals was determined uncorrected to be 261-262^{o}C using the Electro thermal melting point determination equipment.
The Fragmentation patterns obtained from the LMCS spectra, the interpretation of Proton NMR spectra for sample LK006 and by the use of McLafferty rearrangement were used to confirmed the structure of Sample LK006 as 15 – (Acetyloxy) Kaur - 16 - en – 8 – oic acid commonly known as Xylopic acid. The compound is therefore reported in this research work to be one of components responsible for the analgesic properties of the traditional medicinal plant Xylopia aethiopica

KEY WORDS: McLafferty Rearrangement, analgesic, fragmentation patterns, Anti-Inflammatory and Rotary Evaporator

1.0. INTRODUCTION

This research work was geared towards isolation and characterization of compound(s) 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.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 **[1, 2]**. 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 reveal from moderate to high contents of carbohydrates, alkaloid, flavonoids, proteins sterols/terpenes, tannins and phenolic compounds and saponins in the Ethanolic, methanol and aqueous extract [1]. The plant organ investigated was reported to have large amounts of nutrients and rich in **K**, **Ca**, **Mg**, **Al** and **Fe**. The other elements present in smaller quantities were included **Ti**, **Rb**, **SrZr**, **Zn**, **Sc**, **Cu** and **Mo**. The elements detected 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 **[2]**. 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

Xylopia aethiopica is an ever green, aromatic tree belonging to the Annonaceae family and found in most African Countries [**3, 4, 5, 6, 7, 8&9**]**.** 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 **[10]**.

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 **[11]**. 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 **[7, 10, 12, 13, 14, 15, 16&17]**. Aqueous Ethanol Extract of the Fruit of Xylopia Aethiopica (Annonaceae) has been reported to exhibit Anti-Anaphylactic and Anti-Inflammatory Actions in Mice **[16].**

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 **[6, 9].** 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*[16, 17, 18, 19& 20]**.Insect anti feedant, immunomodulatory activities as well as antimicrobial, anti-parasitic, antitumoral **[21, 22& 23]**of theplant has also been reported.

2.0. Materials and Methods

2.1. 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 the Chief Laboratory Technician Department of Botany, Fourah Bay College, University of Sierra Leone, Freetown.

2.2. Preparation of dried plant materials

Plant materials were dried under the shade and not the sun so as to protect the thermo labile components if present from being chemically transformed. It was then reduced in size by crushing it into smaller pieces using a cutlass. The dried plant material was then grounded using a mortar and pestle and kept in specially sealed container until the time of the extraction.

The dried powdered plant material was used to carry out the extraction, isolation and characterization of compound(s) using wet chemical methods and instrumental analysis (Gas and Liquid - MS and NMR spectroscopy)

2.3. 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 Sample LK006 (USA &UK)

Elemental analysis was performed by wet chemical methods and confirmed by the Carlo Elba 1106 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.

The Sample LK006 was labelled as MSQ3AB_15NOV2019SLK_00A – 10 with file name EV-SLK_005and MS file number IM-METCR-AB101-PosNeg, inlet file Number METCR-AB101 using the Open-Lynx equipment.

The specification 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.

3.0. RESULTS AND DISCUSSIONS

Table 1.0: Mass of solvent extracts of the dried powdered fruit with seeds of*Xylopia aethiopica* plants 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

The Petroleum ether extract was concentrated to half of its volume, allowed to cool down and stored in a refrigerator for 48 hours. The brown liquid layer was separated from the mixture and poured into another container and light yellow crystals remaining at the bottom of the flask were collected,air dried, weighed and labelled **LK006.**

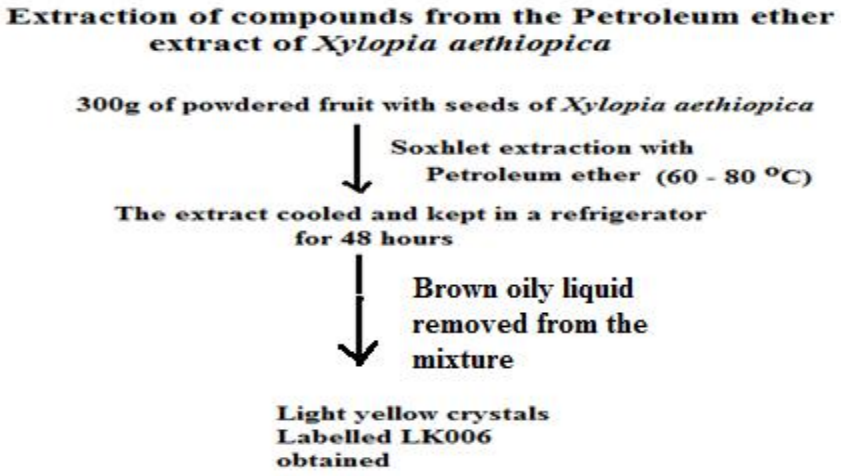

Figure 2.0.: Flow Chart for the extraction of Sample LK006 from Xylopia aethiopica

Mass of powdered leaves = 300g

Mass of Compound isolated = 0.200g

$$\text{Percentage by mass of sample LK006} = \frac{0.200\text{g} \times 100}{300\text{g}} = 0.067\%$$

Mass = 200mg
Recrystallized from ethanol
Nature = light yellow crystals
The melting point of the crystals was determined to be 261-262^{o}Cusing the Electro thermalmelting point determination equipment.
Solubility: Soluble in petroleum ether, water, Ethanol, Chloroform, Dichloromethane and diethyl ether

3.1. Results of Wet Chemical Methods of analysis

The results of wet chemical methods of analysis are reported in Table 4.8.0., below.

Table 2.0.: Wet Chemical Analysis of Sample LK006

Test	Observation	Inference
a. Acid Test – Solutions of sample LK006 was tested with Litmus paper	Blue litmus paper turned red	Sample LK006is acidic
Sample + Ethanol	Smell of ester observed	Contains COOH group
Solution of Sample LK006 + $NaHCO_3$	Effervescence observed and a colourless, odourless gas evolved which turned Lime water Milky	Carbon dioxide gas produced. Sample LK006 is acidic
b. Phenol Test	No reaction observed	Sample LK006 does not contain Phenolic compound
a. Test for unsaturation	The colour of 0.1M $KMnO_4$ solution changes from purple to colourless	Sample LK006 is unsaturated
b. Test for aromaticity	No smoky flame	Sample LK003 is not aromatic
c. Carbohydrate		
Portion of Sample LK006 was strongly heated with in a boiling tube until no	Sample LK006 turned black with a colourless gas and droplets of	Probably carbohydrate present

further change occurred. i. Gas + Lime water i. Liquid + $CuSO_4$	colourless at the mouth of the test tube. Turns lime water milky. Colour changes from white to blue	Presence of Carbon dioxide Presence of water Hence Sample LK006 contains Carbon, Hydrogen and Oxygen
d. The Middleton's test		
5mg of Sample LK006 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		
i. Test for cyanide ions	No Specks of Prussian blue precipitated seen on the filter paper	Nitrogen absent in Sample LK006
i. Test for sulphide ions	No visible reaction seen	Sulphide ions are absent.
i. Test for halides ions	No visible reaction seen	Halides ions are absent

Hence Sample LK006 is a light yellow crystal, slightly soluble in water, Ethanol and Chloroform. Tested positive for unsaturation and contain the elements Carbon, Hydrogen and Oxygen

3.2. Results of Instrumental Methods of Analysis

Table 3.0.: Results of Elemental composition on Sample LK006

Symbol	Element	Atomic weight	Atoms	Mass percent
C	Carbon	12.0107	22	73.37 %
H	Hydrogen	1.00794	32	6.64 %
O	Oxygen	15.9994	4	17.00 %
	Formula	$C_{22}H_{32}O_4$		

Expected Molecular Formula $C_{22}H_{32}O_4$
Molar Mass = 360.5 g/mol.
Proposed Structure of LK006

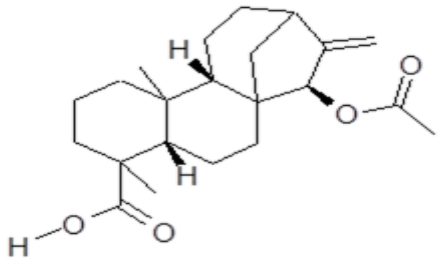

Figure 3.0: Proposed structure of Sample LK006

Physical Characteristics of Sample LK006	**Property Value**
Molecular Weight	360.5 g/mol.
XLogP3-AA	4.5
Hydrogen Bond Donor Count	1
Hydrogen Bond Acceptor Count	4
Rotatable Bond Count	3
Exact Mass	360.23006 g/mol.
Monoisotopic Mass	360.23006 g/mol.
Topological Polar Surface Area	63.6 Å²
Heavy Atom Count	26
Formal Charge	0
Complexity	669
Isotope Atom Count	0

The above Structure was confirmed from the Instrumental analysis sent to USA and The UK

3.3. Results of Proton NMR Spectroscopy (USA) for Sample LK006

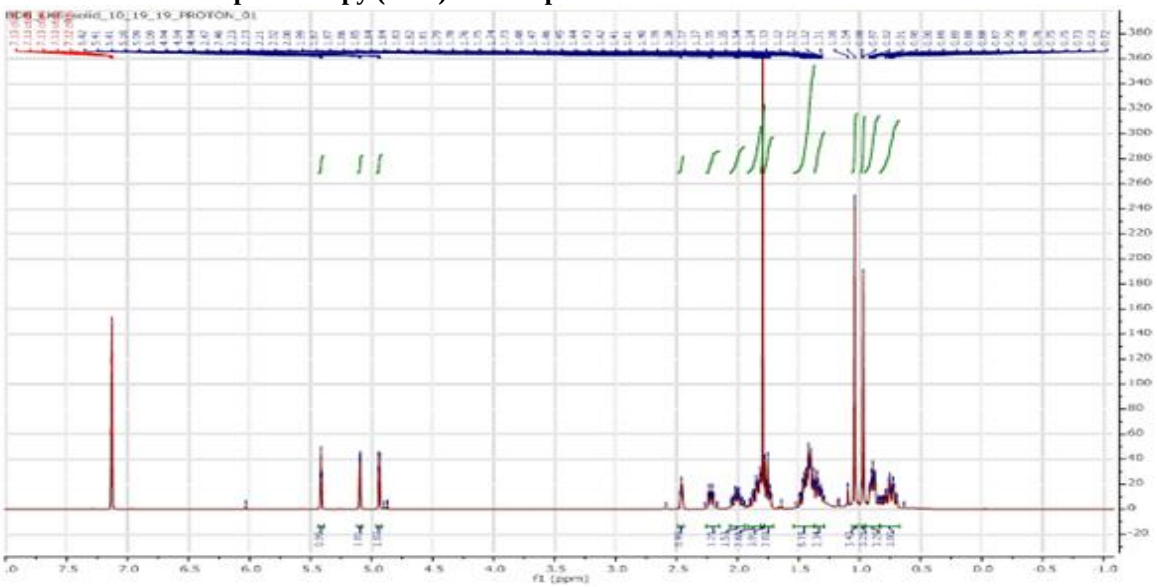

Figure 4. 0: Proton NMR Spectra for Sample LK006

The ^{1}H NMR Spectrum for LK006 is worthy of some comments according to Figure 4.0. The interpretations of δ – values (ppm) for ^{a}H – O, ^{b}H—R, ^{f}H—C_6H_7 ; $^{d}H_3C$, $^{d\,c}$H –C= , ^{e}H – $C_7H_5$and shifts drawn above are shown below;

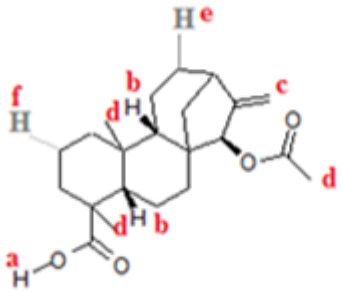

Figure 4.1.: Location of different Proton environments on the proposed structure of Sample LK006

^{a}H – O, δ – values = 5.42 ppm J- value 0.99

^{b}H—R, δ – values = 5.41 ppm J values 1.01, 1.07

^{c}H –C= δ – values = 1.55 ppm J-value 3.91 , 2.07

$^{d}H_3C$ δ – values = 1.0 ppm J-values 3.24, 3.25, 3.29, 3.00

^{e}H – C_7H_5 δ – values = 1.52 ppm J-values 2.07, 3.91

^{f}H—C_6H_7 δ – values = 4.57 ppm J-values 1.07

Solvent peaks δ – values = 7.25 ppm (C6d6)

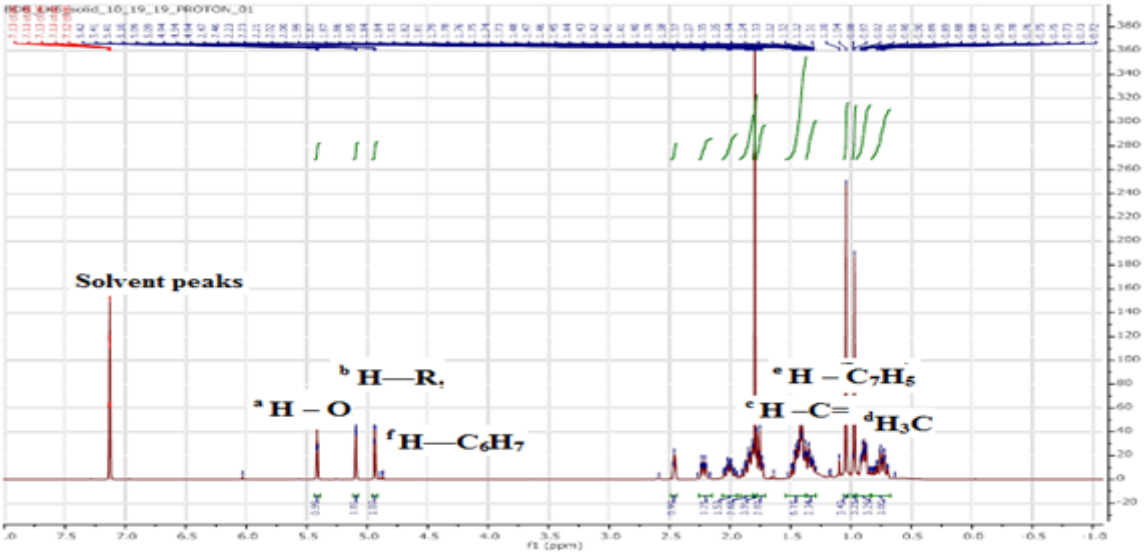

Figure 4.2.: Location of different proton environments on the NMR Spectrum of Sample LK006

The above figure 4.2., clearly indicates the position of Hydrogen atoms in the different environments on the proposed structure of Sample LK006. The confirmation of the structure can be obtained from the mass spectrum obtained from the UK as shown below.

3.4. Results of LCMS/Mass Spectroscopy of Sample LK006

Table 4.0.: ID and Description of Mass Spectrum of Sample LK006

Sample	Vial	ID	File	Date	Time	Description
7	1:15:00 AM	A7	MSQ3AB_15NOV2019SLK_006	11/15/2019	3:54:41 PM	EV-SLK_006

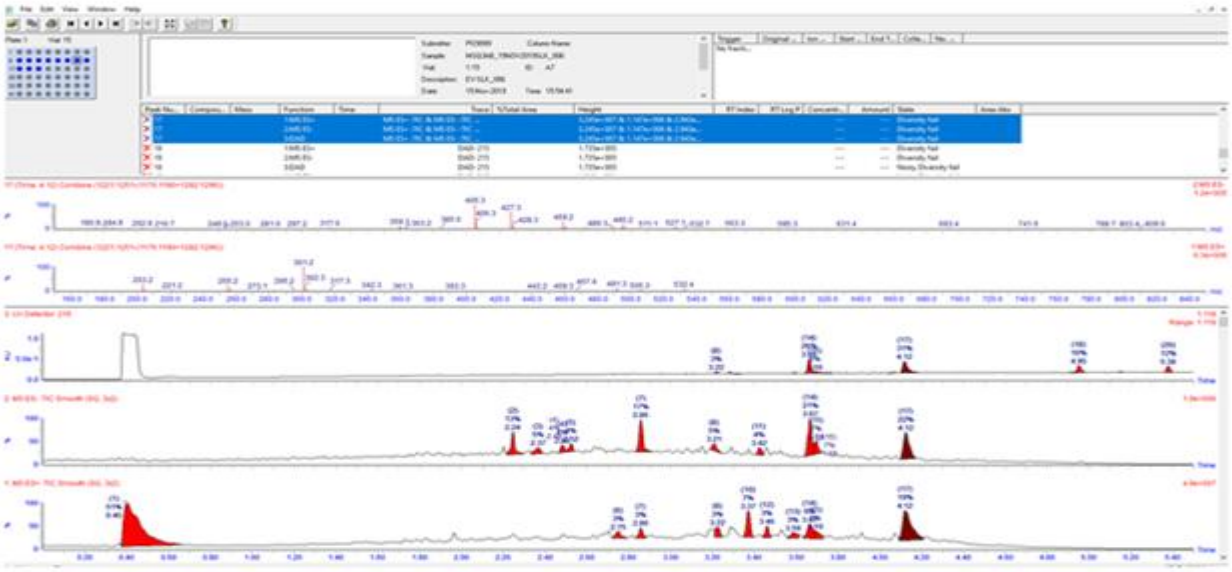

Figure 4.3.: Mass Spectroscopy Spectrum for Sample LK006 from UK

Table 4.1.: Number of Peaks used in obtaining Fragments of Sample LK006

Peak Number	Vial	Function	Trace	BPI	Area Abs.	Area %BP	Width	Height
1	1:15	1:MS ES+	MS ES+ :TIC	8.99E+06	4.00E+06	100	0.296	46645184
2	1:15	2:MS ES-	MS ES- :TIC	1.15E+05	2.00E+04	58.24	0.08	962285.063
3	1:15	2:MS ES-	MS ES- :TIC	1.60E+04	9.00E+03	23.23	0.06	263889.875
4	1:15	2:MS ES-	MS ES- :TIC	7.87E+04	6.00E+03	17.28	0.037	316437.313
5	1:15	2:MS ES-	MS ES- :TIC	6.48E+04	7.00E+03	17.67	0.037	346893.75
6	1:15	1:MS ES+	MS ES+ :TIC	3.99E+05	2.00E+05	5.21	0.073	7407551
7	1:15	1:MS ES+	MS ES+ :TIC	5.79E+05	2.00E+05	5.6	0.05	11033939
8	1:15	1:MS ES+	MS ES+ :TIC	9.25E+05	3.00E+05	6.25	0.04	11069150
9	1:15	1:MS ES+	DAD: 215	1.03E+06	6.00E+02	7.48	0.078	30472.51
10	1:15	1:MS ES+	MS ES+ :TIC	3.17E+06	6.00E+05	13.55	0.06	28440136
11	1:15	2:MS ES-	MS ES- :TIC	4.91E+04	7.00E+03	17.95	0.04	318269.375
12	1:15	1:MS ES+	MS ES+ :TIC	3.30E+05	3.00E+05	5.78	0.04	12499682
13	1:15	1:MS ES+	MS ES+ :TIC	6.43E+05	2.00E+05	4.93	0.053	5993579
14	1:15	1:MS ES+	DAD: 215	1.14E+06	7.00E+03	81.34	0.055	353697.031
15	1:15	1:MS ES+	DAD: 215	1.46E+06	2.00E+03	19.21	0.098	45614.609
16	1:15	1:MS ES+	DAD: 215	1.79E+06	4.00E+02	4.47	0.053	20284.73
17	1:15	1:MS ES+	DAD: 215	6.27E+06	8.00E+03	100	0.118	294280.219
18	1:15	1:MS ES+	DAD: 215	6.41E+04	4.00E+03	49.5	0.113	173539.766
19	1:15	1:MS ES+	DAD: 215	1.22E+03	4.00E+02	5.41	0.077	19118.609
20	1:15	1:MS ES+	DAD: 215	1.15E+03	3.00E+03	37.85	0.065	157414.563

The following results are obtained from twenty peaks with respect to the fragments that could be possibly obtained from Sample LK006 and by McLafferty Rearrangement with ID. NO., obtained from the MSQ3AB_15NOV2019SLK_006 interpreted as shown below;

Table 4.2.: Fragmentation ions obtained from M^+ ion of Sample LK006
Usual fragmentation patterns and by Maclerfty Rule corresponding to molecular ions in the various peak spectrums

M = 360.5gmol^{-1}, M^+ = 359.5gmol^{-1}, O_2C = 44 (O == C== O), $C_6H_9 - CH_3$ = 96, $C_6H_{10}(CH_3)_2$ = 112, C_7H_{12}—CH_2 = 110, C_3H_8 = 44 [CH_3—CH_2 ---CH_3]; CO = 28
[C == O]; $C_2H_4O_2$ = 60 [CH_3COOH]

Ion	Expected Molecular mass	Peak position	Actual Molecular Mass	Intensity
M^+	359.5	1107	359.2	51,928
$M^+ - O_2C$	315.5	695	315.2	487711
$M^+ + O_2C + H$	404.5	494	404.54	12491
$M^+ - 2\ O_2C$	271.5	669	271.1	114245
$M^+ + 2\ O_2C$	447.5	774	447.4	5685.19
$M^+ - 3\ O_2C$	227.5	531	227.3	10289.70
$M^+ + 3\ O_2C - H$	490.5	813	490.3	13354
$M^+ - C_6H_9\ CH_3$	263.5	666	263.15	143164
$M^+ + C_6H_9\ CH_3$	455.5	590	455.4	65517
$M^+ + C_6H_9\ CH_3 + H$	456.5	773	456.4	21204
$M^+ - 2\ C_6H_9\ CH_3$	167.5	513	167.08	841806
$M^+ + 2C_6H_9\ CH_3$	550.5	841	550.7	11028.7
$M^+ - C_6H_{10}(CH_3)_2 + H$	248.5	440	248.13	67839.9
$M^+ + C_6H_{10}(CH_3)_2$	471.5	795	471.3	11085.2
$M^+ + 2C_6H_{10}(CH_3)_2 - H$	582.5	850	582.3	6700.74
$M^+ - C_7H_{12}$—CH_2	249.5	661	249.2	46051
$M^+ + C_7H_{12}$—CH_2	469.5	794	469.3	10535.2
$M^+ + 2\ (C_7H_{12}$—$CH_2) - H$	578.5	849	578.3	8380.67
$M^+ - C_3H_8$	315.5	695	315.2	487711
$M^+ + C_3H_8 + H$	404.5	494	404.5	12491.6
$M^+ + C_3H_8$	403.5	1045	403.3	45244
$M^+ - 2C_3H_8$	271.5	669	471.1	114245
$M^+ + 2C_3H_8$	447.5	774	447.4	5684.19
$M^+ + CO$	387.5	741	387.3	29223.2
$M^+ - CO$	331.5	469	331.2	40606.7
$M^+ + 2CO$	415.5	751	415.2	15888.2
$M^+ - 2CO$	303.5	462	303.6	14867.5
$M^+ + CH_3COOH$	419.5	754	419.4	7497.99
$M^+ - CH_3COOH$	299.5	685	299.2	167727
$M^+ + 2CH_3COOH$	479.5	802	479.3	16818.3
$M^+ - 2\ CH_3COOH$	239.5	658	239.2r	78747.9

The Fragmentation patterns comprising the LMCS and the Proton NMR for sample LK006 and the McLafferty rearrangement confirmed the structure[17, 18, 19&20] of Sample LK006 shown below;

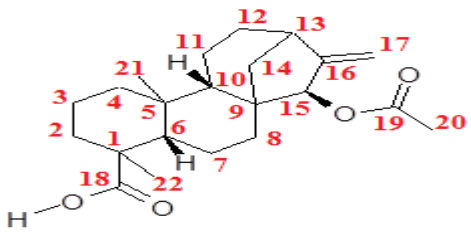

Figure 4.4.: Confirmed structure of Sample LK006 identified as 15-(Acetyloxy) Kaur-16-en-18-oic acid commonly known as Xylopic acid

Hence Sample LK006, whose structure now confirmed to be Xylopic acid, is the main active ingredient responsible for the analgesic activity [**13, 21, 22, 23, 24, 25 & 26]**exhibited by the fruit extracts of *Xylopia aethiopica.*

4.0. CONCLUSION AND RECOMMENDATIONS

Xylopic acid identified as 15-(Acetyloxy) Kaur-16-en-18-oic acid was extracted from the petroleum ether extract of dried powdered fruit with seeds from the traditional medicinal plant Xylopia aethiopica. The petroleum ether extract after Soxhlet extraction was concentrated to one third of its volume and kept in a refrigerator for 48 hours. The oily liquid was separated from the mixture and the crystals remaining on the walls of the flask collected, weighed and labelled as LK006.

The melting point of the crystals was determined uncorrected to be 261-262^{o}C using the Electro thermal melting point determination equipment.The crystals of Sample LK006 were light yellow, slightly soluble in water, Ethanol and Chloroform. It tested positive for unsaturation contains the elements Carbon, Hydrogen and Oxygen during wet chemical methods of analysis.

The Fragmentation patterns obtained from the LMCS spectra, the interpretation of Proton NMR spectra for sample LK006 were analyzed and by the use of McLafferty rearrangement in determining the structure of Sample LK006 as 15 – (Acetyloxy) Kaur - 16 - en – 8 – oic acid commonly known as Xylopic acid. The compound is therefore reported in this research work to be one of the main components responsible for the analgesic properties of the traditional medicinal plant Xylopia aethiopica.

4.1. RECOMMENDATIONS

We there recommend that a further work be done in synthesizing Xylopic acid and using the compound produced to carry out broad spectrum antimicrobial sensitivity testing and analgesic properties.

4.3. SOURCES OF FUNDING

This research received no specific grant from any funding agency be it public, commercial, or not-for-profit sectors.

4.4. CONFLICT OF INTEREST

The authors have declared that no competing interests exist.

4.5. ACKNOWLEDGMENT

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