ANTIBACTERIAL ACTIVITY, ANTIOXIDANT POTENTIAL AND STIGMASTEROL ISOLATION FROM *Laggera aurita* Linn (ASTERACEAE)

ABSTRACT

The study was aimed at the in-vitro investigation of the antibacterial activity, antioxidant potential and bioactive compound isolation from ethyl acetate crude fraction of Laggera aurita (L. aurita) Linn. The crude fraction was tested against five gram negative bacteria (Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Salmonella typhi, Proteus mirabilis) and three gram positive bacteria (Staphylococcus aureus, Enterococcus faecalis and Bacillus subtilis) using macro dilution technique. The antioxidant potentials were evaluated using two different but complementary methods namely ferrous ion chelating activity (FICA) and ferric reducing antioxidant potential (FRAP). Phytocompound isolation was carried out on low pressure open column chromatography. The crude fraction displayed moderate to significant activity against all tested bacteria exhibiting both bacteriostatic and bactericidal effects. The crude fraction showed Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) in the range of 62.50-250 µg/mL and 125-1000 µg/mL respectively. The crude fraction was bactericidal to all tested pathogens except E. coli. The fifty percent effective concentration (EC_{50}) of EDTA standard and the ethyl acetate fraction were obtained as 10.87 µg/mL and 25.77 µg/mL respectively. Similarly the FRAP determinations for ascorbic acid and ethyl acetate fraction yielded 153.63 and 134.40 Fe^{2+} Equivalent per g of fraction indicating 80.06% and 70.61% FRAP units respectively. The isolated phytocompound coded LAE was obtained as a white crystalline solid with melting point of 136-138°C and Rf of 0.56 in hexane:ethylacetate (6:4). The isolated compound was identified by spectroscopic data analysis from FT-IR, GC-MS, 1D and 2D NMR and in comparison with literature. The compound was identified as stigmasterol. The observed significant antibacterial and antioxidant properties demonstrated by L. aurita in this study validate its widespread use in traditional medicine. Therefore the study had shown that L. aurita

contains bioactive principles and may serve as a source for potential an*tibacterial and oxidative str ess therapeutic agents.*

Key words: L. aurita, Antibacterial, Antioxidant, Isolation, Stigmasterol.

1.0 INTRODUCTION

Natural products from plants, animals and minerals had been used by mankind for ages either as dietary supplements or for therapeutic purposes. Recently focus had shifted to natural products as sources for the discovery of new therapeutic agents to fight diseases [1]. Medicinal plants are considered as the richest source of ingredients for use in drug development and synthesis. They contain bioactive constituents that had been exploited for decades in the treatment of various diseases and ailments [2].

Synthetic therapeutics had over the years developed problems of toxicity, microbial drug resistance, allergy and drug addiction [3]. On the other hand, new drugs of plant origin which are constantly synthesized by plants in response to environmental change are much more compatible and safer for human body systems than synthetic drugs [4]. Several phytochemicals have been found to possess a wide range of activities, which may help in the protection and management of diseases. For example, Saponins can protect against hypercholesterolemia and microbial infections. Alkaloids, flavonoids, steroids, saponins, tannins and triterpenoid had reportedly demonstrated various medicinal properties such as anti-inflammatory, anti-diabetic and analgesic activities and also central nervous system activity [5].

The genesis of diseases can also be attributed to other factors such as free radicals which may accumulate as by-products of biochemical processes in our bodies [6]. They are capable of attacking the healthy cells of the body leading to loss of structure and function [7]. The reactive oxygen species (ROS) and reactive nitrogen species (RNS) collectively constitute the free radicals. The accumulation of free radicals in the body generates a phenomenon called oxidative stress. Oxidative stress can arise when cells cannot adequately destroy the excess free radicals formed. In other words, oxidative stress results from an imbalance between formation and neutralization of ROS/RNS [8]. Antioxidants are naturally occurring molecules that combat oxidative damage in biological systems by scavenging free-radicals. An antioxidant achieves this by slowing or

preventing the oxidation process that can damage cells in the body [9]. Increased intake of antioxidants can neutralize the free radicals and protect the body from cell damage. The antioxidative effect may be mainly due to the presence of phenolic components such as flavonoids, phenolic acids, tannins and diterpenes [10]. Others may include cardiac glycosides, alkaloids and saponins [11].

Several compounds had been isolated from extracts of other species of the genus *Laggera.* These include fatty acid derivatives, eudesmane sesquiterpenes, flavonoids, sterols, phenolic acid, triterpenoid ester, alkaloids [12]. Compounds isolated from *L. pterodonta* include taraxasteryl acetate; a pentacyclic triterpenoid ester [13], flavonoids; artemitin, quercetin, chrysosplenetin B, luteolin, apigenin, pinostrobin [14] and the sterols; stigmasterol, β-sitosterol [15]. Some compounds isolated from *L. crispate* include Laggeric acid, 4, 5-seco-eudesmane sesquiterpene acid [16]. Other compounds isolated from *L. alata* include eudesmane; 3β-angeloyloxy-4β-hydroxy-eudesm-7, 11-en-8-one and 3β- angeloyloxy-4β-acetoxy-11-hydroxy eudesm-6-en-8-one [17]. From *L. tomentosa* the following comounds were also isolated; artemitin, quercetin, chrysosplenetin B, luteolin, apigenin, 3, 4',5-trihydroxy-6,7-dimethoxyflavone, and pinostrobin [14]. A sesquiterpene and flavone, namely 3-(3'-acetoxy-2'-hydroxy-2'-methyl butyryl)- cuauhtemone and 3',5,6-trihydroxy-3,6-dimethoxyflavone respectively were also isolated from *L. tomentosa* [18].

L. aurita Linn. (Asteraceae) is a widely used medicinal plant in African countries like Nigeria, Senegal, Tanzania and Ghana. It had been reportedly used in traditional medicine for the treatment of fever, epilepsy, dyspepsia, indigestion, rheumatic pain, wounds, constipation, dysentery, chronic ulcers and as insect repellant [19, 20, 21]. *L. aurita* was also reportedly used in the treatment of asthma, bronchitis, malaria, stomatitis, inflammation and nasal congestion [12]. The causes of most of these ailments are pathogens including bacteria, virus and fungi [4]. Likewise oxidative stress is associated with the development of several chronic disorders such as cancer, rheumatoid arthritis, inflammation and gastric ulcers [8]. Phytochemical screening of *L. aurita* revealed the presence of important secondary metabolites such as flavonoids, tannins, alkaloids, terpenoids, phenols, saponins and cardiac glycosides [20, 22]. Various biological properties of *L. aurita* had been

reported such as repellent and oviposition deterrent activities against mosquito vectors [23], antibacterial activities [24, 25], analgesic and anti-inflammatory activities [20, 22] and anti-hyperalgesic [26] but there was no attempt at isolation and characterization of bioactive phytocompounds [27]. Furthermore, the biological and pharmacological properties of phytocompounds can be affected by several factors such as geographical locations, phenological age of plant, harvest time and extraction method used [28]. Consequently, there is need to carry out the antibacterial activity, antioxidant potential and isolation of bioactive compound from *L. aurita* (Asteraceae).

2.0 EXPERIMENTAL

2.1 Plant Collection, Identification and Preparation

The plant *L. aurita* was collected from Gombe State University Campus, Gombe Nigeria. The plant was identified by a Botanist in Biological Science Department of Gombe State University. The sample was compared with a previously deposited specimen and Voucher No. GSUH-34 was allocated. The plant was allowed to dry under shade and pulverized to coarsely powder using motorized miller. The powdered plant material was kept in black polythene bags refrigerated until required for use.

2.2 Extraction, Fractionation and Isolation Procedures

About 1.5 Kg of coarsely powdered *L. aurita* was extracted at room temperature by exhaustive maceration method using methanol for a period of 7 days with occasional shaking, whereby plant-to-solvent ratio was 1:10. The macerated plant sample was filtered using Whatman no. 1 filter paper. The combined filtrate was concentrated with a rotary evaporator (BÜCHI R-100, Switzerland) under reduced pressure at 45^{o}C. One hundred grams (100 g) of the methanol crude extract of *L. aurita* was suspended in 10% methanol in distilled water in a separatory funnel (1000 mL) and successively partitioned with hexane (HEX), ethyl acetate (EtOAc) and n-butanol (BuOH) in this order. Each extraction process was repeated thrice with 250 mL of each solvent to get

sufficient quantity fractions. The hexane, ethyl acetate and n-butanol portions were evaporated under reduced pressure using rotary evaporator.

The ethyl acetate fraction was subjected to column chromatography gradient elution procedure using silica gel 60 (70-230 mesh), performed with hexane/ethyl acetate and ethyl acetate/methanol at 5% increase in volume of the eluting solvents (100:0-100:20 v/v) in the following manner; (100:00), (95:05), (90:10), (85:15), (80:20), (75:25), etc. to yield several fractions of 100 mL each. Each fraction was collected separately in conical flasks and numbered consecutively. Seventy one fractions were obtained and were pooled together based on similarities in TLC profile to give 14 sub-fractions. Furthermore, a compound from fraction 10-13 which showed one spot was purified by recrystallization and designated as LAE with R_f value of 0.56 in hexane: ethyl acetate (6:4) solvent system.

2.3 Antimicrobial Assays

2.3.1 Source and Maintenance of Organism

Eight microbes consisting of five Gram negative bacteria; *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia, Salmonella typhi, Proteus mirabilis* and three gram positive *Staphylococcus aureus, Enterococcus faecalis* and *Bacillus subtilis* were obtained and confirmed at the Research Laboratory of the Department of Medical Microbiology and Parasitology, Federal Teaching Hospital, Gombe, Nigeria. They were maintained and subcultured on Muller-Hinton agar (MHA) (Oxoid, UK) to obtain pure colonies.

2.3.2 Preparation of McFarland Standard

About 0.5 mL of 0.0448M $BaCl_2$ (1.17% w/v $BaCl_2.2H_2O$) was added to 99.5 mL of 0.18M H_2SO_4 (1% v/v) with constant stirring. The standard was distributed into screw capped McCartney bottles of same size as those used in growing the bacterial pure colonies. The tubes were sealed tightly to prevent solvent loss by evaporation and stored protected from light [29].

2.3.3 Preparation of the Inoculums

A loopful of test bacteria grown on Mueller Hinton Agar at 37 °C for 18 h was taken and suspended in normal saline solution (0.85 %, w/v) NaCl. The density of the organism suspension was adjusted until it matches the turbidity of 0.5 M McFarland standard which approximates to 1.0 x 10^6 cfu/mL of bacteria.

2.3.4 Minimum Inhibitory Concentration (MIC) using Broth Dilution Method

MIC of extract was carried out using broth dilution standard method with little modifications [30]. Nine serial two-fold dilutions of extract and the conventional antibiotic (gentamicin) were made from the stock concentration to obtain final concentration range of 500 µg/mL to 3.9 µg/mL. The nineth test tube (No. 9) served as negative control (broth + inoculums). For each bacterium specie three columns of nine test tubes were used. Each test tube got culture medium (2 ml) + plant extract or gentamicin (1.8ml) + 0.2 mL standardized bacterial inoculums (10^6cfu/mL). The test tubes were covered and incubated at 37^{o}C for 24 hours and observed for turbidity or growth. The lowest concentration with no turbidity among the test-tubes was recorded as the minimum inhibitory concentration (MIC).

2.3.5 Minimal Bactericidal Concentration (MBC)

Minimum bactericidal concentration (MBC) was considered as the lowest extract concentration killing 99.9% of the bacterial inocula after 24 h incubation at 37°C [27]. MBC values were determined by subculturing contents of test tubes without turbidity or no visible growth from MIC determinations unto neutral sterile Mueller Hinton Agar plates. The plates were labeled indicating each test bacterium. The microbes were spread evenly over the surface of a prepared MHA media with the aid of a swab stick. The plates were incubated at 37^{o}C for 24 hours for all bacteria and observed for colony growth. The MBC was the plate with the lowest concentration of the plant extract or gentamicin without colony growth. All determinations were carried out in triplicate.

2.4 Antioxidant Activity Test

2.4.1 Ferrous Iron Chelating Activity (FICA)

The ferrous iron chelating activity of the plant extract was carried out according to a standard method with minor modification [31]. Ethyl acetate fraction and standard (0.2 ml) were added to 0.336 mL of Tris HCl (0.1 M, pH7.4) at five different concentrations (100, 50, 25, 12.5, 6.25 µg/ml) followed by the addition of 0.40 mL of 0.9% NaCl w/v and 0.1 mL of 2 mM $FeSO_4$. The mixture was incubated for 5 minutes after which 0.25 mL of 0.25% aqueous 1, 10-Phenanthroline was added. The absorbance was read at 510 nm against a control consisting of the same reaction mixture, except plant extract solution. EDTA was used as a positive control. Chelating activity was calculated using the following formula:

Iron Chelating Activity = $(A_{control} – A_{sample})/A_{control}$ x 100.; where $A_{control}$ is the absorbance of control reaction (without plant extract), and A_{sample} is the absorbance in the presence of a plant extract (i.e. iron chelator). The 50% effective concentration (EC_{50}) is defined as the concentration of crude extract or EDTA required to chelate 50 % of ferrous ions was computed from the linear regression equation of the plot of iron chelating activity against fraction' concentration using excel spreadsheet, y = mx+ c. Where y = 50% maximum % chelating activity, x = concentration at which 50% chelation activity can be achieved, m = Slope and c = intercept.

2.4.2 Ferric Reducing Antioxidant Power (FRAP) Assay

The Ferric reducing antioxidant power assay was performed in accordance with standard procedure with little modification [27]. Five different concentrations of ethyl acetate crude fraction (100, 80, .60, 40, 20, µg/mL) were taken in a test tube and 2.5 mL of phosphate buffer (pH 6.8) and 2.5 mL of potassium ferricyanide solution was added to a test tube. The mixture was incubated for 20 min at 50^{o}C for reaction completion. About 2.5 mL trichloroacetic acid (10%) solution was added to the test tubes. The total mixture was centrifuged at 3000 rpm for 10 minutes and 2.5 mL of the supernatant solution was withdrawn from the mixture and mixed with 2.5 mL of distilled water. About 0.5 mL of freshly prepared ferric chloride (0.1%) solution was added to the diluted reaction

mixture and the absorbance of the solution was read at 700 nm against blank. A typical blank solution contains the same solution mixture without plant extract. $FeSO_4$ was used to produce the calibration curve while ascorbic acid was used as standard. The iron (III) reducing activity was performed in triplicate and expressed in µg/mL $FeSO_4$ Equivalent per g extract fraction. The reducing power was also expressed as an increase in A700 after blank subtraction. FRAP value of the sample was calculated using the equation $y = 0.0052x + 0.0072$ (R^2=0.9991) obtained from the standard curve of $FeSO_4$.

2.5 Spectroscopic Analysis of Isolated Compound

Characterization of isolate was performed using infrared spectrophotometer (IR), Nuclear Magnetic Resonance Spectroscopy (1H NMR and 13C NMR), GC-MS spectra data and Melting Point determination. Infrared spectrum of the sample was obtained and recorded on Agilent Technologies Happ-Genzel FT-IR spectrometer Cary 630, while the 1H NMR and 13C NMR spectra were recorded on 400 MHz Agilent Technologies premium compact + AR. with the sample initially dissolved in deuterated chloroform ($CDCl_3$). Chemical shift values (δ) were reported in parts per million (ppm) relative to TMS standard. The GC-MS analysis for molecular mass determination was carried out on Agilent Technologies GCMSD, USA. The complete analysis of the sample was carried out in Multi-user Reaserach Science Laboratories, Ahmadu Bello University (ABU), Zaria, Nigeria. Melting points were determined using glass capillaries on a Buchi-535 melting point apparatus.

3.0 RESULTS AND DISCUSION

3.1 Antibacterial Activity

Table 1 below shows the Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the ethyl acetate fraction.

Table 1: MIC and MBC of ethyl acetate fraction of *L. aurita*

Bacteria	*L. aurita* MIC (µg/mL)	Gentamicin MIC (µg/mL)	*L. aurita* MBC (µg/mL)	Gentamicin MBC (µg/mL)
S. aureus	125	15.63	125	15.63
E. faecalis	62.50	15.63	125	31.32
B. subtilis	62.50	7.81	125	15.63
E. coli	250	62.50	1000	125.0
P. *aeruginosa*	125	62.50	500	125.0
K. pneumonia	250	31.25	500	62.50
S. *typhi*	250	31.25	500	62.50
P. mirabilis	125	31.25	250	62.50

The antibacterial activity of micro organisms are reportedly classified based on MIC values as follows; 24.4≥MIC≤ 78.2 µg/mL are considered significant, while 100 < MIC = 625 µg/mL are moderate or weak (MIC > 625 µg/mL) against various resistant pathogens [30]. On the basis of such classification, the ethyl acetate fraction of *L. aurita* displayed moderate to relatively significant activity against the gram-positive and gram-negative pathogens with MIC values ranging between 62.50 µg/mL and 250 µg/mL depending on the tested bacterium except *E. coli*. The observed broad spectrum of antibacterial activity of the crude extract may be due to the presence of different classes of secondary metabolites acting in synergy. *Bacillus subtilis* and *Enterococcus faecalis* were the most susceptible to the extract at MIC of 62.50 µg/mL.

Table 2: Bacteriocidal/Bacteriostatic capacity of the ethyl acetate fraction

Bateria	**Ethyl acetate fraction**	**Gentamicin**
S. aureus	1 (+)	1 (+)
E. faecalis	2 (+)	2 (+)
B. subtilis	2 (+)	2 (+)
E. coli	4 (-)	4 (-)
P. *aeruginosa*	3 (+)	3 (+)

K. pneumonia	2 (+)	2 (+)
S. *typhi*	2 (+)	2 (+)
P. mirabilis	2 (+)	2 (+)

(+): Bactericidal effect, (-) Bacteriostatic effect. Nd= Not determined

If the ratio MBC/MIC <4, the effect is considered as bactericidal but if the ratio MBC/MIC > 4, the effect is considered as bacteriostatic (Table 2) [32]. The ethyl acetate fraction was bactericidal against all the tested pathogens except *E. coli* which showed less susceptibility to both the ethyl acetate fraction and the control (gentamicin). This implies that the crude extract could potentially be used in the management of infections or diseases associated with the tested organisms. Generally it was observed that the results indicated that the crude extract was more active against Gram-positive bacteria compared to Gram-negative bacteria. This was further confirmed by previous studies that described Gram-positive bacteria as having less complicated cell wall structure compared to the gram negative bacteria [27, 30]. The antibacterial activity of the crude extract of *L. aurita* Linn reported in this study are generally higher relative to that reported in literature [27]. This may be due to differences in geographical locations, age of plant, time the plant was harvested, environmental factors or method of extraction [28].

3.2 Ferrous Ion Chelating Activity (FICA)

The ferrous ion chelating effect of ethyl acetate fraction and standard EDTA at different concentrations of 6.25, 12.5, 25, 50, and 100 µg/mL are presented in Table 3.

Table 3: Ferrous iron chelating activity (FICA) of ethyl acetate fraction

Concentration (µg/ml)	EDTA %FICA	Ethyl acetate fraction %FICA
100	90.22±0.03	82.25±0.02
50	75.02±0.04	66.63±0.04
25	61.63±0.04	55.67±0.04
12.5	53.04±0.01	46.07±0.02

6.25	38.86±0.02	30.78±0.04

The EDTA standard and ethyl acetate fraction at100 µg/mL exhibited maximum iron chelating activities of 90.22% and 82.25% respectively.

3.2.1 Determination of EC_{50} of the ethyl acetate fraction

The fifty percent effective concentration (EC_{50}) is a quantitative measure that indicates how much of a particular substance is needed to achieve 50% of a given biological activity [33]. The EC_{50} chelating activities of fraction and EDTA are shown in Figure 1.

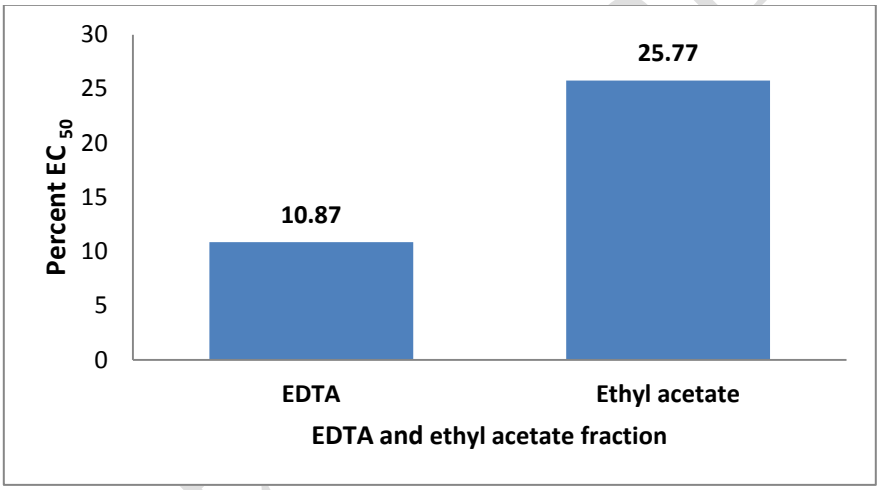


Figure 1: *EC_{50} Chelating power of ethyl acetate fraction and EDTA*

The EC_{50} chelating activity of EDTA and crude extract are 10.87µg/mL and 25.77µg/mL respectively. The lower the EC_{50} value, the higher the chelating activity of the compound or crude extract. These results show that the ethyl acetate fraction is also a good metal chelating agent relative to the EDTA standard.

3.3 Ferric Reducing Antioxidant Power (FRAP)

FRAP Units and percentages FRAP of the ethyl acetate fraction and the standard ascorbic acid are shown in table 4.

Table 4: Ferric Reducing Antioxidant Power of the **ethyl acetate fraction** of *L. aurita*

FRAP Units (Fe^{2+} Equivalent per g fraction)			Percentage FRAP	
Concentration (µg/ml)	Ascorbic acid (Fe^{2+} µg/ml)	Ethyl acetate fraction (Fe^{2+} µg/ml)	Ascorbic acid	Ethyl acetate fraction %
100	153.63±0.01	134.40±0.04	80.06±0.01	70.61±0.04
80	132.21±0.04	122.96±0.02	69.47±0.04	64-66±0.02
60	124.00±0.05	119.03±0.02	65.20±0.05	62.62±0.02
40	103.97±0.01	79.23±0.03	54.79±0.01	41.92±0.03
20	89.62±0.02	52.46±0.01	47.32±0.02	20.80±0.01

Data presented as mean of three independent determinations ± SD.

In the FRAP assay, a linear increase in reducing power was observed over the concentration range of 20 – 100 µg/mL of the fraction and ascorbic acid (Table 4). The FRAP values for ascorbic acid and ethyl acetate fraction are 153.63 and134.40 µg/mL Fe^{2+} Equivalent per gram of fraction respectively. The percentage FRAP of the fraction showed 70.61% FRAP units while that of the ascorbic acid standard was 80.06% FRAP units at 100 µg/mL.

The observed high antioxidant activity of the crude extract may be indicative of the synergistic effect of the various phytocompounds such as flavonoids, saponins, polyphenols, cardiac glycosides, coumarins and tannins which had been reported to possess antioxidant activities [34].

3.4 Spectroscopic Characterization

3.4.1 FT-IR (KBr) $ú_{max\ cm}^{-1}$: A broad peak at 3421 cm^{-1} and moderately intense peak at 1047 cm^{-1} observed indicates O-H bond vibrations of hydroxyl group. The out of plane C-H vibration of unsaturation was observed at 838 cm^{-1}. The corresponding C=C bond vibrations was observed at

1654 cm^{-1} as a weak peak of C=C olefinic stretching. The stretching and bending vibrations of methyl groups were observed as an intense peak at 2933 cm^{-1} and as medium intensity peak at 1461 cm^{-1}. The vibrations of the methylene component were observed at 2862 cm^{-1} and 1367 cm^{-1}. The moderately intense peak at 738 cm^{-1} can be attributed to the rocking movement of methylene groups. The corresponding C-O vibration was observed as a weak intense peak at 1047 cm^{-1}. These absorption frequencies are in agreements with those of stigmasterol as reported in other studies [35, 36, 37, 38].

Table 5: FT-IR Spectrum Data of LAE

SN	Frequency (cm^{-1})	Type of Vibration
1	3421	O-H stretching
2	2933	C-H stretching.(CH_3)
4	2862	C-H stretching (CH_2)
5	1654	C=C stretching
6	1461	CH_3 bending
7	1367	CH_2 bending
8	1241	CH_2 bending
9	1047	C-O stretching
10	838	CH bending

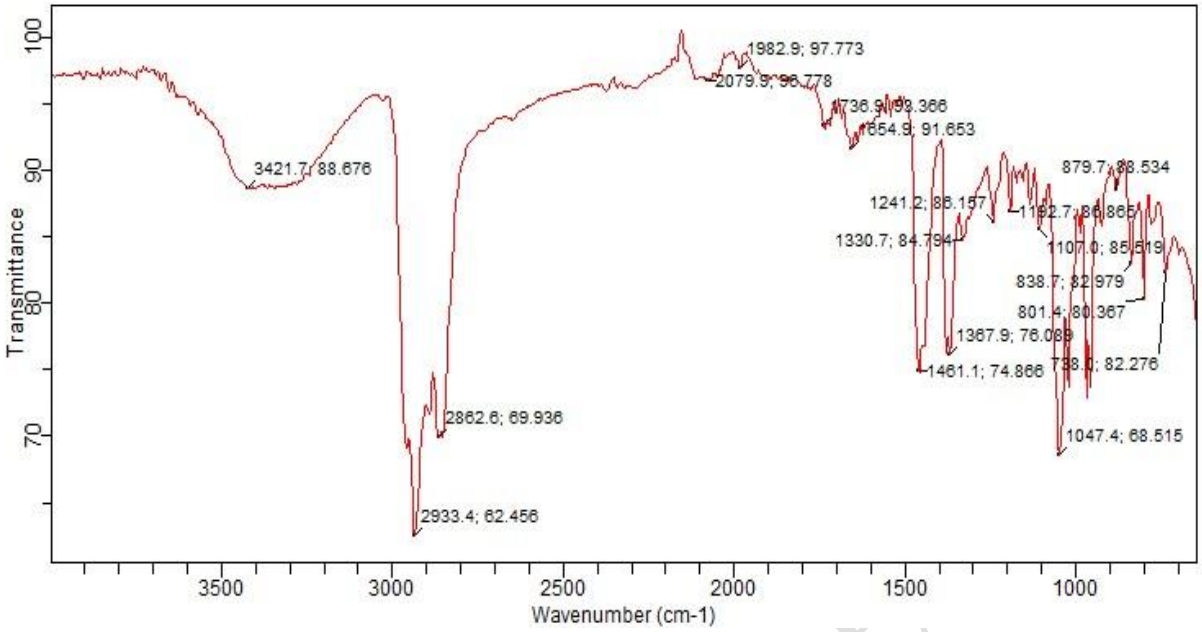


Figure 2: FT-IR spectrum of LAE

3.4.2 Gas Chromatography-Mass Spectrometry (GC–MS) Spectrum

Mass spectrum of isolated the compound LAE showed parent molecular ion [M^+] peak at mlz 412 amu (Figure 3). The percentage fragment of other peaks relative to the base peak are m/z - 412(15.15), 379 (2.02), 351 (5.05), 300(8.08), 271(12.12), 241(4.04), 213(7.58), 161 (19.19), 133(20.20), 83(48.48), 55(100) and 43(65.66). The above fragmentation pattern of $C_{29}H_{48}O$ is consistent with literature and suggests that the compound is stigmasterol as shown in Figure 3 below.

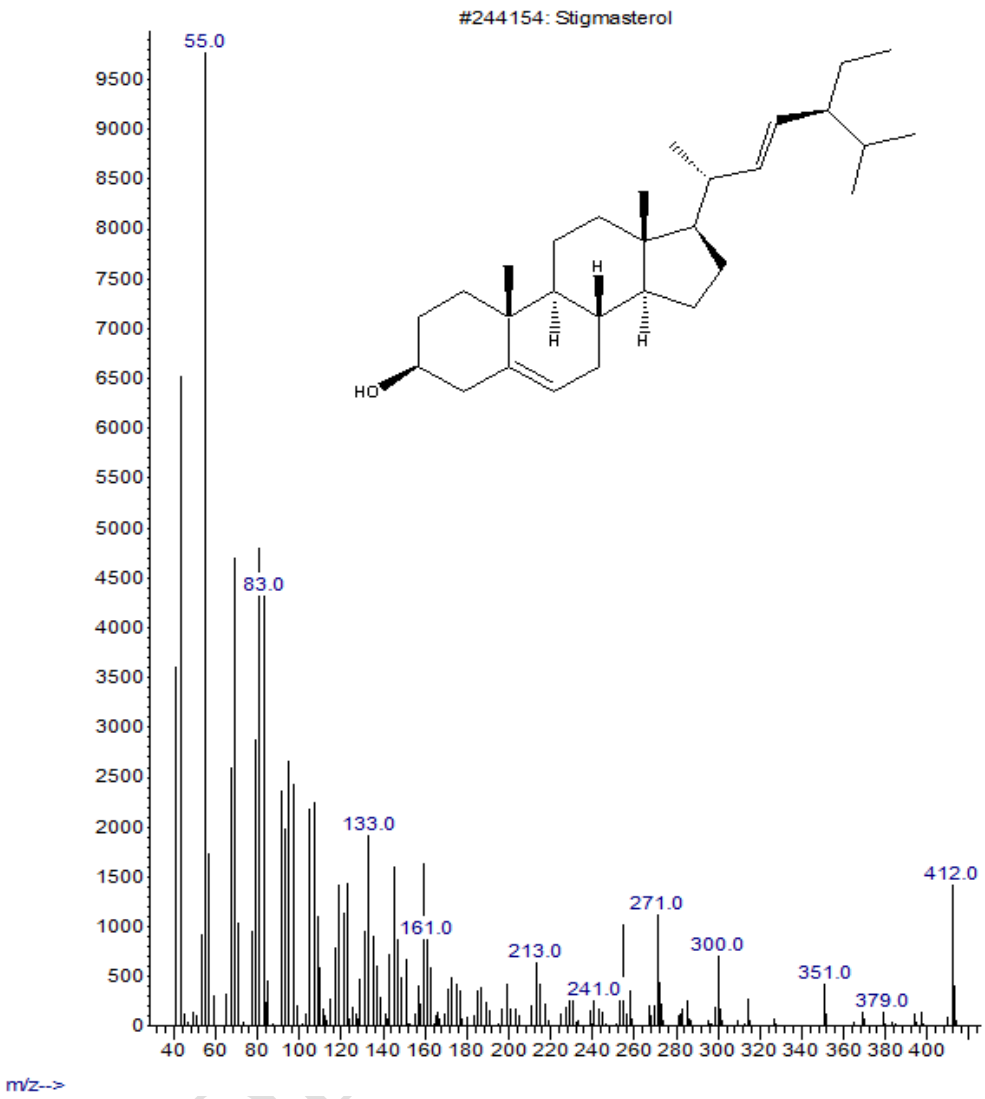
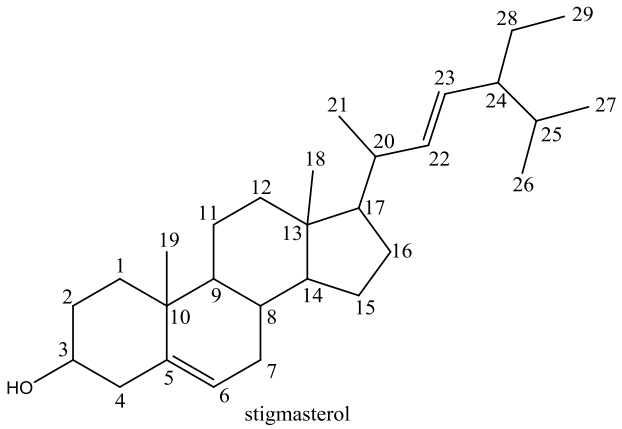


Figure 3: GC-MS spectra of LAE

3.4.3 NMR Analysis of LAE

Close examination of the 1H-NMR spectrum of LAE (400Hz, $CDCl_3$; appendix 1) reveals a typical steroidal nucleus with three basic regions/environments of the steroids showing signals at 0.6 ppm – 2.2 ppm representing the methyl, methylene and methine protons overlap at 3.5 ppm. There are three olefinic resonances at 5.339, 5.122 and 5.022 ppm. A proton on an oxygenated carbon at H-3 appears as a multiplet at 3.521 ppm. The peak due to OH is shown at 4.964 ppm. Angular methyl protons were also noticed at 0.678, 1.034 and 0.804 ppm corresponding to C-18 and C-19 and C-21 respectively.

The 13C-NMR and DEPT result (appendix 2 and 3) showed a molecular compound consisting of 29 carbon atoms, comprising 6 methyl (CH_3) groups (all singlet), 9 methylene (CH_2), 11 methine (CH) and 3 quaternary (C) carbon atoms further indicating the steroidal nature of the compound. The downfield resonances at δ 140.714 ppm and δ 121.702 ppm for (C-5, C-6) and δ 138.310 ppm and δ 129.224 ppm for (C-22, C-23) indicates the presence of unsaturation. The C5, C6, C22 and C23 are alkene carbons. The signals at δ 21.196 ppm and δ 12.020 ppm correspond to angular carbon atom of C-19 and C-18 respectively. The signals at 140.714, 37.213, and 42.264 ppm correspond to the quartenary carbons of C-5, C-10 and C-13. The resonance at δ 71.786 ppm is due to C-3 attached to β-hydroxyl group. Based on literature evidence, the observed frequencies are consistent with those of stigmasterol.



stigmasterol

The 1H and 13C NMR values for all protons and carbons were assigned on the basis of COSY, and HMBC. The 1H-1H COSY (appendix 4) established the correlations between protons that are situated in the same environment; major correlations observed include: δH 5.3 and δH 2.1; δH5.2 and δH 2.05; δH 3.5 (H-3) and δH 2.0; δH 3.52 (H-3) and δH 2.27 (H-4); δH 5.03 and δH 5.3. The HMBC spectrum (appendix 5) allowed establishing the long range connectivity between the various units of the molecule. Some major correlations observed between protons and carbon include; proton at (2.29) correlated with δ31.624 (C2), δ71.786 (C3), and δ121.702 (C6); proton at (5.339) showed correlation with C10 (δ50.106) and C12 (39.640); and (5.2) showed correlation with δ 56.824, 129.224 and 40.497 for C17 C20 and C23 respectively.

Table 6 below showed a good match and further confirms the structure of stigmasterol as reported in literature [35].

Table 6: 1H &13C-NMR data of LAE (Stigmasterol) comparison with literature [35]

Carbon atom	13C NMR Experimental	13C NMR Literature	1H NMR Experimental	1H NMR Literature	DEPT
C-1	37.213	37.26	1.840	1.85	CH_2
C-2	31.624	31.67	1.430	1.46	CH_3
C-3	71.786	71.81	3.521	3.52 m	CH
C-4	42.264	42.31	2.291	2.27	CH_2

C-5	140.714	140.76	-	-	C=C
C-6	121.702	121.71	5.339	5.35 br	C=CH
C-7	31.859	31.90	1.987	1.96	CH_2
C-8	31.624	31.70	1.477	1.48	CH
C-9	50.105	50.16	0.941	0.93	CH
C-10	36.477	36.51	-	-	C
C-11	21.196	21.21	1.477	1.49	CH_2
C-12	39.640	39.68	1.164	1.16	CH_2
C-13	42.180	42.22	-	-	C
C-14	55.899	55.96	1.034	1.05	CH
C-15	24.336	24.36	1.578	1.56	CH_2
C-16	28.916	28.92	1.763	1.70	CH_2
C-17	55.899	55.96	1.138	1.13	CH
C-18	12.020	12.05	0.678	0.69s	CH_3
C-19	21.083	21.08	1.034	1.03 s	CH_3
C-20	40.497	40.49	2.008	2.02	CH
C-21	24.334	23.07	1.034	1.02	CH_3
C-22	138.310	138.31	5.100	5.10	C=C
C-23	129.224	129.28	5.002	5.03	C=C
C-24	51.212	51.24	1.523	1.53	CH
C-25	28.916	29.15	1.653	1.65	CH
C-26	18.952	18.98	0.821	0.82	CH_3
C-27	19.384	19.40	0.784	0.78	CH_3
C-28	25.398	25.40	1.114	1.15	CH_2
C-29	12.248	12.25	0.804	0.80	CH_3

To the best of our knowledge, the present study is the first time stigmasterol, a phytosterol was isolated and characterized from the ethyl acetate fraction of *L. aurita* Linn. Phytosterols are group

of steroidal alcohols. They play important roles as structural component in cell membranes and maintaining membrane stability. There are many structural forms of phytosterol but the most important are β-sitosterol, stigmasterol, campesterol and brassicasterol. They have been reported to show anti-inflammatory, antibacterial and antitumor activities [38]. Stigmasterol is reported to exhibit a broad spectrum of pharmacological activities against various disease conditions such as inflammation, arthritis, diabetes, cardiovascular ailments, renal disorder, anti-hepato toxicity, antimicrobial and anti-cancer activities [39].

3.5 CONCLUSION

This study had established that the ethyl acetate fraction of *L. aurita* could be a potential source of raw material for the development of new antibacterial and oxidative stress therapeutic agents. Results from this research validate the ethnomedicinal uses of *L. aurita* Linn in the treatment of several ailments by traditional medicine practitioners. The compound isolated in this study had previously been isolated from other plants with reported antibacterial, antitumor and anti-inflammatory activities. However the isolation of stigmasterol from *L. aurita* Linn is being reported for the first time based on available literature and to the best of our knowledge. Therefore, this study had contributed to literature on natural products research.

NOTE:

The study highlights the efficacy of "traditional medicine" which is an ancient tradition, used in some parts of India. This ancient concept should be carefully evaluated in the light of modern medical science and can be utilized partially if found suitable.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of

interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

Ethical considerations

Ethical issues (including plagiarism, falsification, double publication or redundancy) have been completely observed by the authors.

REFERENCES

1. Breijyeh Z, Jubeh B, Karaman R. Resistance of Gram-Negative Bacteria to Current Antibacterial Agents and Approaches to Resolve It. *Molecules.* 2020; 25(1340); 1-23.
2. Ameh GI. Evaluation of the phytochemical composition and antimicrobial Properties of crude methanolic extract of leaves of *Ocimum gratissimum. Journal of Natural Sciences, Engineering and Technology.* 2010; 9(1): 147-152.
3. Adamu HM, Ushie OA, Lawal DS, Oga IA. Phytochemical Screening of Fruit of *Azanza garckeana* and Root of *Acacia macrothyrsa. International Journal of Traditional and Natural Medicines*. 2013; 3(1):19-25.
4. Agyare C, Mensah AY, Asante O. Antimicrobial activity and phytochemical studies of some medicinal plants from Ghana. *BLACMA.* 2006; 5(6): 113-117.

5. Mir AM, Sawhney SS, Jassal SMM. Qualitative and quantitative analysis of phytochemicals of *Taraxacum officinale. Wudpecker Journal of Pharmacy and Pharmocol.* 2013; 2(1):1-5.
6. Boligon AA, Machado MM, Athayde LM. Technical Evaluation of Antioxidant Activity. *Med chem*. 2014; 4(7): 517-522.
7. Lobo V, Patil A, Phatak A, Chandra N. Free radicals, antioxidants and functional foods: Impact on Human health. *Pharmacogn Review*. 2010; 4(8):118-126.
8. Pham-Huy AL, Hua He Pham-Huy C. Free Radicals, Antioxidants in Disease and Health. *Int J Biomed Sci*. 2008; 4(2): 89–96.
9. Baunthiyal M, Singh V, Dwivedi S. Insights of Antioxidants as Molecules for Drug Discovery. *International Journal of Pharmacology.* 2017; 13 (1):874-889*.*
10. Agbor GA, Kuate D, Oben JE. Medicinal Plants can be a good source of antioxidants: Case study in Cameroon. *Parkistan Journal of Biological Sciences*. 2007; 10(4): 534-544.
11. Bajpai VK, Kang SC. Isolation and characterization of biologically active secondary metabolites from *Metasequoia glyptostroboides* Miki ex Hu. *J Food Safety*. 2011; 1(31): 844-848.
12. Getahun T, Sharma V. Gupta N. The Genus *Laggera (Asteraceae)* Ethnopharmacological information, chemical composition as well as biological activities of i ts essential oils and extracts: A review. 2019; 16(8): 1-23.
13. Kuljanabhagavad T, Suttisri R, Pengsuparp T, Ruangrungsi N. Chemical Structure and Antiviral Activity of Aerial Part from *Laggera pterodonta*. *Journal of Health Research.* 2009; 23(4): 175-177.
14. Ping L, Yang G Mui CZ. Chemical Constituents from L*aggera Pterodonta*. *Zhong Yao Cai*,. 2014; 2:12-17.
15. Liu B, Zhang T, Zhang X, Ye W. Chemical constituents of *Laggera pterodonta*. *China journal of Chinese material medica*. 2010; 35(5): 602-606.

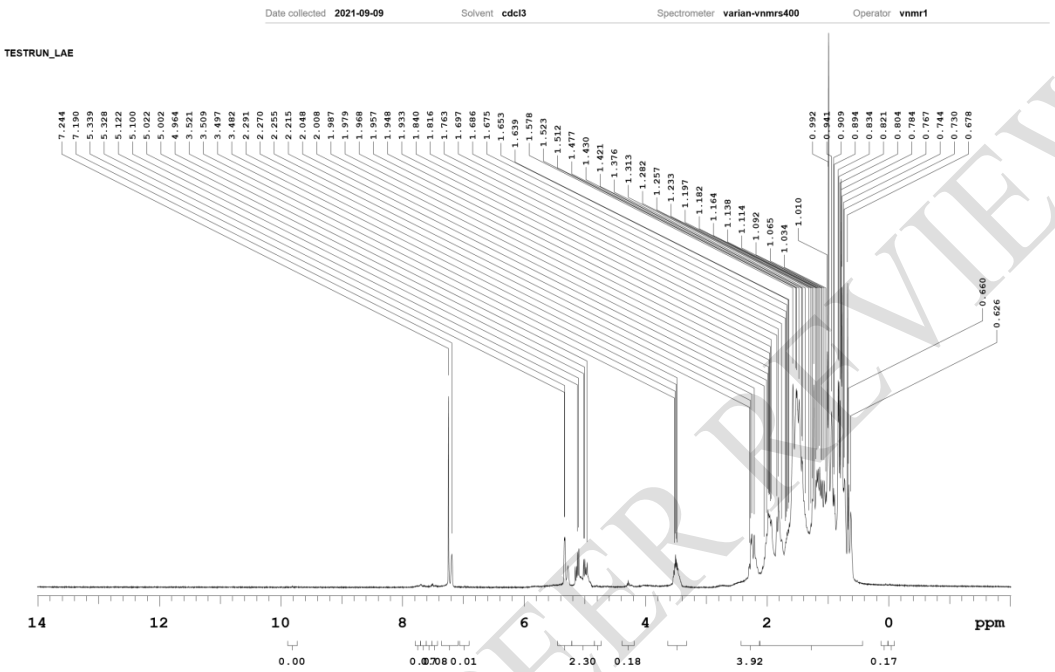
16. Yi-Hua Q, Yang Y, Tan J, Tan C. Chang J. Laggeric Acid, a Novel Seco-Eudesmane Sesquiterpenoid From *Laggera Crispata. J Asian Nat Prod Res*. 2014; 16(3):318-322.
17. Ollengo AM, Vulule MV, Matasyoh CJ. Isolation, Structure Elucidation and Larvicidal Activity of *Laggera alata* Extracts. *Research Journal of Pharmacognosy and Phytochemistry.* 2016; 8(3):153-164.
18. Haile A. Phytochemical Investigation on the Leaves of *Laggera Tomentosa* (Ethanol Extract). A Graduate Project Submitted to the Office a Research and Graduate Programme of Addis Ababa University. 2007.
19. Burkill HM. The Useful Plants of West Tropical Africa, Vol.1, Families A-D. Royal Botanic Gardens Kew. 1985; 479-481.
20. Shehu A. Analgesic and Anti-Inflammatory Effects of Methanol Extract and Fractions of *Laggera Aurita* Linn F (Compositae) In Mice and Rats. A Thesis Submitted to the School of Postgraduate Studies, Ahmadu Bello University Zaria, In Partial Fulfilment of the Requirements for the Award of a Master Degree in Pharmacology Department of Pharmacology and Therapeutics, Faculty of Pharmaceutical Sciences Ahmadu Bello University, Zaria, Nigeria. 2014.
21. Muhammad JS, Sani M. Evaluation of Haematological Parameters and Blood Glucose After a 28-day Oral Administration of Standardized Extract of *Laggera aurita* (Linn) in Rats. *Nigerian Journal of Pharmaceutical and Biomedical Research.* 2018; 3(2): 96-101.
22. Fulata AM, Usman H, William A, Ibrahim S, Zadva I. Phytochemical, Analgesic and Anti-inflammatory Evaluation of the Crude Ethanol and *n*-hexane extracts of the aerial Parts of *Laggera aurita* Linn. *Chemistry Research Journal*. 2017; 2(5): 22-28.
23. Singh SP, Mittal PK. Mosquito repellent and oviposition deterrent activities of *Laggera aurita* plant extract against malaria vector *Anopheles stephensi. Entomology and Applied Science Letters*. 2015; 2 (1): 18-22.
24. Egharevba HO, Okwute S. K. Some Bioactive Fatty Derivatives from *L. Pterodonta. Nature and Science*. 2014; 12(1):79-86.

25. Shahwar D, Ahmad N, Khan MA, Ullah S, Ahmad N. Chemical composition and biological activities of the essential oil of *Laggera aurita* Linn (DC.) grown in Pakistan. *Turkish Journal of Biochemistry–Turk J Biochem*; 2012; 37 (3): 329–335.
26. Olurishe OT, Mati GF. Anti hyperalgesic potentials of *Laggera aurita* in Swiss Albino mice. *Pak. J. Pharm. Sci.* 2014; 27(1):169-172.
27. Dibala CI, Konaté K, Diao M, Ouédraogo M. Dicko MH. Phytoconstituents Analysis, Antioxidant Capacity and Antimicrobial Properties of Extracts from *Laggera Aurita* L. (Asteraceae). *International Journal of Pharmacy and Pharmaceutical Sciences*. 2014; 6(7): 172-178.
28. Musyimi DM, Ogur JA, Muema PM. Phytochemical Compounds and Antimicrobial Activity of Extracts of Aspilia Plant (*Aspilia mossambicensis)* (Oliv) Wild. *International Journal of Botany Volume*. 2018; 4 (1): 56-61.
29. Andrews JM. Determination of minimum inhibitory concentrations. *Journal of Antimicrobial Chemotheraphy*. 2001; (48): 5-16.
30. Kwaji A, Adamu HM. Chindo IY. Isolation, characterization and biological properties of betulin from Entada Africana Gull. and per. (Mimosaceae). *Journal of Apllied and Advancd Research*. 2018; 2(3): 28-31.
31. Essuman EK, Nagajyothi PC, Tettey CO. Antioxidant, Ferric Iron Chelation and Antimicrobial Activities of Extracts of *Pseudocydonia sinensis* (Chinese Quince) Fruit. *Journal of Medicinal Plants Studies*. 2017; 5(5): 175-179.
32. Mogana AR, Adhikari A, Tzar MN, Ramliza R, Wiart C. Antibacterial activities of the extracts, fractions and isolated compounds from Canarium patentinervium Miq. Against bacterial clinical isolates. *BMC Complementary Medicine and Therapies*. 2020; 20(55):1-11.

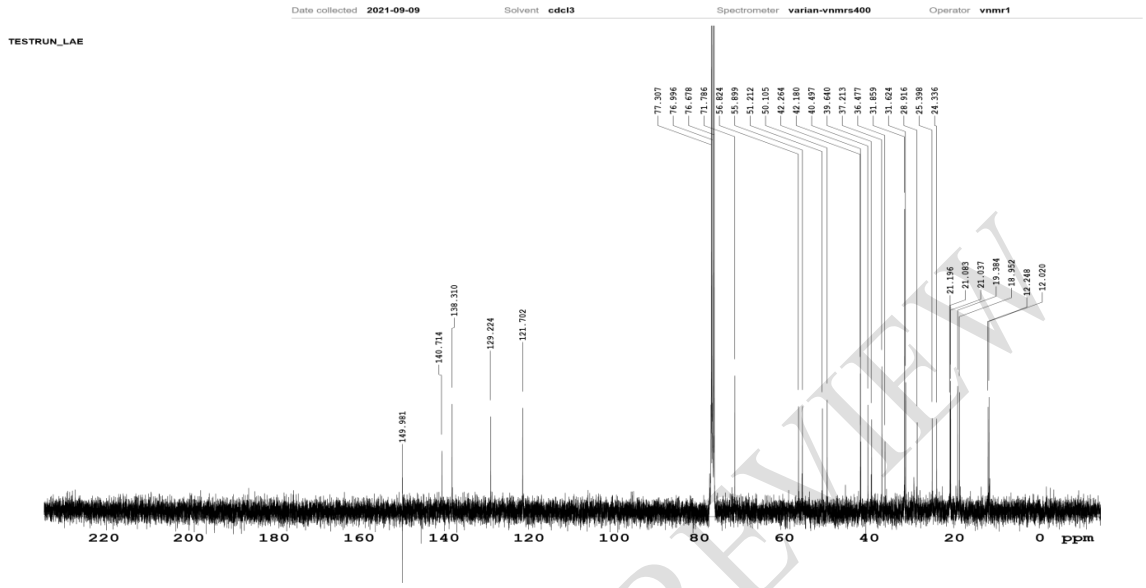
33. Hoetelmans RM. "IC_{50} versus EC_{50}". PD-PD relationships for anti-retroviral drugs. Amsterdam: slotervaart. Hospital. Archived from the original on 2017-05-28-via U.S. Food and Drug Administration. 2017.
34. Teke NG, Lunga PK, Wabo KH, Kuiate J,Vilarem G, Giacinti G, Kikuchi H, Oshima Y. Antimicrobial and antioxidant properties of methanol extract, fractions and compounds from the stem bark of Entada abyssinica Stend ex A. Satabie. *BMC Complementary and Alternative Medicine*. 2011; 11 (57): 1-8.
35. Yusuf AJ, Abdullahi, MI, Haruna AK, Idris AY, Musa AM. Isolation and Characterization of Stigmasterol and Bis-(5,7-diacetyl-catechin-4'-α-rhamnopyranoside) from the Stem bark of *Neocarya macrophylla* (Sabine) Prance (Chrysobalanaceae). *Nigerian Journal of Basic and Applied Science*. 2015; 23(1): 15-22.
36. Pierre LL, Moses MN. Isolation and Characterisation of Stigmasterol and β-Sitosterol from *Odontonema Strictum* (Acanthaceae), *Journal of Innovations in ZPharmaceuticals and Biological Sciences*. 2015; 2 (1): 88-96.
37. Olawumi, O.O. and Koma, S.O. (2019). Stigmasterol and Stigmasterol Glycoside: Isolated Compounds from *Tetrapleura Tetraptera* extracts. *Journal of Biochemistry International*, 6(1): 21-48.
38. Jiang K, Gachumi G, Poudel A, Shurmer B, Bashi Z, El-Aneed A. The establishment of tandem mass spectrometric fingerprints of phytosterols and tocopherols and the development of targeted profiling strategies in vegetable oils. *American Society for Mass Spectrometry.* 2019; 30 (1):1700-1712.
39. Nayak SP, Kar D M, Nayak P S. Isolation and Characterization of Stigmasterol from Chloroform Fraction of Aerial Part of *Argemone Mexicana* L. *International Journal of Pharmacy and Pharmaceutical Sciences.* 2015; 7 (12): 25-29.

APPENDIX

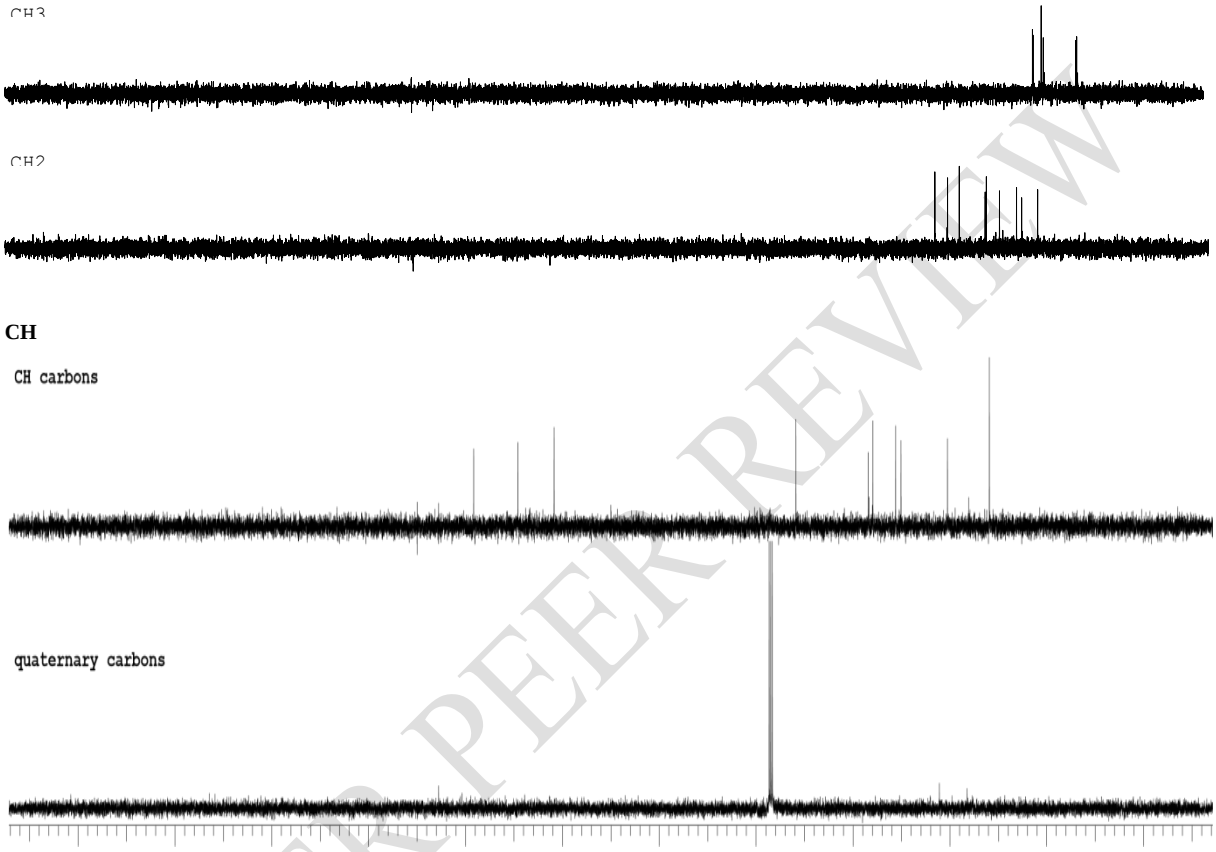
1: 1H of LAE



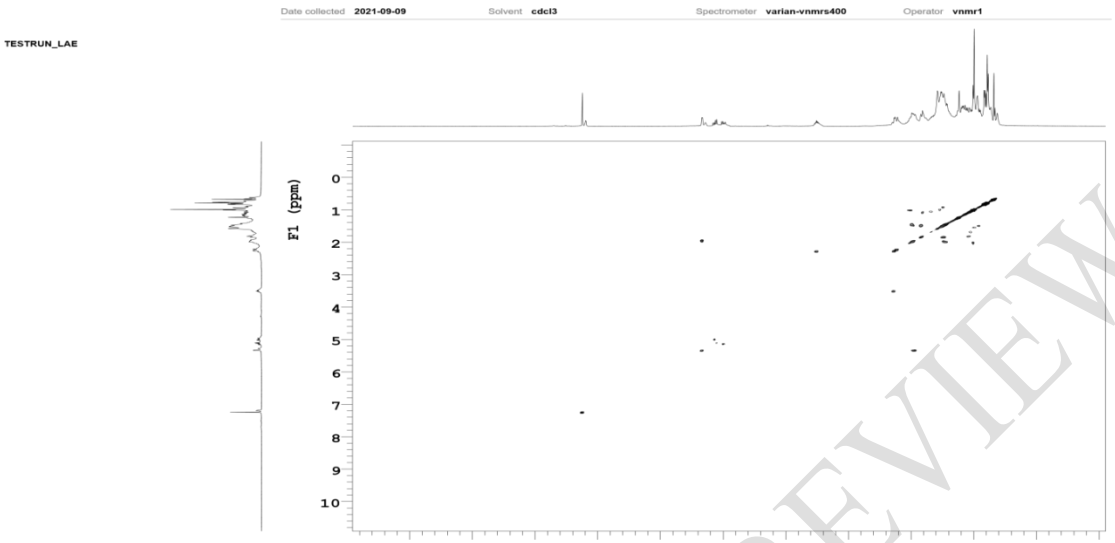
2. 1H of LAE



3: DEPT of LAE



4. COSY



5.0 HMBC