

**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 extract fraction of *Laggera aurita* (*L. aurita*) Linn. The crude extract 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 extract displayed moderate to significant activity against all tested bacteria exhibiting both bacteriostatic and bactericidal effects. The crude extract 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 extract was bactericidal to all tested pathogens except *E. coli*. The fifty percent effective concentration (EC_{50}) of EDTA standard and the ethyl acetate crude extract 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 R_f 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 antibacterial and oxidative stress therapeutic agents.

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

UNDER PEER REVIEW

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. 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, chryso-splenetin 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; 3 β -angeloyloxy-4 β -hydroxy-eudesm-7, 11-en-8-one and 3 β - angeloyloxy-4 β -acetoxy-11-hydroxy eudesm-6-en-8-one [17]. From *L. tomentosa* the following compounds were also isolated; artemitin, quercetin, chryso-splenetin 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, pain, epilepsy, dyspepsia, indigestion, rheumatic pain, wounds, constipation, dysentery, chronic ulcers and as insect repellent [19, 20, 21]. *L. aurita* was also reportedly used in the treatment of asthma, bronchitis, malaria, stomatitis and nasal congestion [12]. 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 phytochemicals [27]. Furthermore, the biological and pharmacological properties of phytochemicals 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 for a period of 7 days with occasional shaking. 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°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 $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$) 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 1.0×10^6 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 ninth 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 (10^6 cfu/mL). The test tubes were covered and incubated at 37°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°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 crude extract 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 FeSO₄. 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 = $(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}} \times 100$.; 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 (EC₅₀) 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 extract (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°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. FeSO₄ 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 FeSO₄ 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 FeSO₄.

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 (CDCl₃). 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 Research 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 DISCUSSION

3.1 Antibacterial Activity

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

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

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

The antibacterial activity of micro organisms are reportedly classified based on MIC values as follows; $24.4 \geq \text{MIC} \leq 78.2$ µg/mL are considered significant, while $100 < \text{MIC} = 625$ µg/mL are moderate or weak ($\text{MIC} > 625$ µg/mL) against various resistant pathogens [30]. On the basis of such classification, the ethyl acetate fraction crude extract 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

| Bacteria | Ethyl acetate | Gentamicin |
|----------------------|---------------|------------|
| <i>S. aureus</i> | 1 (+) | 1 (+) |
| <i>E. faecalis</i> | 2 (+) | 2 (+) |
| <i>B. subtilis</i> | 2 (+) | 2 (+) |
| <i>E. coli</i> | 4 (-) | 4 (-) |
| <i>P. aeruginosa</i> | 3 (+) | 3 (+) |

| | | |
|---------------------|-------|-------|
| <i>K. pneumonia</i> | 2 (+) | 2 (+) |
| <i>S. typhi</i> | 2 (+) | 2 (+) |
| <i>P. mirabilis</i> | 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 crude extract 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 crude extract 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 | Crude extract %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 at 100 µg/mL exhibited maximum iron chelating activities of 90.22% and 82.25% respectively.

3.2.1 Determination of EC₅₀ of the ethyl acetate crude extract

The fifty percent effective concentration (EC₅₀) is a quantitative measure that indicates how much of a particular substance is needed to achieve 50% of a given biological activity [33]. The EC₅₀ chelating activities of fractions are shown in Figure 1.

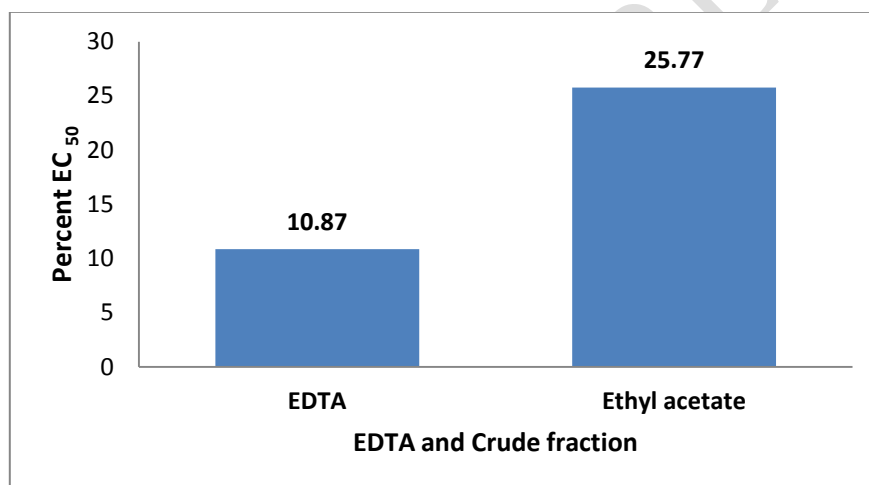


Figure 1: EC₅₀ Chelating power of ethyl acetate crude fraction and EDTA

The EC₅₀ chelating activity of EDTA and crude extract are 10.87µg/mL and 25.77µg/mL respectively. The lower the EC₅₀ value, the higher the chelating activity of the compound or crude extract. These results show that the crude extract is also a good metal chelating agent relative to the EDTA standard.

3.3 Ferric Reducing Antioxidant Power (FRAP)

The ferric reducing activities: FRAP Units and percentages FRAP of the crude extract and the standard ascorbic acid are shown in table 4.

Table 4: Ferric Reducing Antioxidant Power of the fractions of *L. aurita*

| FRAP Units (Fe ²⁺ Equivalent per g fraction) | | | Percentage FRAP | |
|---|--|--|-----------------|---------------|
| Concentration (µg/ml) | Ascorbic acid (Fe ²⁺ µg/ml) | Crude extract (Fe ²⁺ µg/ml) | Ascorbic acid | Crude extract |
| 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 crude extract and ascorbic acid (Table 4). The FRAP values for ascorbic acid and ethyl acetate crude extract are 153.63 and 134.40 µg/mL Fe²⁺ Equivalent per gram of crude extract respectively. The percentage FRAP of the crude extract 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 phytochemicals 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) \hat{u}_{\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 |

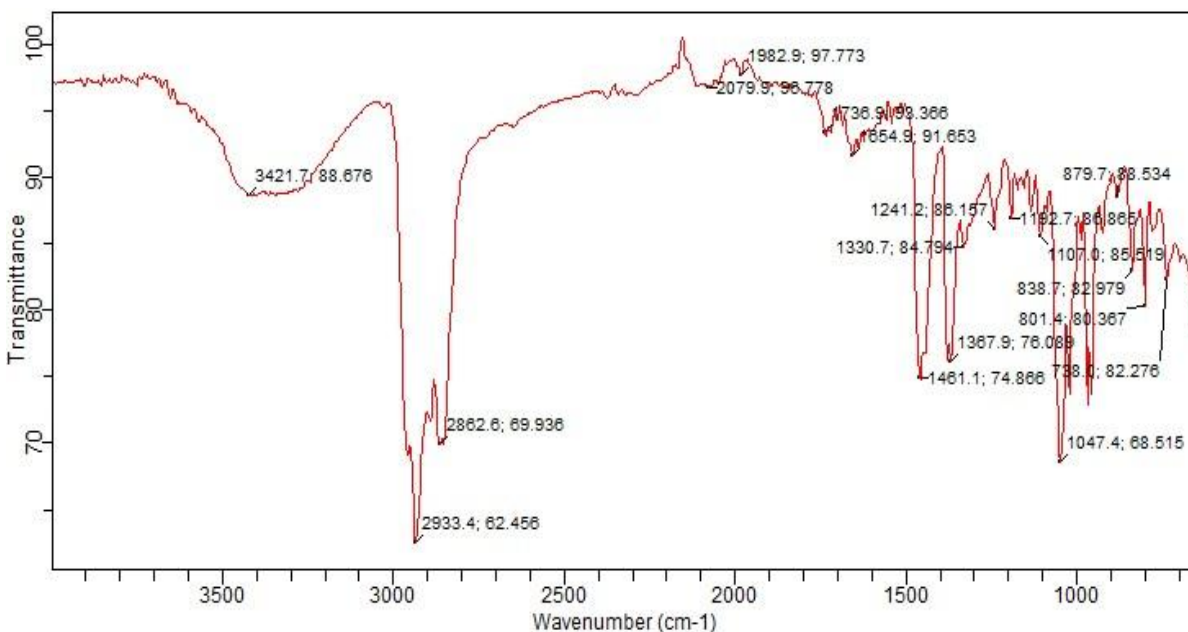


Figure 2: FT-IR spectrum of LAE

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

Mass spectrum of isolated compound LAE showed parent molecular ion $[M]^+$ peak at m/z 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.

Abundance

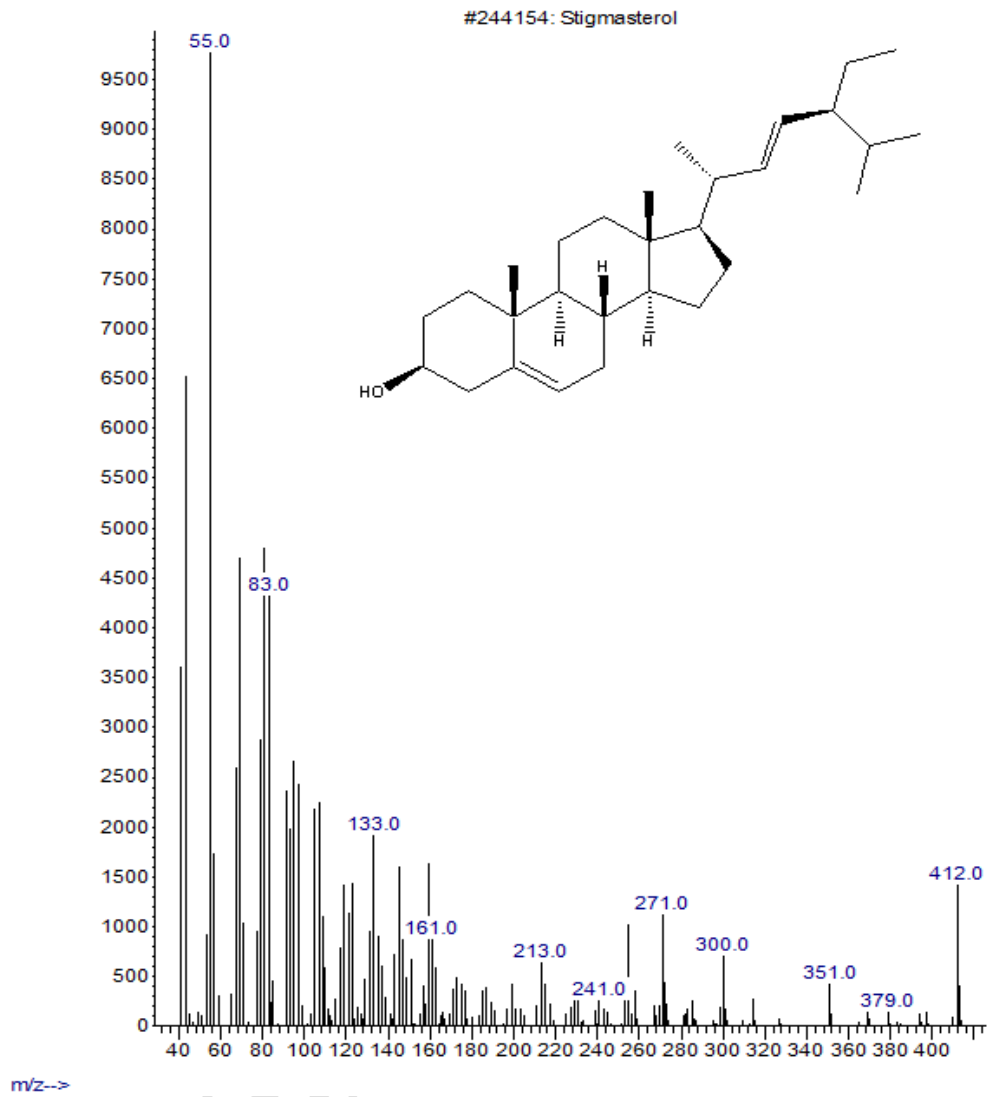
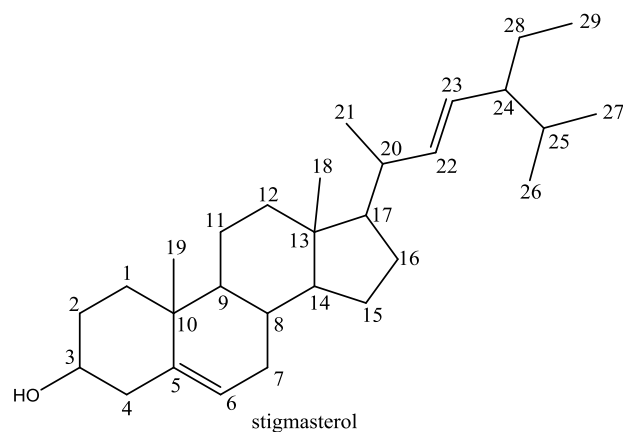


Figure 3: GC-MS spectra of LAE

3.4.3 NMR Analysis of LAE

Close examination of the $^1\text{H-NMR}$ 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 $^{13}\text{C-NMR}$ 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 quaternary 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 stigmaterol.



The ^1H and ^{13}C NMR values for all protons and carbons were assigned on the basis of COSY, HMQC and HMBC. The ^1H - ^1H COSY (appendix 4 and 5) established the correlations between protons that are situated in the same environment; major correlations observed include: δH 5.3 and δH 2.1; δH 5.2 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 6) 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 δ 31.624 (C2), δ 71.786 (C3), and δ 121.702 (C6); proton at (5.339) showed correlation with C10 (δ 50.106) 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 & ^{13}C -NMR data of LAE (Stigmasterol) comparison with literature [35]

| Carbon atom | ^{13}C NMR Experimental | ^{13}C 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 crude extract 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.

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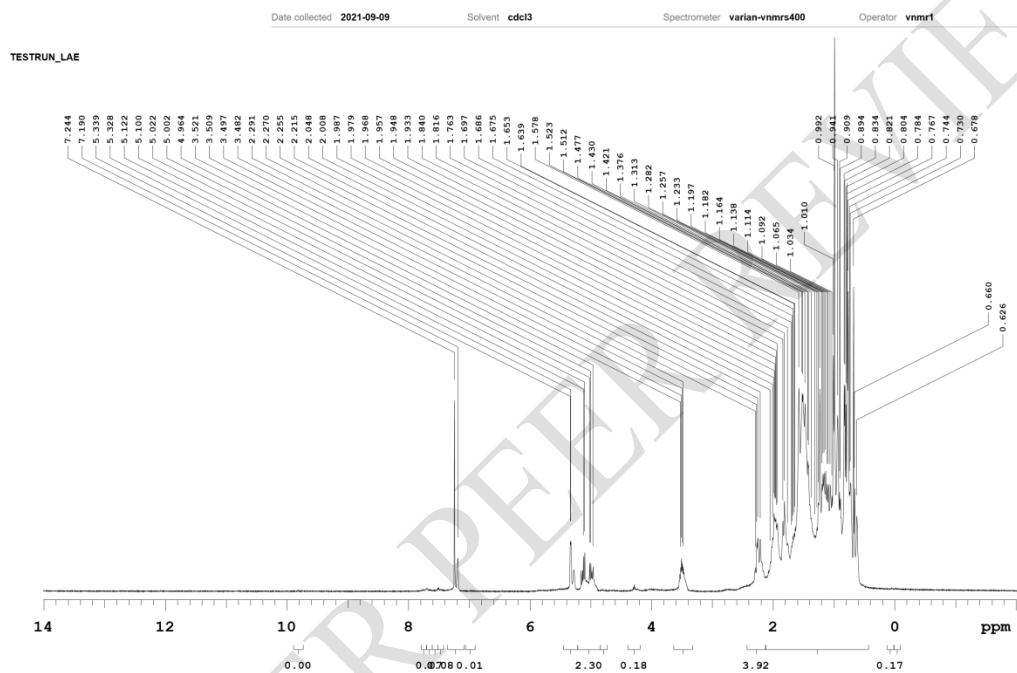
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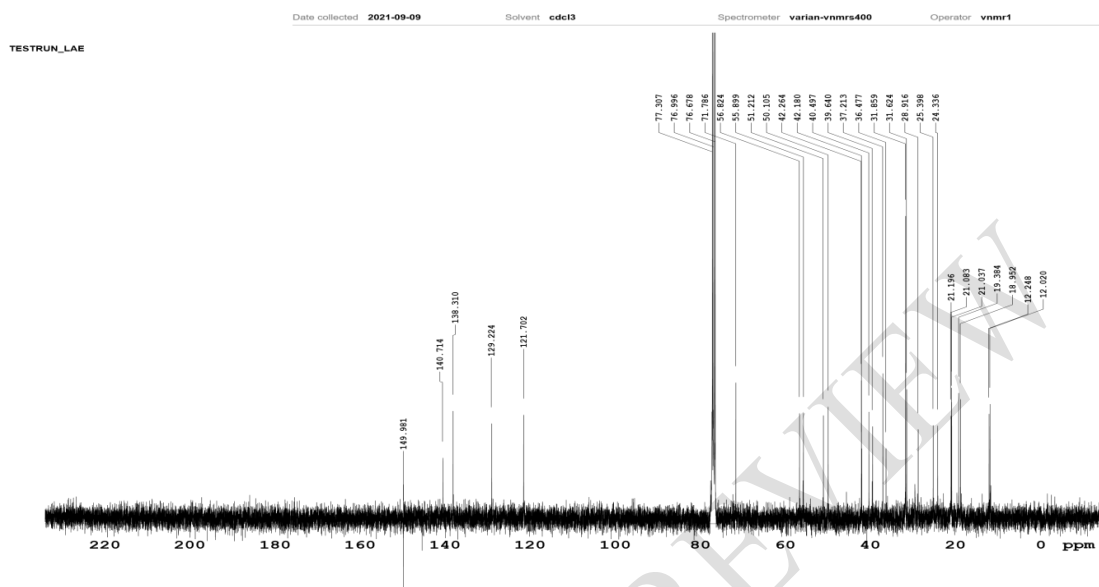
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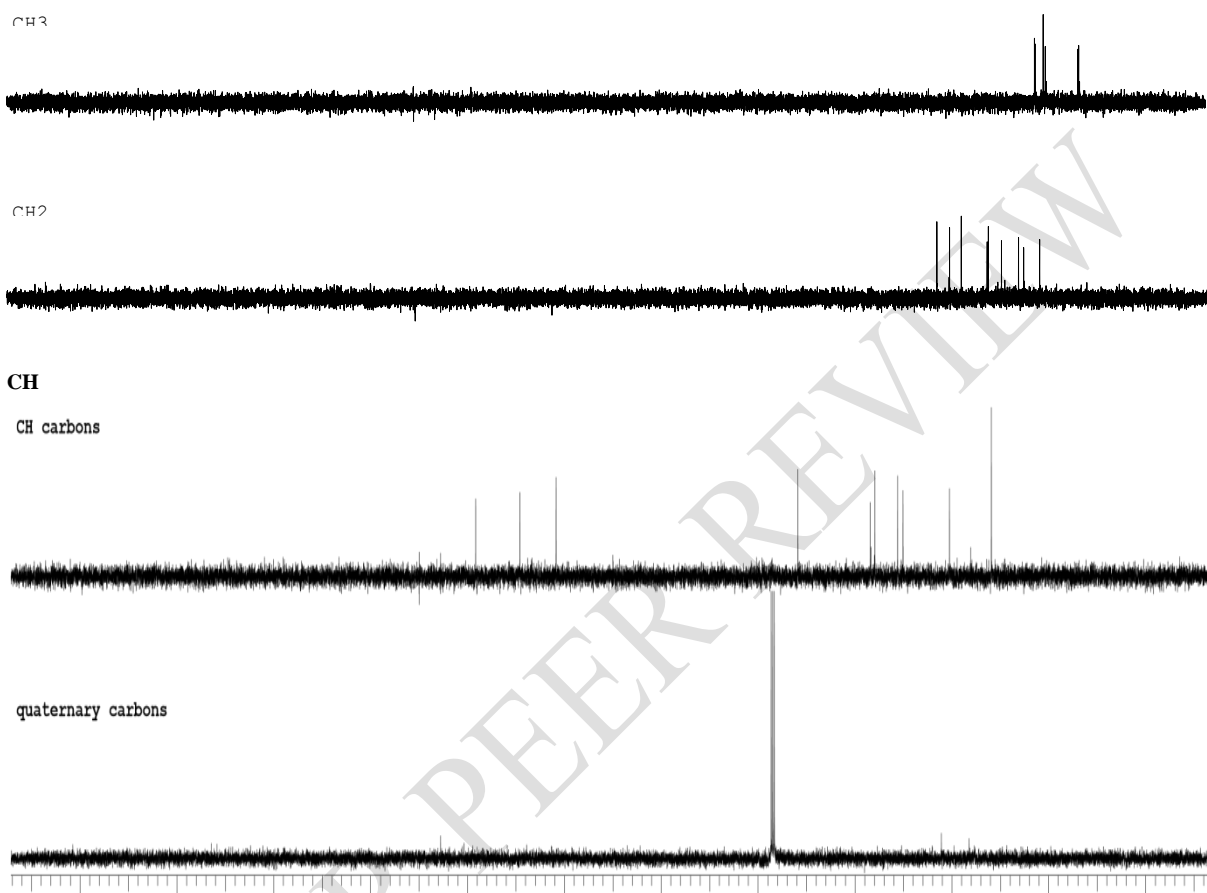
APPENDIX 1: ¹H of LAE



2. ¹H of LAE

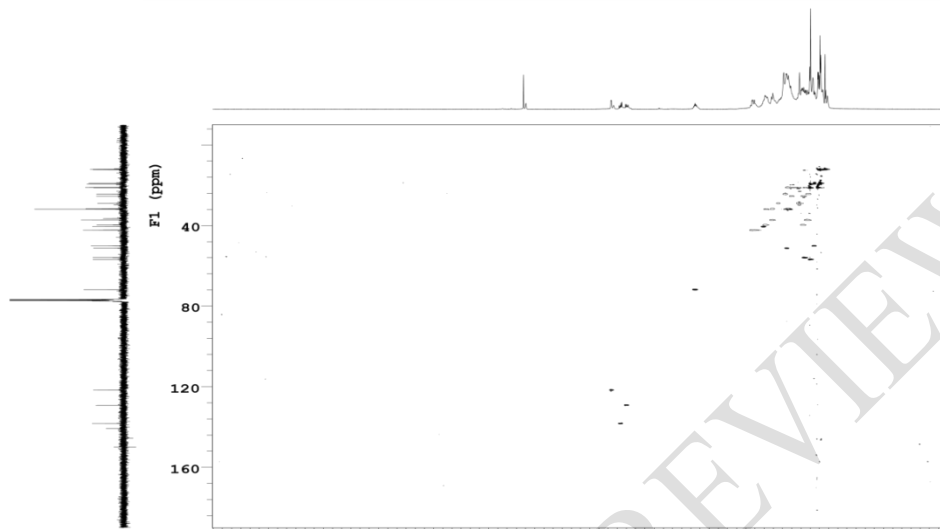


3: DEPT of LAE



TESTRUN_LAE

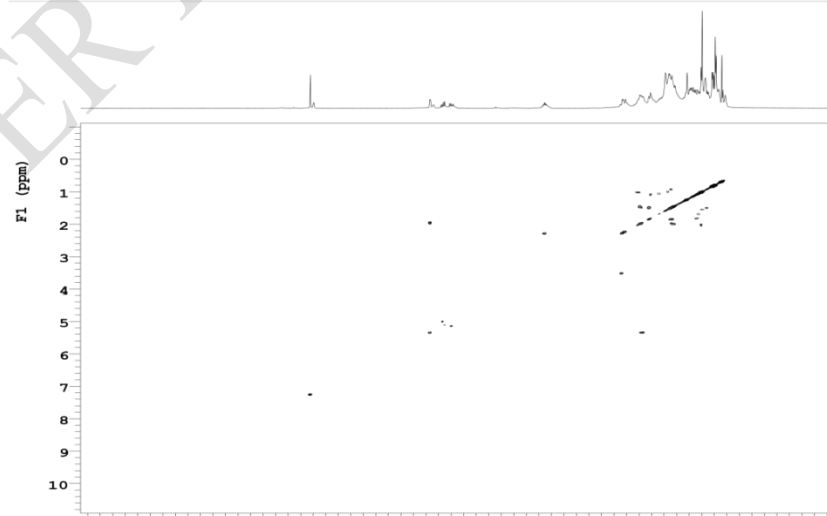
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4. HMBC

TESTRUN_LAE

Date collected 2021-09-09 Solvent cdcl3 Spectrometer varian-vnmrs400 Operator vnmr1



5. HMQC

UNDER PEER REVIEW