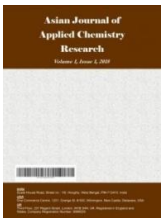

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Isolation and Characterisation of Hydrolysable Tannin from Ethyl Acetate Portion of the Aerial Part of *Phyllanthus amarus* Schum. & Thonn

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Authors' contributions

This work was carried out in collaboration between all authors. Author HAU designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors HU and MBA manage the statistical analysis of the study. Authors BFM, MB and MAS managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

The present work involves extraction of phytochemicals from aerial part of Phyllanthus amarus Schum. & Thonn with n-hexane and 85% methanol. The isolation and characterization of Phytoconstituents was done from the methanol extract through portioning with chloroform and ethyl acetate. Fractionation and isolation (using column and thin layer chromatography respectively) of ethyl acetate column pooled portion afforded a compound coded as E-3.3C. The structure of the isolated compound was established on spectroscopic evidences (IR, 1HNMR, MS), which revealed the compound as 1-de (oxygalloyl)-2ˈ,3ˈ,-di-methoxy-amariin a hydrolysable tannins.

Keywords: Phyllanthus amarus; fractionation; fragmentation; soxhlet extraction; spectroscopy.

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1. INTRODUCTION

The usefulness of certain herbs as therapeutic agents has been known for thousands of years through the enormous contribution of primitive man worldwide who virtually ate plants in loci to ascertain their use [1,2]. It is believed that the cure to any debilitating human ailments and diseases may be found among the World's flora in natural pharmacy [3]. Today, natural products derived from plants are being tested for the presence of new drug with new modes of pharmacological effect; many of which are efficacious and contains compounds that are potential drug that require further examination [4,5]. The use of traditional medicines and medicinal plants in most developing countries as therapeutic agents for the maintenance of good health has been widely observed [6]. Medicinal plants produce bioactive compounds used mainly for medicinal purposes. These compounds either act on different systems of animals including man, and/or act through interfering in the metabolism of microbes infecting them. The microbes may be pathogenic or symbiotic, In either way the bioactive compounds from medicinal plants play a determining role in regulating host-microbe interaction in favour of the host; so the identification of bioactive compound in plants, their isolation and characterization of active ingredients in crude extracts by various analytical methods is important.

Phyllanthus amarus Schum. & Thonn. is a small herb belonging to the *Euphorbiaceae* (Phyllanthaceae) family; this plant is a branching annual glabrous herb which is 30-60 cm high and has slender, leaf-bearing branchlets, distichous leaves which are subsessile, elliptic-oblong, obtuse, rounded base [7]. Flowers are yellowish, whitish or greenish, auxillary, males' flowers in groups of 1-3 whereas females are solitary. Fruits are depressed-globose like smooth capsules present underneath the branches and seeds are trigonous, pale brown with longitudinal parallel ribs on the back [8]. The plant species is well known for its medicinal properties and widely used worldwide. The plant *Phyllanthus amarus* is widely distributed in all tropical regions of the planet. This plant may be indigenous to the tropical Americas [9,10,11], the plant is a common weed of cultivated fields and spreads widely in West Africa and other parts of the world and has been discovered some years ago in some part of Nigeria [12]. *Phyllanthus amarus* has been reported to possess lignans namely phyllanthin and hypophyllanthin obtained from the leaves of the plant that has been noted to enhance the cytotoxic responses with cultured multidrug-resistant cells [13,14]. In addition, a di-dehydrohexahydroxyldiphenoyl hydrolysable tannin named amariin. geranin, corilagin, 1,6-digalloylglucopyranoside, were isolated from the polar fraction of aerial parts of *Phyllanthus amarus* [15]. In this study, the polar fraction of the aerial parts of *phyllanthus amarus* was investigated, for isolation of possible compound(s).

2. METHODOLOGY

2.1 Sample Collection and Identification

The plant material were collected from Federal College of Education Okene, Otite, Okene Local Government area of Kogi State, Nigeria and was identified and authenticated by a plant Taxonomist in the department of Biological sciences, University of Maiduguri. The voucher number 16/003A was given and the herbarium specimen deposited in the research laboratory, department of Chemistry University of Maiduguri. The aerial part were collected and air-dried under shade until dryness; they were ground into coarse powder using wood mortar and pestle.

2.2 Extraction of Plant Material

Soxhlet extraction method was adopted in this study [16]. One thousand two hundred gram (1200g) of the powdered plant material was sequentially, exhaustively extracted with n-hexane, then 85% methanol as described by [16]. The crude extracts were filtered and concentrated at low pressure. The crude methanol extract was subjected to solvent partitioning with chloroform and ethyl acetate, they were concentrated and evaporated after which they were coded as PACF and PAEA (*Phyllanthus amarus* chloroform portion and *Phyllanthus amarus* ethyl acetate) respectively. The ethyl acetate portion of the crude methanol extract was chromatographed on silica gel (60-120G mesh) using a solvent system (chloroform and ethyl acetate) in a mix-ratios of 0% through 100% increasing polarity by 10%. The elution afforded a total of 11 fractions i.e., E1-E11. All the Fractions obtained were subjected to thin layer chromatography (TLC), similar fractions were pooled on the basis of their *Rf* values and 6 fractions (E1-E6) were obtained. Fractions were concentrated on rotary evaporator with temperature of the water bath not exceeding 40ºC.

2.3 Isolation and Purification of Ethyl Acetate Portion of Aerial Part of *Phyllanthus amarus*

Fraction E-3 was purified over sephadexLH-20 using Methanol to afford five sub fractions coded as E3.1, E3.2, E3.3, E3.4 and E3.5. They were evaporated and condensed under reduced pressure. Since there was very less yield in sub-fractions E-3.1, E-3.2, E-3.4 and E-3.5; E-3.3 was selected and again subjected to PTLC (preparative thin layer chromatography). using a solvent system of toluene: Ethylformide: Formic acid in a ratio of 50:40:10 which provided three distinct and prominent spots coded as E-3.3A, E-3.3B, and E-3.3C with *Rf values* of 0.4, 0.47 and 0.52 respectively, Methanol was used for washing and purification. Based on the amount obtained, E-3.3C with the highest yield was subjected to spectroscopy analysis. The structure of the isolated compound was established on the basis of spectroscopic evidences (IR, UV, ^{1}HNMR and MS).

3. RESULTS

The IR, ^{1}HNMR spectrum and Mass spectrometric data of the isolated compound are showed in Tables 1, 2 and 3 respectively.

4. DISCUSSION

4.1 Structure Elucidation and Characterization of Sub-fraction E-3.3C

The ethylacetate portion of the aerial part of *Phyllanthus amarus* was subjected to a series of chromatographic techniques, leading to the isolation of a compound E-3.3 Cyield (5 mg). Compound E-3.3C is amorphous substance and greenish yellow in colour, obtained from column pooled fraction of ethyl acetate chromatographed on Sephadex LH-20. The best mobile phase selected by PTLC was tolune: Ethylformide: Formic acid (50:40:01) and its *Rf* value was determined as 0.52. It was further subjected to PTLC for purification using a solvent system of toluene:ethylacetate:formic acid (50:30:6) and a single spot was observe. The IR spectrum for the isolated compound indicated the presence of hydroxyl group at 3358, tannins are known to absorb at that region [17]. The strong absorption band at 2925 and 2854 cm^{-1} were suggestive of aliphatic C-$H_{asymmetrical}$ and C-$H_{symmetrical}$ stretching vibrational frequencies respectively which coincide with the reports by Coates & Pantoja and Gonzalez [18,17]. The band at 1729 cm^{-1} is an indicative of the carbonyl functionality feature in the compound, especially for the esters as supported by Silverstein et al. & Stuart, [19,20] and suggested tannins. The medium absorption band around 1457 cm^{-1} was assigned to aromatic C-$C_{streatching}$ vibration, as also reported by Pantoja and Gonzalez [17]. The absorption band at 1368$cm^{-1}$was assigned to aliphatic methene C-$H_{rocking}$ vibration, these data agrees with the earlier reports of [18,17,21,22]. Similarly, peaks at 1283 followed by absorption at 1210and 1116$cm^{-1}$are of the esters/ether linkage C-$O_{stretching}$ vibration and also absorption at 1210cm^{-1}were assigned to aromatic C-H in plane bending vibration [17,18].Absorption due to the presence of sugar functional groups are within the range of 1200 and 950 cm^{-1}, more specifically thepeaks observed at 1155, 1116, 1070 and 1014 cm^{-1}appeared as evidence for polyphenols, especially aromatic C-$O_{stretching}$ vibrations [17] and were supported by the findings of [23] who worked on different tannins with similar IR spectral data. Absorptions at 860.28 cm^{-1} is an indicative of 1,4-disubstituted aromatic ring, bands at 756.37 cm^{-1} is due to aromatic C-H out of plane bending vibration [23,24,18]. Also absorption seen at 697cm^{-1} is due to C=$C_{stretching}$ vibrations specifically Meta substitution in a ring, which also agrees with the report of [25] as shown in Table 2. The HNMR spectrum showed chemical shift singlet at δ_H 8.41 for two methylene protons at (C'5 and C'5) of α,β-unsaturated esters protons and two methine at (C''5 and C''5) of the aromatic protons, this assignment were in line with the findings of [26] where he reported that, α,β-unsaturated esters show a large chemical shift downfield resulting from the various β- and γ-carbonyl interactions with the protons, while the proton γ to the carbonyl and close to the same plane for the aromatic methine, will lead to quite large downfield shifts. Due to gamma-gouch interaction which is a long distance coupling as explained by [27], a multiplet at δ_H 4.12 was assigned to the methyl proton of the sugar unit attached to the carboxylate at (C-6) as supported by Jag and Hans [28,26]. Two singlets at δ_H 3.70 and 3.26 were assigned to the two methoxy protons that are attached to the cyclohexenone ring (C'3 and C'2) which are in line with the findings of [29]. The ^{1}H NMR spectrum showed an upfield ^{1}H intensity, a multiplet at δ_H 2.31 was assign to the anomeric carbon atom (C-1) of the sugar unit while a singlet at δ_H 1.8 and 1.24 was assign to the four C-CH-O of the sugar unit at (C-2, C-3, C-4 and C-5) respectively the reasons

for the smaller chemical shift expressed by the sugar protons could be due to various factors like solvent, temperature, pH and nature of the compound as reported by Baker and Cairns & Hans [30,26]. The spectrum at δ_H 0.82 ppm is due to two methyl protons at (C'1 and C'1) as shown in Table 3,The mass spectrum of the proposed compound showed a molecular ion peak at 828 (M+ at 828 m/z) which corresponds to the molecular formula $C_{36}H_{28}O_{23}$. Peak at 800 (M-28) is due to the loss of CO, indicating the presence of carbonyl group and also an evidence of an ester group [28]. Also Peak at 701 is as a result of removal of the deoxysugar unit (M-128) and formation of acid anhydride, which confirms the presence of sugar moiety present in the proposed compound. There are peak at 678 (M-150), 662 (M-166) and formation of the based peak at 647 (M-181) which are cleavage due to the loss of aromatic esters and formation of ether bond between ring B and the sugar this kind of cleavage was explained by Jag [28] as shown in Figs. 1 and 2. The structure of the proposed compound was affirmed based on the information obtained from the spectra data and literature as 1-de(oxygalloyl)-2', 3',-di-methoxy-amariin with a molecular mass of 828, a hydrolysable tannin having the same nucleus with a compound isolated previously from the aerial part of *Phyllanthus amarus* named Amariin.

Table 1. IR spectroscopy data for E-3.3C

S/No	Group	Vibration	Absorption frequency(cm^{-1})	References
1	Arom CH_2-OH	$OH_{stretch}$	3358.89	[17]
2	Methyl C-H	$C\text{-}H_{asym.stretch}$	2854.11	[17]
3	Methyl C-H	$C\text{-}H_{sym.stretch}$	2925.22	[17]
4	Esters C=O	$C{=}O_{stretch}$	1729.88	[20]
5	Arom CH_2-OH	$CH_{2stretch}$	1457.19	[17]
6	Methyl C-H	$C\text{-}H_{rock}$	1368.25	[17]
7	Esters/etherC-O	$C\text{-}O_{stretch}$	1283.00	[21]
8	Arom C-H	$C\text{-}H_{inplane}$	1210.00	[21]
9	Arom C-O	$C\text{-}O_{stretch}$	1155-1014	[17]
10	Arom C-H	$C\text{-}H_{out\text{-}of\text{-}plane\ bend}$	860-756.37	[24]
11	Arom C-H	$C\text{-}H_{bend}$	697.00	[25]

Key; Arom = Aromatic, stretch = stretching, bend = Bending

Table 2. ^{1}HNMR spectroscopy data for isolate E-3.3C in D2O

S/No	Carbon no	Type of Proton	Chemical Shift (ppm)	References
1	C 5, C 5	=CH, arom methine	8.41(s)	[26]
2	C'5,C'5	O=C-C=CH, esters	8.41(s)	[26]
3	C6	$\text{-O-}CH_2$,sugar	4.12(m)	[28] [26]
4	C'2,C'3	$\text{C-O-}CH_3$,methoxy	3.70. 3.26(s)	[29]
5	C1	$\text{O-}CH_2$,anomericsugar	2.31(m)	[28] [26]
6	C2,C3,C4,C5	O-CH, sugar	1.84(s)-1.24(s)	[28] [26]
7	C'1C'1	CH,methyl	0.82(s)	[29]

Key; s= singlet, m= multiplet

Table 3. Summary of fragments identified by mass spectrometric data for the isolate (E-3.3C)

S/N	M/Z	LostFragment	Peaks observed	Molecular formula
I	$M\text{-}e^-$	e^-	828	$C_{36}H_{28}O_{23}$
2	M-28	CO	800	$C_{35}H_{28}O_{22}$
3	M-128	$C_6H_8O_3$	701	$C_{30}H_{20}O_{20}$
4	M-150	$C_7H_2O_4$	678	$C_{29}H_{26}O_{19}$
5	M-166	$C_7H_2O_5$	662	$C_{29}H_{26}O_{18}$
6	M-181	$C_8H_5O_5$	647	$C_{28}H_{23}O_{18}$

Key: e^-= electron lost, M= molecular ion peak, M/Z= mass to electron ratio

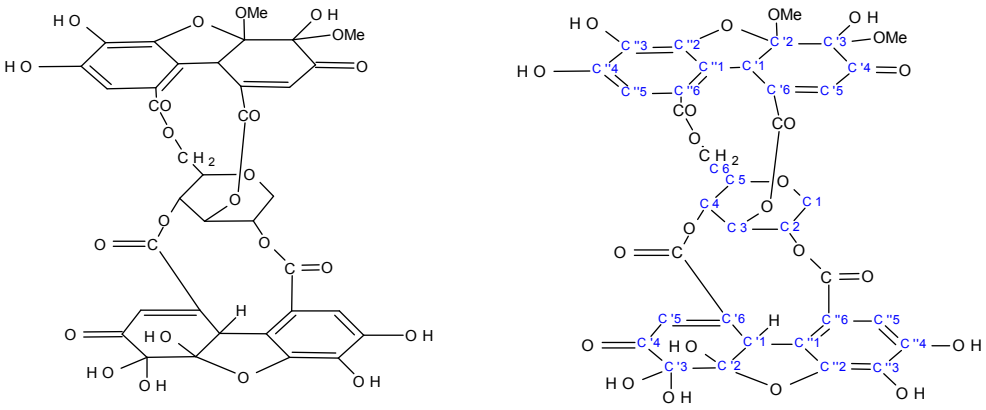

Fig. 1. Structure of compound E3.3C ($C_{36}H_{28}O_{23}$) numbering system

1-de(oxygalloyl)-2', 3',-di-methoxy-amariin; 1-deoxy-bis-[di-2',2'-epoxy]-2',3'-di-methoxy–[di-dehydroxylpentahydroxyldiphenoyl-(O-2,O-3,O-4,O-6)-glucopyranoside; Molecular mass (828)

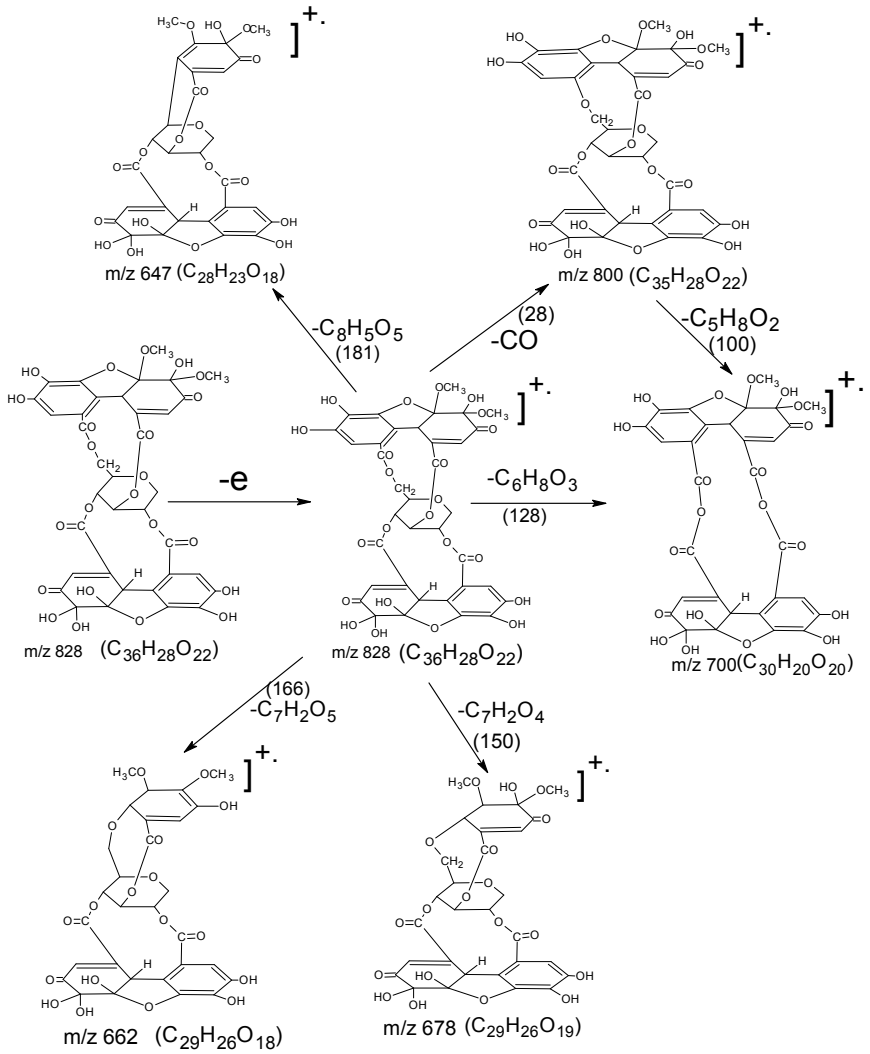

Fig. 2. Fragmentation parttern of 1-de (oxygalloyl)-2', 3',-di-methoxy-amariin

5. CONCLUSION

Hydrolysable tannins was isolated and characterized from ethyl acetate portion of aerial part of *Phyllanthus amarus*. Therefore, it may be concluded that the activity exhibited by *Phyllanthus amarus* aerial part may be due to the isolated compound from the ethyl acetate pooled column portion (E-3.3C) identified as 1-de[oxygalloyl]-2′3′,-di-methoxy-amariin via interpretation of its IR, ^{1}HNMR chemical shifts and Mass spectrometry.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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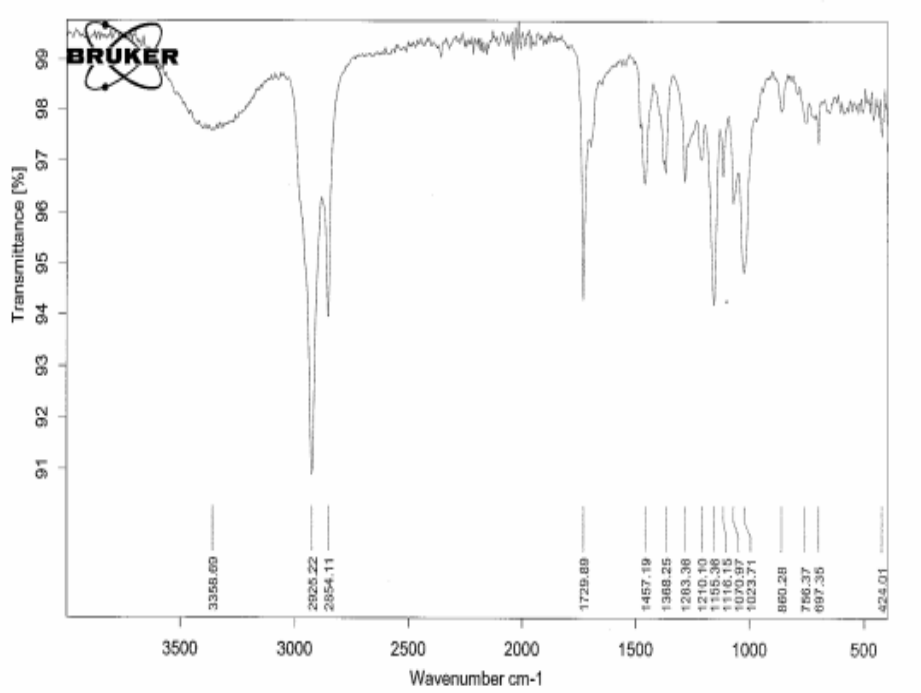
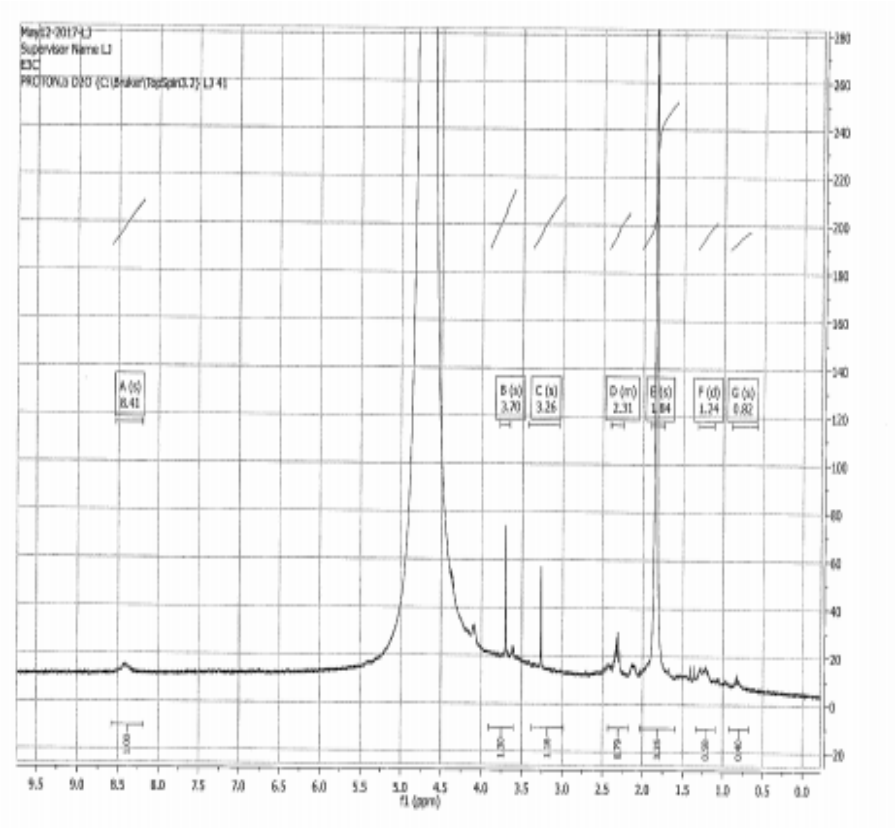
APPENDIX I. FTIR spectra data for isolate E3.3C



Appendix II. ^{1}HNMR Spectra data for isolate E3.3C



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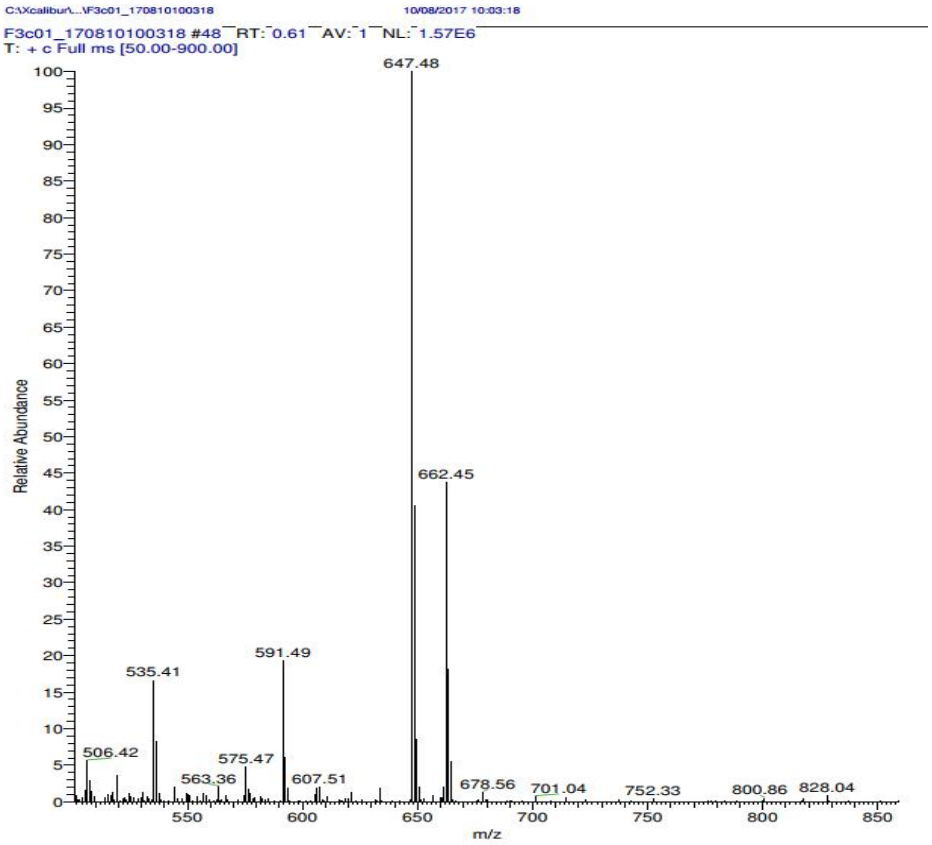

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