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BIOACTIVITY AND FREE RADICAL SCAVENGING ABILITY OF AN ISOLATED TRITERPENOID SAPONIN FROM THE ROOT BARK OF *MASSULARIA ACUMINATA SPP*

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ABSTRACT

A triterpenoid saponin was isolated from the methanol extract of the root bark of *Massularia acuminata spp*. The compound was identified as *3-O* – β- D- glucopyranosyl – (1-4)- β- D- glucopyranosyl – (1-4)- β- D- glucopyranosyl- olean -12-en-28-oic acid on the basis of chemical and spectral evidence of IR and NMR (^{1}H, ^{13}C, DEPT, ^{1}H-^{1}H COSY, HSQC and HMBC) data. The compound showed good inhibitory activity against the microorganisms, *Salmonella spp*, *Staphylococcus aureus, Escherichia coli*, *Aspergillus niger* and *Candida albicans* indicating that it is a bioactive compound. It was also found to possess antioxidant property by virtue of its high DPPH free radical scavenging ability of 73.09% at 100µg/ml, property that can make it relevant in stress management and cosmetic industry.

KEYWORDS: *Massularia acuminata*, Triterpenoid saponins, Antimicrobial, Antioxidant, Isolation, Glycoside, Spectroscopy.

INTRODUCTION

Triterpenoid saponins are saponins having triterpenoid nucleus as the aglycone. They are glycosides of triterpenes having surfactant property (Garai, 2014). The aglycone nucleus is either tetracyclic or pentacyclic with modified side chains (Negi *et al*., 2013). However, the most abundant are the pentacyclic aglycone structures and they have been widely investigated in plant kingdom and are known to be present in over 500 plant species (Kharkwal *et al*., 2012). The structural complexity arising from varied substituents and side chains on the aglycone gives rise to a number of physical, chemical and biological properties of this class of phytochemicals (Negi *et al*., 2013). Sun *et al.* (2006) have also observed that the diverse activities of triterpenoid saponins are attributed to the different substructures in the A-, C- and E rings or other positions. These diverse activities include bioactivities which have made this class of phytochemicals to be relevant in many areas including pharmaceutical industry, agriculture, cosmetics and feeds thereby generating interest in the study of these compounds. In recent years, there have been discovery of novel triterpenoid saponins with important biological activities (Garai, 2014). Many of the pharmacological and biological activities of these triterpenoid saponins are well documented in many works such as those of Sun *et al*. (2006), Tsuzuki *et al*. (2007), Hassan *et al*. (2010), Man *et al*. (2010), Jyothi and Seshangiri (2012), Negi *et al*., (2013) and Lunga *et al*.(2014) among a number of others.

The root bark of *Massularia acuminata* has been found to be rich in saponins and Terpenoids as phytochemicals of the plant part (Ukekpe *et al*., 2015). In the search for novel triterpenoid saponins, this work describes the isolation and structural elucidation of a triterpenoid saponin from the methanol extract of the root bark of *Massularia acuminata*. The isolated compound was assayed for its antimicrobial activity and antioxidant property and both have been reported.

Experimental

Plant Material

The root bark of *Massularia acuminata* was obtained by peeling- off the bark from washed fresh roots. This was air dried and ground to fine powder.

Extraction and Isolation

The plant part was extracted exhaustively in sequence by soxhlet method with the solvents, hexane, chloroform, ethyl acetate, acetone, ethanol and methanol in that order to yield crude extracts.

Solvent fractionation of the methanol crude extract was carried out with the solvents, hexane, chloroform, ethyl acetate, acetone, ethanol and methanol sequentially. The hexane suspension was taken as the hexane fraction of methanol extract (HFME). HFME was subjected to column chromatography by the method described by Teke *et al*. (2010) and Ode *et al*. (2011) using hexane – methanol (1:4) solvent system as the eluent. This afforded compound HFME1, a white amorphous powder as the first column fraction.

Determination of Melting Point
The melting point of the isolated compound was determined using Graffin- Gallenkamp 350BM25 melting point apparatus in a capillary tube.

Infra-red (IR) Spectroscopic Analysis
The IR spectrum of the compound was recorded on FTIR – ATR Bruker Alpha- p spectrometer.

Nuclear Magnetic Resonance (NMR) Spectroscopic Studies
The 1D- and 2D- NMR experiments (^{1}H, ^{13}C, DEPT, ^{1}H-^{1}H COSY, HSQC and HMBC) of the compound in DMSO-d6 were performed on Bruker 600 MHz machine to obtain the spectra.

Acid hydrolysis/Sugar identification
The acid hydrolysis of the compound was achieved with the method described by Li *et al*. (2010) and Xu *et al*. (2010). The compound (10 mg) was refluxed in 10 cm^3 2M methanolic HCl for 3 hours. The hydrolysate was diluted with distilled water and the aglycone component extracted in chloroform layer. The aqueous layer was then neutralized with conc. Na_2CO_3 and TLC analysis carried out using CH_2Cl_2 - MeOH - H_2O (15:6:1) as the developing reagent. The plate was then sprayed with 50% ethanolic H_2SO_4, allowed to dry and then heated at 100 ^{0}C to visualize the spot. Comparison was made of the R_f value with that of standard sugars to identify the sugar moieties.

Antimicrobial Activity
The antimicrobial activity of compound HFME1 was assayed against *Salmonella spp*, *Staphylococcus aureus* and *Escherichia coli* for the antibacterial activity, and *Aspergillus niger* and *Candida albicans* for the antifungal activity by the modified plate-well diffusion method (Uduak and Kola, 2010, Babu *et al*., 2007). Wells were made in nutrient agar and sabourand dextrose agar (SDA) plates which had been previously incubated with bacteria and fungi respectively. The sample solutions of different concentrations (0.2cm^3 each) were introduced into the wells and left to pre-diffuse for 30 minutes. Distilled water was used as negative control and standard drugs, streptomycin for bacteria and fulcin for fungi, were used as positive controls.

The bacterial plates were incubated at 37°c for 24h, while the fungi plates were incubated at 25°c for 72 h.

The degree of inhibition was determined by the size of the zone of inhibition measured in mm and was taken as evidence of antimicrobial activity of the compound.

Antioxidant Assay
The antioxidant activities of the isolated compound was determined by the modified 1,1- diphenyl -2-picrylhydrazyl (DPPH) free radical scavenging method of Hemalatha *et al*. (2010), Khalaf *et al*. (2008) and Walia *et al*. (2010).

DPPH solution (0.1mM) was prepared in 95% methanol and solutions of different concentrations (20, 40, 60, 80 and100µg/ml) of each of the isolated compounds were prepared also in 95% methanol. Each compound's solution, 3cm^3 was added to 1cm^3 of the DPPH solution and the absorbance measured at 517nm after 30 minutes using JENWAY 6305 spectrophotometer. The absorbance of the blank (3cm^3 95% methanol added to a 1ml DPPH solution) was also measured.

The radical scavenging activity of each of the Compound's solution was expressed in percentage of the ratio of lowering of the absorption of DPPH by the compound's solution relative to the absorption of DPPH $\{(A_{DPPH} - A_{Sample})/ A_{DPPH}\}$ x 100%, (Saha *et al*., 2008), where A is the absorbance. The IC_{50} was obtained by regression and the method of Rajan *et al*. (2011).

RESULTS AND DISCUSSION

Compound HFME1 ^{1}H and ^{13}C Spectra Data

Carbon (Aglycone)	^{13}C (δ)	DEPT	^{1}H(δ)	Carbon (Sugar moiety)	^{13}C (δ)	DEPT	^{1}H(δ)
1	38.6	$>CH_2$	1.31, 1.56 m	C_1'	103.21	>CH-	5.35 d
2	24.4	$>CH_2$	1.45, 1.70 m	C_2'	83.1	>CH-	4.21
3	89.2	>CH-	2.77 d	C_3'	78.4	>CH-	3.49
4	39.6	>C<	-	C_4'	71.6	>CH-	3.90
5	55.6	>CH-	0.98 t	C_5'	75.8	>CH-	3.69
6	18.5	$>CH_2$	1.38, 1.63 m	C_6'	62.8	$>CH_2$	3.60, 3.66
7	33.3	$>CH_2$	1.31, 1.56 m	C_1''	101.78.	>CH-	4.70 d
8	39.8	>C<	-	C_2''	77.0	>CH-	3.56
9	48.3	>CH-	1.07 s	C_3''	78.1	>CH-	2.96
10	37.9	>C<	-	C_4''	71.3	>CH-	3.31
11	24.2	$>CH_2$	1.31, 1.52 d	C_5''	78.4	>CH-	3.13
12	123.6	>CH-	5.19 t	C_6''	62.8	$>CH_2$	3.01, 3.11
13	142.9	>C<	-	C_1'''	96.78	>CH-	5.25 d
14	41.9	>C<	-	C_2'''	72.3	>CH-	3.79
15	28..3	$>CH_2$	1.13, 1.38 m	C_3'''	74.8	>CH-	3.60
16	23.8	$>CH_2$	1.29, 1.54 m	C_4'''	68.8	>CH-	3.50
17	46.5	>CH-	-	C_5'''	77.49	>CH-	3.50
18	41.7	>CH-	2.82 t	C_6'''	63.21	$>CH_2$	3.41, 3.47
19	46.9	$>CH_2$	1.20, 1.45 m				
20	30.9	>C<	-				
21	34.2	$>CH_2$	1.31, 1.56 m				
22	33.4	$>CH_2$	1.65, 1.90 m				
23	28.3	$-CH_3$	0.94 s				
24	16.9	$-CH_3$	0.89 s				
25	15.4	$-CH_3$	0.67 s				
26	17.8	$-CH_3$	1.12 s				
27	25.8	$-CH_3$	0.88 s				
28	179.0	>C<	-				
29	34.1	$-CH_3$	0.84 s				
30	22.9	$-CH_3$	0.78 s				

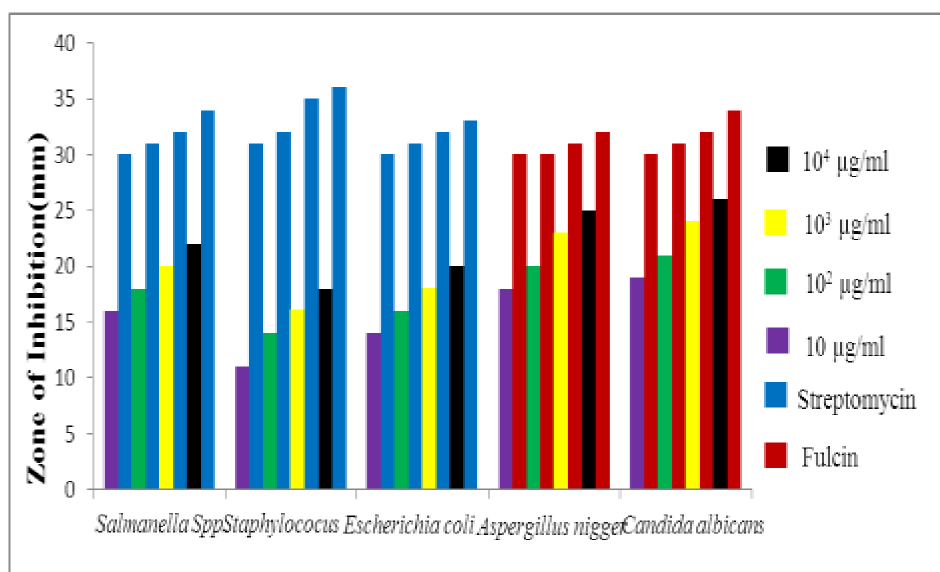


Figure 1: Antimicrobial Activity of Compound HFME1.

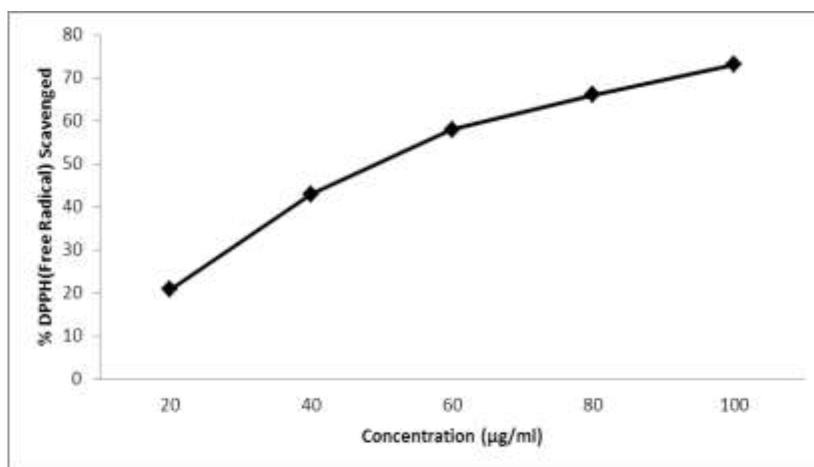
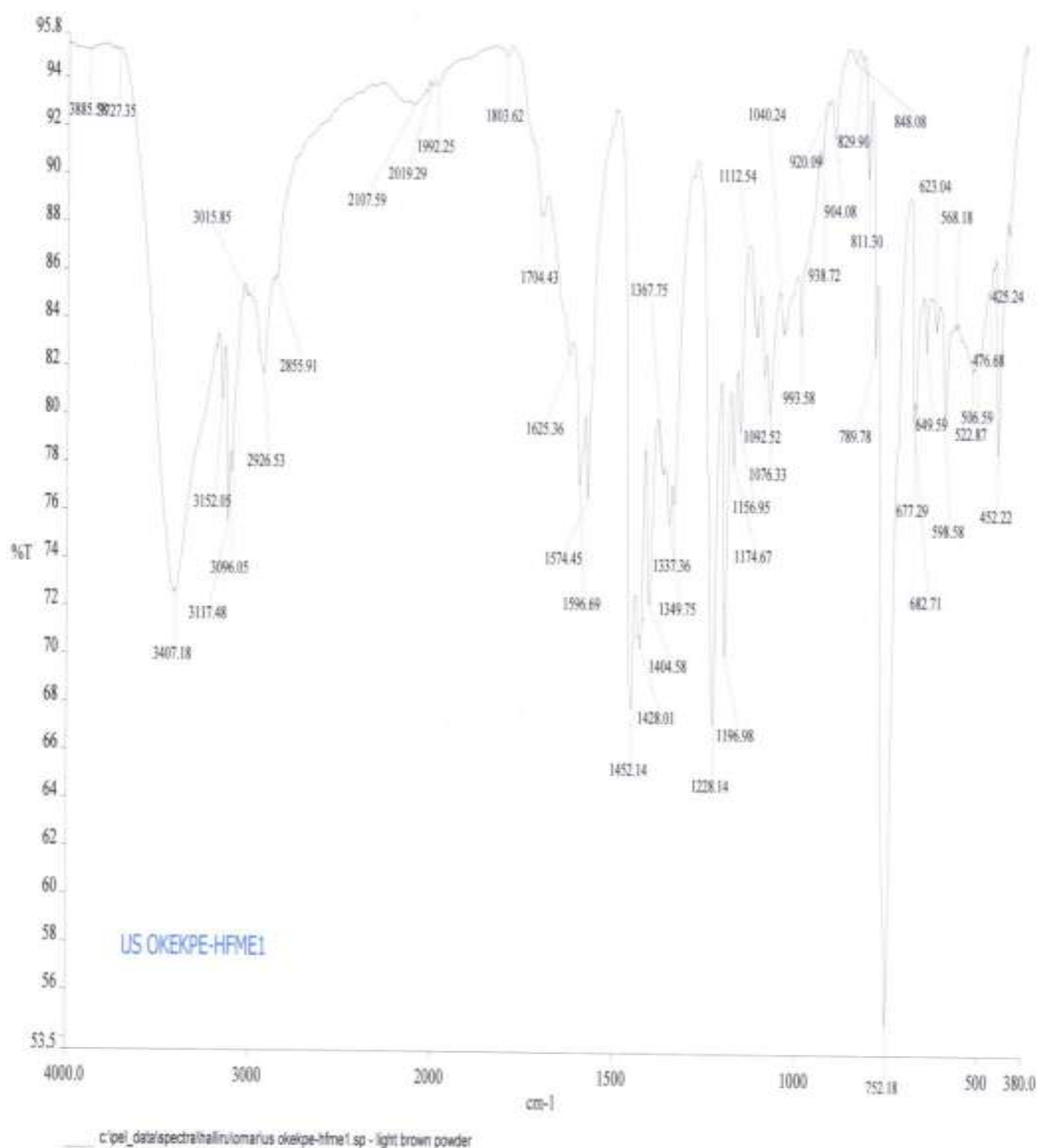
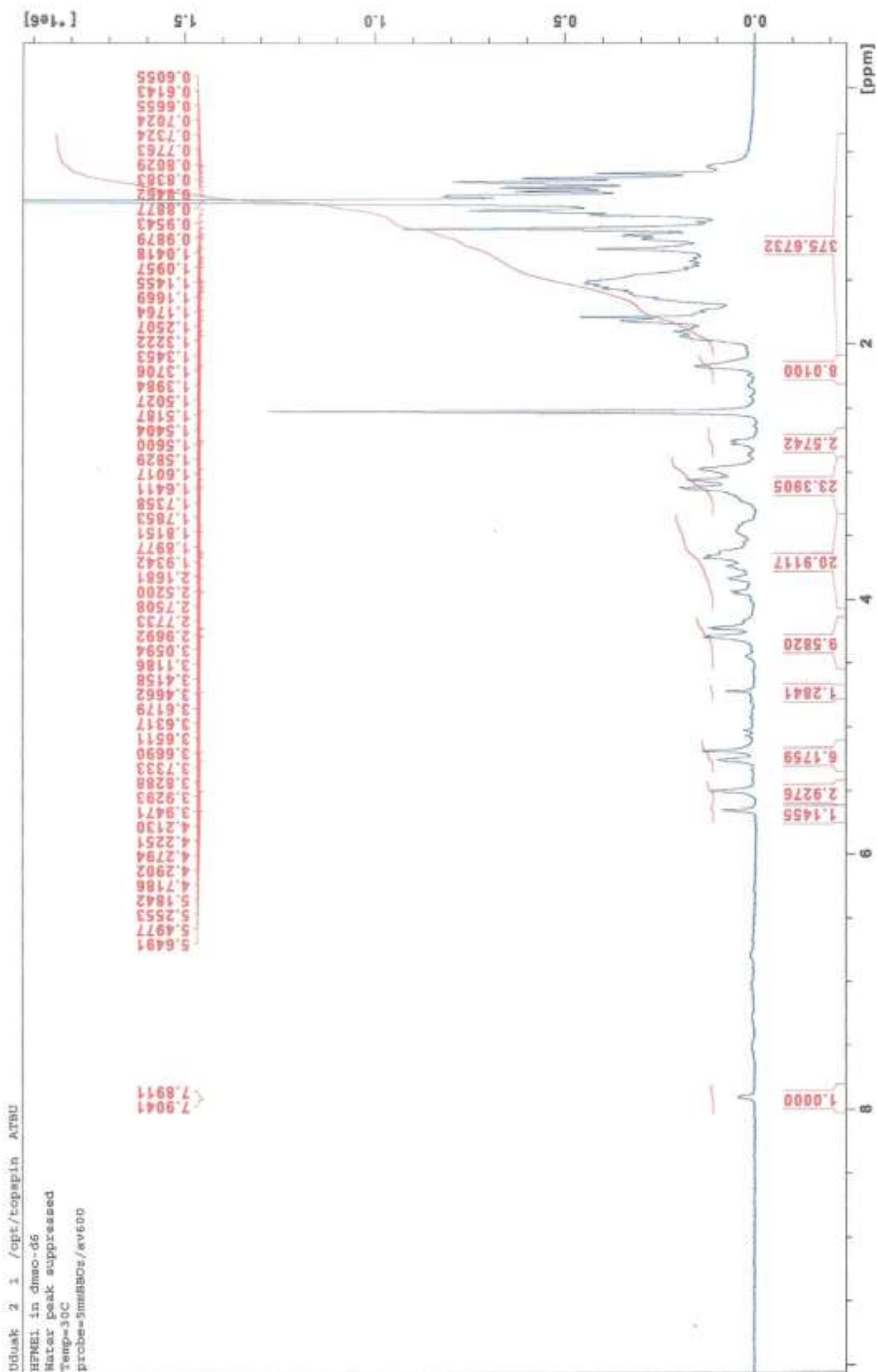


Figure 2: Free Radical Scavenging Ability of Compound HFME1.

Supporting spectra of the isolated compound.

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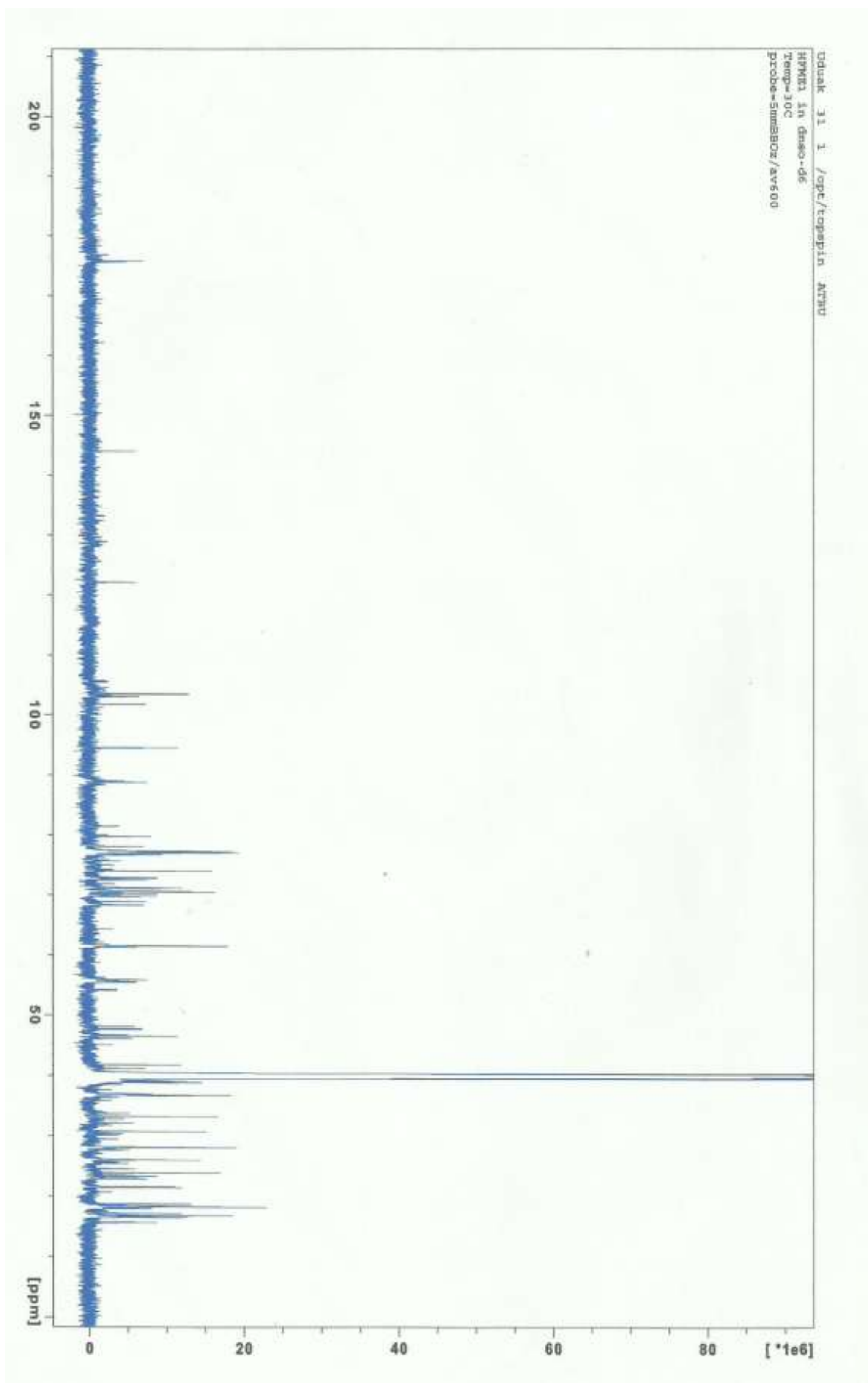
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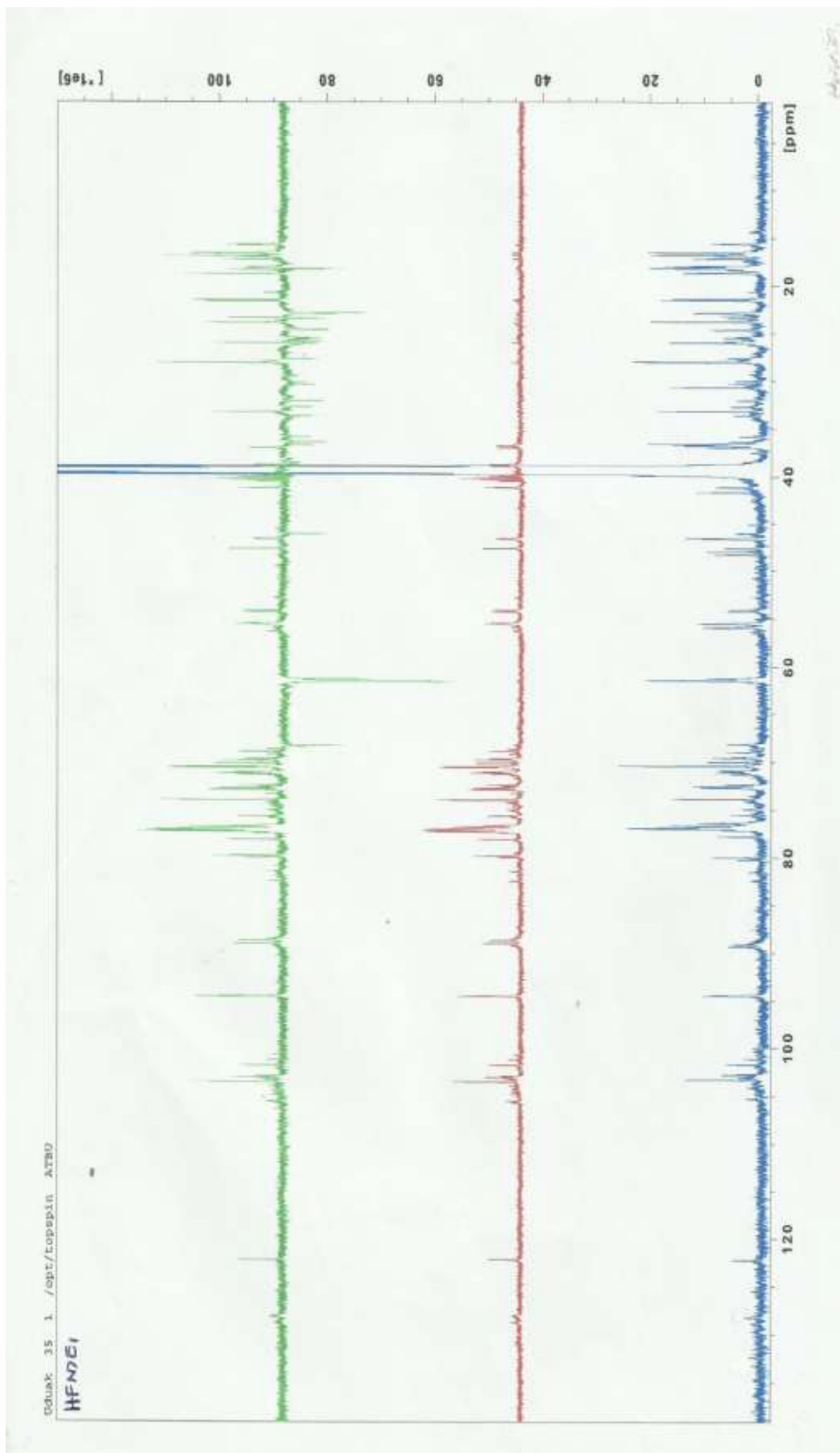
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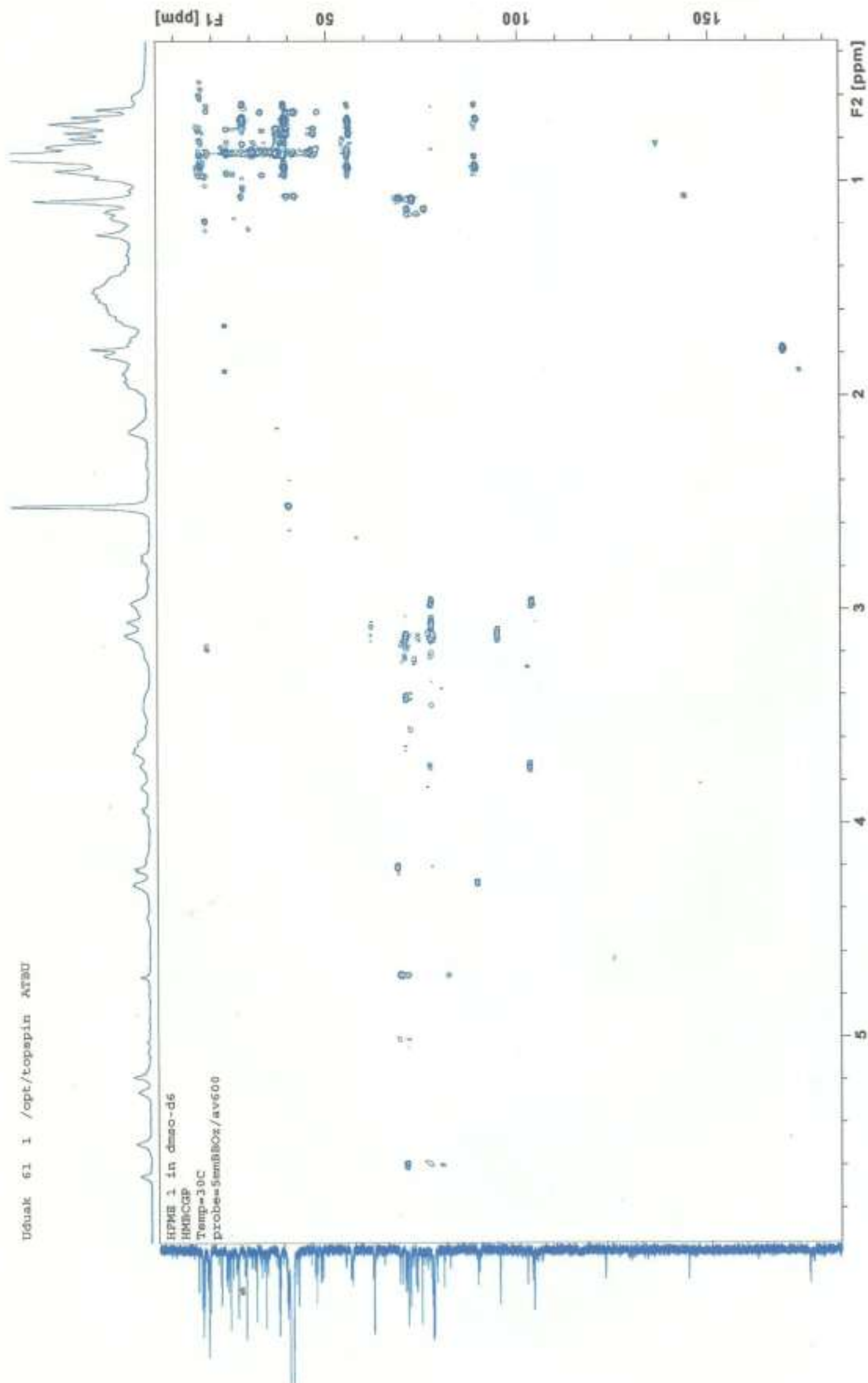
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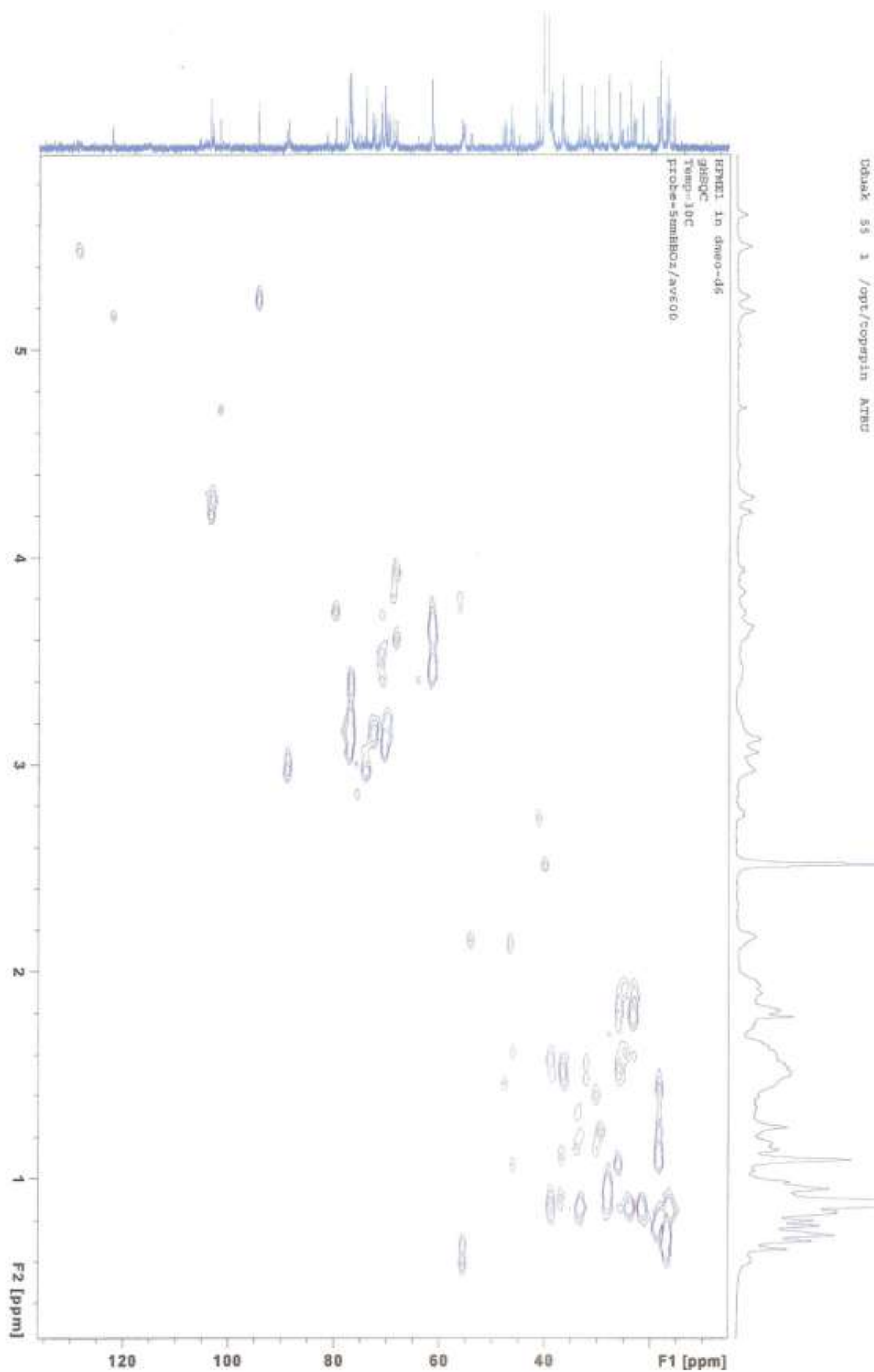
F2 [ppm]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120
100
80
60
40
F2 [ppm]
5
4
3
2
1

Compound HFME1 was obtained as the first column fraction (R_f 0.883) of hexane fraction of the methanol crude extract of the plant part. Compound HFME1 was isolated as a white amorphous powder with a melting point of 201-210 ^{0}C.

A study of the IR spectrum of the compound showed absorption bands at 3047 cm^{-1} (–OH), 2926 cm^{-1} (C-H stretching), 1704 cm^{-1} (C=O stretching), 1625 cm^{-1} (C=C stretching), 1228 cm^{-1} (C-O stretching) and 752 cm^{-1} (C-H bending). The glycosidic nature of the compound is evidenced by the broad band at 3047 cm^{-1} (Khan *et al.*, 2010). The C=C absorption band and the C-H bending band at 752 cm^{-1} are observed characteristic absorption bands of olefinic bond of a pentacyclic triterpenoid saponins.

The structural elucidation of compound HFME1 was achieved through the spectra study of 1D (^{1}H, ^{13}C, DEPT) and 2D (COSY, HSQC and HMBC) NMR experiments of the compound. Comparison of the spectral information with literature values showed the data of the compound to be in good agreement with spectra data of olean – 12- en- 28 - oic (Oleanolic) acid aglycone (Teng *et al.*, 2003, Seebacher *et al.*, 2003, Shah *et al.*, 2009), while the glycone portion was identified to be glucose as TLC analysis of acid hydrolysis sugar products revealed glucose as the only sugar.

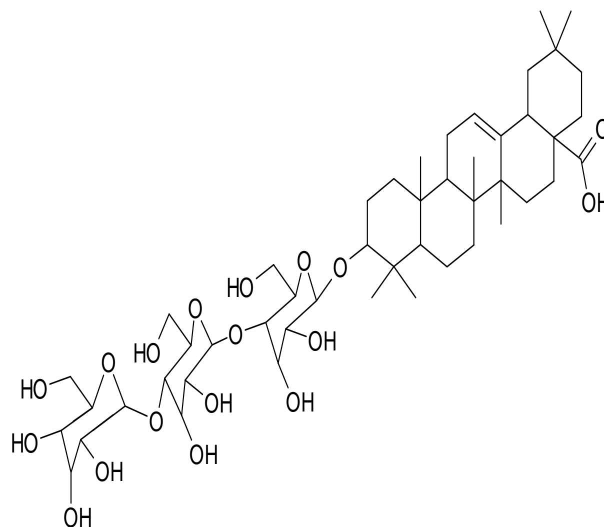
The ^{1}H NMR showed seven methyl singlet at δ1.12, 0.94, 0.89, 0.88, 0.84, 0.78, 0.67 and vinyl proton at δ5.46, suggesting that the aglycone is an olean-12-ene skeleton (Debella *et al.*, 2000).. Three anomeric proton signals were observed as doublet at δ5.25 (J = 6.48), 5.35 (J= 7.8) and 4.70 (J = 7.26) which suggests the presence of three sugar moieties (Zhoa, 2010).

The ^{13}C NMR spectra data showed C-3 signals of the compound at δ 89.2, which is a down field shift from δ 78.5 of oleanolic acid's C-3 (Güvenalp *et al.*, 2006) indicating glycosylation at the point (Alvarez *et al.*, 2003, Mehta *et al.*, 2004). The glycosylation at this point was further confirmed by the HMBC experiment which showed correlation of the anomeric proton C_1' with C-3 of the aglycone as the only glycosidic linkage with the aglycone, suggesting the compound to be monodesmosidic (Sun *et al.*, 2006, Kareru *et al.*, 2008 and Mohammad *et al.*, 2012).

The C-12 and C-13 chemical shifts at δ 123.6 and δ 142.9 respectively suggest the presence of olefinic carbons (Mehta *et al*, 2004, Xu *et al.*, 2010), and as suggested by the C=C absorption band on IR spectrum. This confirms the aglycone to be of olean-12-ene skeleton and therefore olean-12-en-28-oic acid.

The presence of three sugar moieties is evidenced in the observed signals at δ103.21, 101.78 and 96.35 for anomeric carbons (Mehta *et al.*, 2004, Zhoa, 2010) corroborating the anomeric proton signals on the ^{1}H spectrum. The sequencing of the sugar units and the attachment to the aglycone was confirmed by the HMBC experiment (Teng *et al.*, 2003, Shah *et al.*, 2009, Li *et al.* 2010).

Thus, on the basis of the chemical and spectroscopic analyses, compound HFME1 has been established to be a monodesmosidic trisaccharide triterpenoid saponin of olean-12-en-28-oic acid aglycone and has been identified as *3-O* – β- D- glucopyranosyl – (1-4)- β- D- glucopyranosyl – (1-4)- β- D- glucopyranosyl- olean -12-en-28-oic acid ($C_{48}H_{78}O_{18}$).



Compound HFME1 (*3-O* – β- D- glucopyranosyl – (1-4)- β- D- glucopyranosyl – (1-4)- β- D- glucopyranosyl- olean -12-en-28-oic acid) Structure.

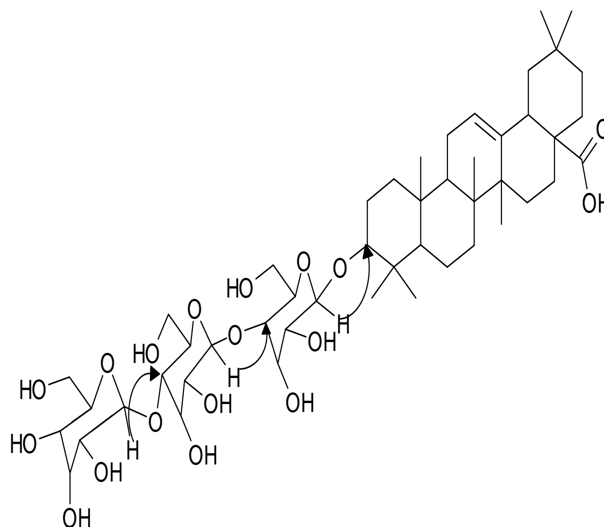


Figure 11: Key HMBC Correlation of Compound HFME1.

Antimicrobial Activities of the Isolated Compound

The isolated compound, HFME1 showed appreciable inhibitory activity against the tested organism, *Salmonella spp, Staphylococcus aureus*, *Escherichia coli*, *Aspergillus nigger* and *Candida albicans*. However,

it exhibited higher antifungal activity than antibacterial. The higher antifungal activities of the compounds is as also observed by Barile *et al*. (2007) for five saponins isolated from *Allium minutiflorum* which show significant antifungal activity with Minutoside B and C, having activities that may be comparable with that of common natural antibiotics and synthetic fungicides. The high activity against fungi is as expected as Garai (2014) has pointed that saponins exhibit high toxicity against fungi because of their ability to complex with sterols and cause membrane permeabilisation in fungi.

The antimicrobial activity of the isolated compound clearly shows that the compound is bioactive in nature and its presence in the plant, among other chemical substances, is evidence that *Massularia acuminata* as a medicinal plant.

Antioxidant properties of the isolated compound
The result of the free radical scavenging assay showed the isolated compound to be comparatively a good free radical scavenging agent. Its scavenging ability of 73.09% at 100μg/ml (IC_{50} - 50.06μg/ml) makes it a good antioxidant as the result is comparable with that of the common antioxidant standard, ascorbic acid reported by Khalaf *et al*. (2008).

Antioxidant activity is one of the properties of triterpenoid saponins (Man *et al*., 2010 and Negi *et al*., 2013). This has been shown by Elekofehinti *et al*. (2012) in their work which showed saponins from *Solanum anguivic* fruits to exhibit antioxidant and anti peroxidative properties in Wister rats. The free radical scavenging ability of the compound can make the compound to be relevant in stress management, cosmetic industry and in checking rancidity/lipid peroxidation.

CONCLUSION

Compound HFME1 has been isolated from methanol extract of the root bark of *Massularia acuminata* as a white amorphous powder. The compound has been identified to be a triterpenoid saponin and established to be *3-O* – β- D- glucopyranosyl – (1-4)- β- D-glucopyranosyl – (1-4)- β- D- glucopyranosyl- olean -12-en-28-oic acid. The compound is bioactive as it has a good inhibitory activity against *Salmonella spp, Staphylococcus aureus*, *Escherichia coli*, *Aspergillus nigger* and *Candida albicans*. It has been found to possess a comparatively good free radical scavenging ability and therefore possesses antioxidant property.

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