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UV, IR and NMR Characterization of a novel pentasaccharide Isolated from the Root Bark of *Phyllanthus muellerianus* (Kuntze) Excell (*EUPHORBIACEAE*)

Okolo Ephraim Ayegba*[a], Pateh Umar Usman[b], Sule Mohammed Ibrahim[b] and Odiba O. Jephthah[b]

[a]*The Department of Chemistry, Kogi State University, Anyigba, Nigeria*
[b]*The Department of Pharmaceutical and Medicinal Chemistry, Ahmadu Bello University, Zaria, Nigeria*

ABSTRACT

The methanol extract of the root bark of Phyllanthus muellerianus was phytochemically investigated using various chromatographic techniques. This led to the isolation of a novel pentasaccharide (PA), α-D-Glc$_p$-(2→6)-[α-D-Fru$_f$(1→2)]α-D-Fru$_f$-(1→4)β-D-Glc$_p$-(2→2)β-D-Fru$_f$. Complete NMR analysis using ^{1}H NMR, ^{13}C NMR, DEPT, COSY, HSQC, HMBC and NOESY was carried out to unequivocally resolve their structures. The UV and IR analyses were also carried out. Phyllose is the trivial name proposed for the pentasaccharide.

Keywords: Methanol extract, root bark, chromatographic techniques, isolation, NMR analysis, UV and IR analyses, pentasaccharide.

INTRODUCTION

From theoretical and practical standpoints, carbohydrates as a group are compounds that are generally considered to be of highest importance and interest. They are the products of photosynthesis – a process that sustains all life forms on earth. Carbohydrates are a great source of energy either as food for man and animals or after geological transformations as coal and peat. Their chemistry is very rich, especially their diverse stereochemical forms. [1]

They have been widely isolated from plants. Gentianose is a trisaccharide isolated from the root of the yellow gentian while stachyose is a tetrasaccharide from the manna of the ash tree and is found in the seeds of many leguminous plants. [1] More recently, novel oligosaccharides isolated from plants are: stellariose, a pentasaccharide from *Stellaria media*; [2] and a heptasaccharide and an octasaccharide isolated from *Paris polyphylla*. [3]

There is abundant literature concerning the biological activity of natural polysaccharides and oligosaccharides. ASLP is a polysaccharide isolated from a marine source that is known to affect the immune system. It does this by increasing the proliferation of splenocyte. Oligosaccharides with antioxidant, antitumor and immune-stimulation activities are known. They are obtained from fungi, land plants or algae and could contain only neutral sugars such as laminari-oligosaccharides corresponding to β-(1→3)-D-glucans. [4], [5] Fondaparinux the first synthetic pentasaccharide anticoagulant was derived from a natural polysaccharide, heparin. [6], [7]

The pharmacological actions of *Phyllanthus sp.* are numerous including analgesic and anti-inflammatory actions among others. [8] Additionally the antimicrobial properties of the extracts of different parts of *Phyllanthus* species

are widely published. *Phyllanthus muellerianus* stem bark extracts using methanol and water as solvents have been found to have activity against *Clostridium sporogenes*, *Streptococcus mutans*, and *Streptococcus pyogenes*. The traditional use against wound infections and tetanus is validated by its activity against *Streptococcus pyogenes* and *Clostridium sporogenes*. [9] A number of *Phyllanthus sp.* has been phytochemically investigated and many compounds have been isolated and identified. These compounds include alkaloids, flavonoids, terpenoids, steroids, lipids and sugars. Sugars isolated from *Phyllanthus sellowianus* are galactose, glucose, laevulose and saccharose. [8] With respect to the subject matter here, a novel pentasaccharide has been isolated from the root bark of *Phyllanthus muellerianus*.

MATERIALS AND METHODS

2.1 *Materials*

All solvents used were of analytical grade; including methanol (Fluka Sigma Aldrich, Switzerland), n-butanol (Kermel, China), ethylacetate (Qualikems, India), chloroform (Kermel, China) and distilled water obtained from the Department of Pharmaceutics and Pharmaceutical Microbiology, Ahmadu Bello University, Zaria, Nigeria. TLC plates (aluminium), silica gel (60-120 mesh), sephadex, chromatographic tanks and columns were used as chromatographic materials. The UV and IR analysis of the isolated compounds were performed on the thermoelectron UV machine at the Department of Pharmaceutical and Medicinal Chemistry, Ahmadu Bello University, Zaria, Nigeria; and Shimadzu FTIR-8400S spectrophotometer at the National Research Institute for Chemical Technology, Zaria respectively. The NMR spectra were obtained using Bruker AVANCE III NMR spectrometer at the School of Chemistry and Physics, University of Kwa-Zulu Natal, Durban, South Africa.

2.2 *Methods*

2.2.1 *Collection and identification of plant material*

The root of *Phyllanthus muellerianus* was collected in December, 2012 from Agojeju, Ejule in Kogi State, Nigeria. It was confirmed and authenticated by U.S. Gallah of the Herbarium Section of the Department of Biological Sciences, Ahmadu Bello University, Zaria by comparing it with a standard specimen with voucher number 900351.

2.2.2 *Extraction and partitioning*

The root bark of the plant was dried, powdered and extracted with methanol using the maceration method. It was then partitioned in distilled water using n-butanol to afford the partitioned n-butanol fraction of the methanol extract.

2.2.3 *Thin layer chromatography of n-butanol fraction*

Pre-coated TLC plates were used to carry out thin layer chromatography by the one way ascending technique. Capillary tubes were used to manually apply spots and the chromatograms were developed in air-tight chromatographic tanks at room temperature, employing different solvent systems. The spots were visualized after been sprayed with 10% sulphuric acid followed by heating in an oven. One of the solvent systems used is ethyl acetate-methanol-water (10:5:1).

2.2.4 *Column chromatography of n-butanol fraction*

The column was packed using the wet slurry method. Cotton wool was placed at the bottom end of the column and solvent was added. The tap was then opened to be sure it runs. Silica gel was mixed with the solvent and stirred thoroughly after which it was packed into the column and allowed to settle. Cotton wool was also placed on the bed of the silica gel. The n-butanol fraction was dissolved in methanol, and silica gel was added to form a powdered mixture which was allowed to dry. The dry mixture was then packed on the cotton wool on the silica gel bed.

The chromatograph was gradiently eluted starting from chloroform (100%) through ethylacetate (100%) to ethyl acetate-methanol (85:15) before the collection of the target compounds in a less complicated mixture, as monitored by thin layer chromatography. Different column fractions collected were pooled together into six major combinations depending on the similarity of their TLC profiles. The six different combinations of the fractions are: 31-33, 34-35, 36-41, 42-44, 45-48, and 49-60.

2.2.5 Isolation of PA

Column fractions 36-41 was mounted on a small column packed with sephadex and then eluted with methanol. This gave rise to sephadex fractions SE1-SE14 of which SE3-14 were pooled together. Column fractions 42-44 was also mounted on a small column packed with sephadex and then eluted with methanol. This gave rise to sephadex fractions S1-S8.

The sephadex fractions SE3-14 and S8 were separately subjected to preparative thin layer chromatography using a pre-coated TLC plate. The plates were developed using ethyl acetate-methanol-water (10:5:1) as solvent system. The bands containing the compounds of interest were scraped off and washed repeatedly with methanol to afford a brown compound. The respective compounds from the preparative TLC were subjected to co-TLC and their R_f values were found to be the same, hence, they were merged to afford compound PA. The spot of the pure compound was developed in different solvent systems to ascertain its purity. The melting point of PA was then taken using an electrothermal apparatus. The isolated compound was subjected to UV, FTIR, 1D- and 2D-NMR spectroscopy.

RESULTS AND DISCUSSION

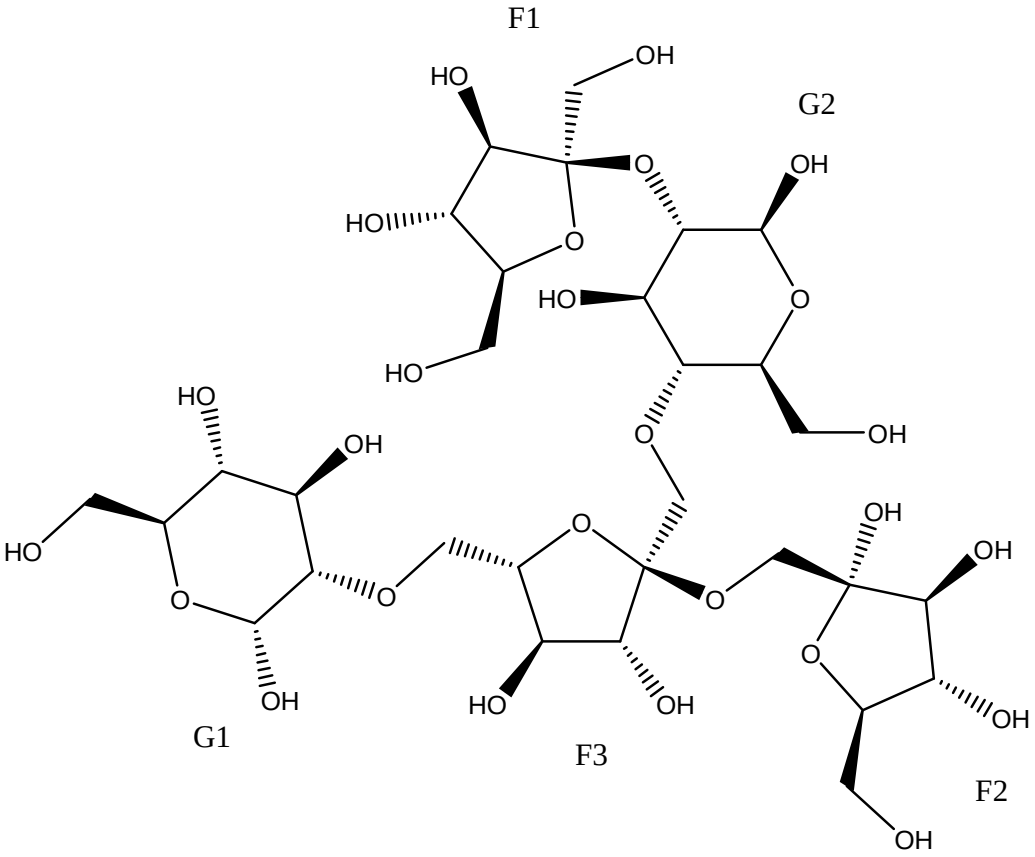
Table 1.0

^{1}H, ^{13}C, HSQC and DEPT NMR Spectral Data of the Pentasaccharide PA

α-D-Glucopyranose G1				β-D-Glucopyranose G2				β-D-Fructofuranose F1			
S/No	δ_C	δ_H	DEPT	S/No	δ_C	δ_H	DEPT	S/No	δ_C	δ_H	DEPT
1	94.0	5.14	CH	1	98.2	4.50	CH	1	64.6	3.60	CH_2
2	73.0	3.37	CH	2	76.3	3.16	CH	2	99.2	-	C
3	73.9	3.81	CH	3	77.5	3.99	CH	3	78.0	3.32	CH
4	69.4	3.78	CH	4	76.8	4.08	CH	4	71.2	3.82	CH
5	71.7	3.82	CH	5	71.9	3.84	CH	5	83.2	3.98	CH
6	62.6	3.69	CH_2	6	62.8	3.80	CH_2	6	62.9	3.73	CH_2
α-D-Fructofuranose F2				α-D-Fructofuranose F3							
S/No	δ_C	δ_H	DEPT	S/No	δ_C	δ_H	DEPT				
1	65.1	3.60	CH_2	1	65.9	3.50, 3.68	CH_2				
2	103.2	-	C	2	105.9	-	C				
3	78.0	3.32	CH	3	78.1	3.32	CH				
4	71.9	3.82	CH	4	74.9	3.70	CH				
5	83.3	3.98	CH	5	84.2	4.03	CH				
6	64.2	3.73	CH_2	6	64.5	3.53, 3.65	CH_2				

The TLC chromatograms of the pure compounds were developed in ethyl acetate-methanol-water (EtOAc:MeOH:H_2O – 10:5:1), ethyl acetate-chloroform-methanol-water (EtOAc:$CHCl_3$:MeOH:H_2O – 10:2:5:1) and the upper layer of n-butanol-acetic acid-water (n-BuOH:AcOH:H_2O – 4:1:5). The melting point of PA was determined to be 158-160 ^{0}C.

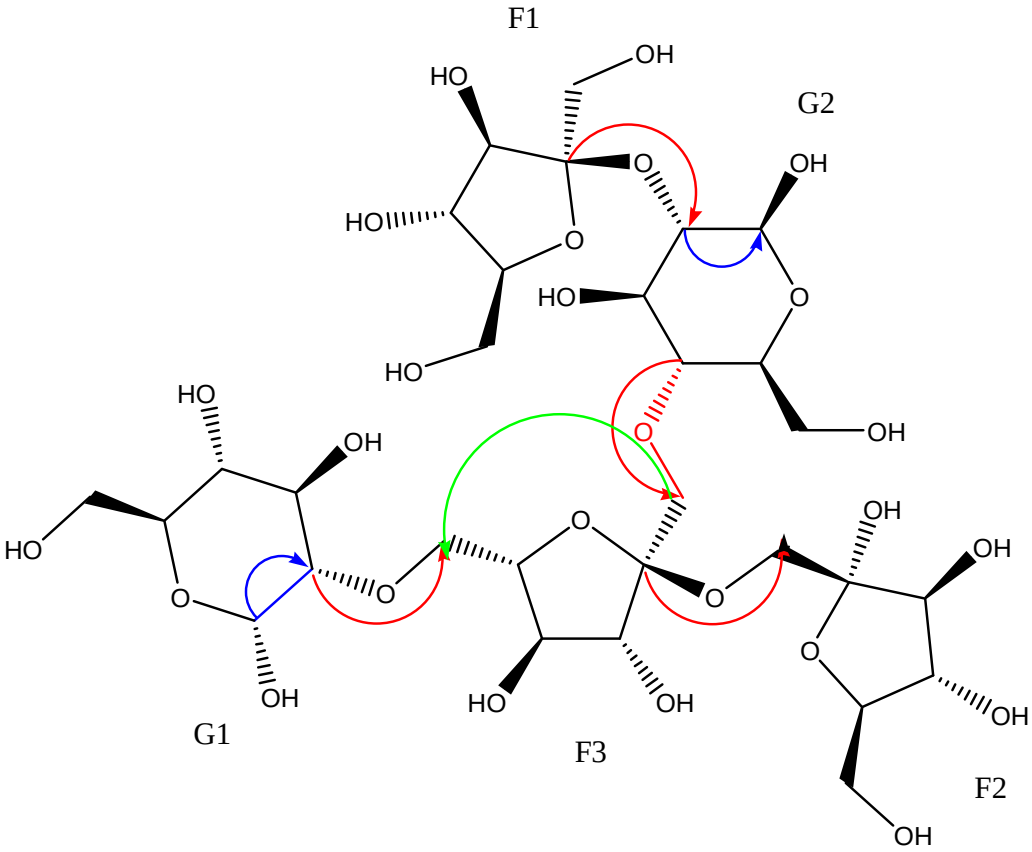
The UV spectrum of PA indicates 214 nm as the wavelength of maximum absorption. This is as a result of $n \rightarrow \sigma^*$ transitions. The IR values at 3391 cm^{-1}, 2928 cm^{-1} and 1066 cm^{-1} represent O-H, C-H and C-O stretching vibrations respectively. The proton NMR of PA is characteristic of sugars. The peaks at 5.14 ppm and 4.50 ppm are characteristic of glucose while the oxymethine and oxymethylene peaks are found between 3.00 ppm and 4.00 ppm. Thirty peaks appear in the ^{13}C NMR spectrum within the range of sp^3 hybridized carbon atoms attached to highly electronegative elements (δ_C 62.6-105.9 ppm) such as oxygen. Five of these carbon peaks are anomeric corresponding to an α-D-glucopyranose G1 (anomeric δ_C 94.0 ppm), a β-D-glucopyranose G2 (anomeric δ_C 98.2 ppm), a β-D-fructofuranose F1 (anomeric δ_C 99.2 ppm) and two α -D-fructofuranose units, F2 (anomeric δ_C 103.2 ppm) and F3 (anomeric δ_C 105.9 ppm) respectively. [10], [11] The DEPT spectrum indicates the fact that among the 30 carbon atoms, 3 are quartenary (δ_C 99.2, 103.2 and 105.9 ppm), 19 are methine (δ_C 69.4-98.2 ppm) while 8 are methylene (δ_C 62.6-65.9 ppm) carbon atoms. The HSQC correlations indicate the hydrogen atoms that are bonded to their respective carbon atoms and their chemical shifts. The HMBC spectrum unravels the way the sugar molecules are linked: C-2 of F3 is correlated to C-1 of F2, C-4 of G2 is correlated to C-1 of F3, C-2 of F1 is correlated to C-2 of G2, and C-2 of G-1 is correlated to C-6 of F3. Based on all these data, the proposed structure of PA is:

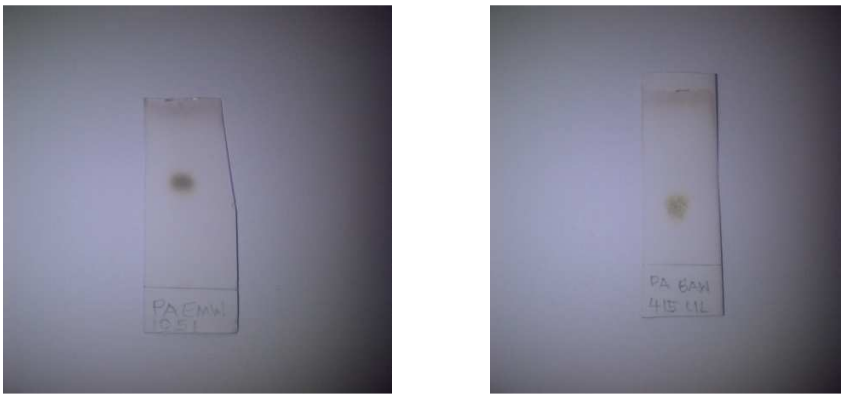


α-D-Glc$_p$-(2→6)-[α-D-Fru$_f$(1→2)]α-D-Fru$_f$-(1→4)β-D-Glc$_p$-(2→2)β-D-Fru$_f$

The COSY correlations: H-1, H-2 of G1; H-1, H-2 of G-2; H-5, H-6 of F3 and the NOESY correlation H-1, H-6 of F3 support the proposed structure.

Some of the HMBC, COSY and NOESY correlations are depicted below.





EtOAc:MeOH:H_2O – 10:5:1
R_f = 0.53

n-BuOH:AcOH:H_2O – 4:1:5 (Upper layer),
R_f = 0.30

Figure 1 Thin layer chromatograms of the pentasaccharide (PA)

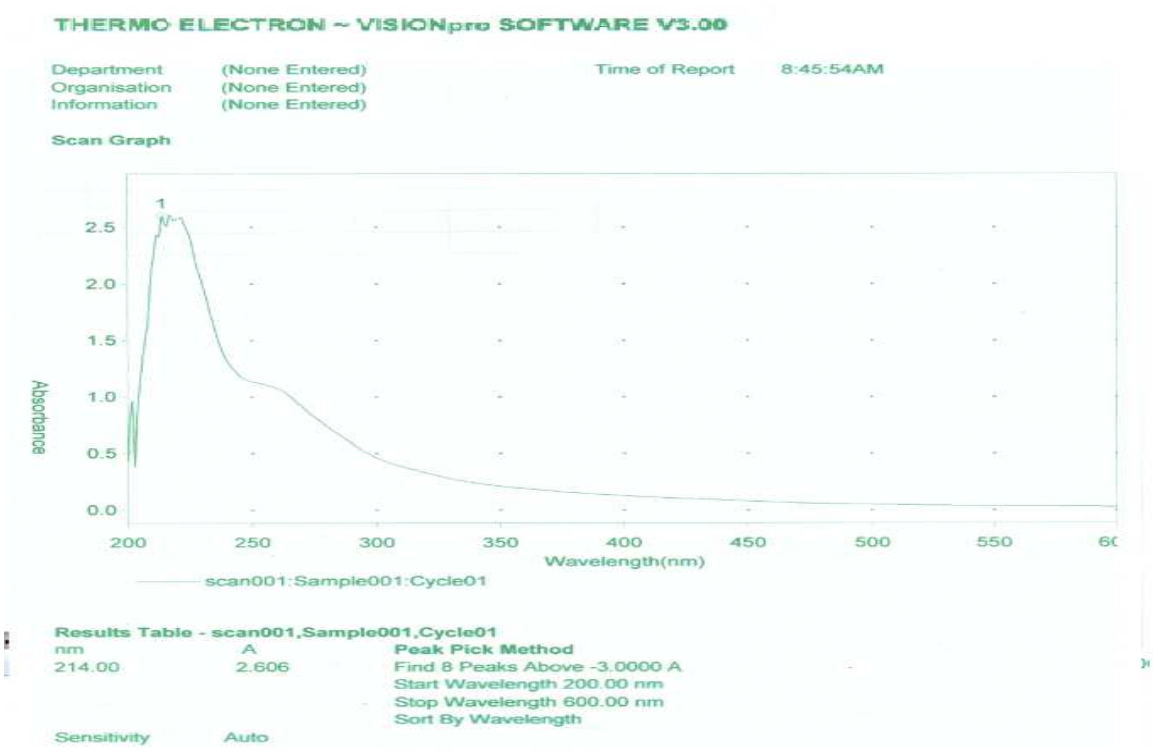
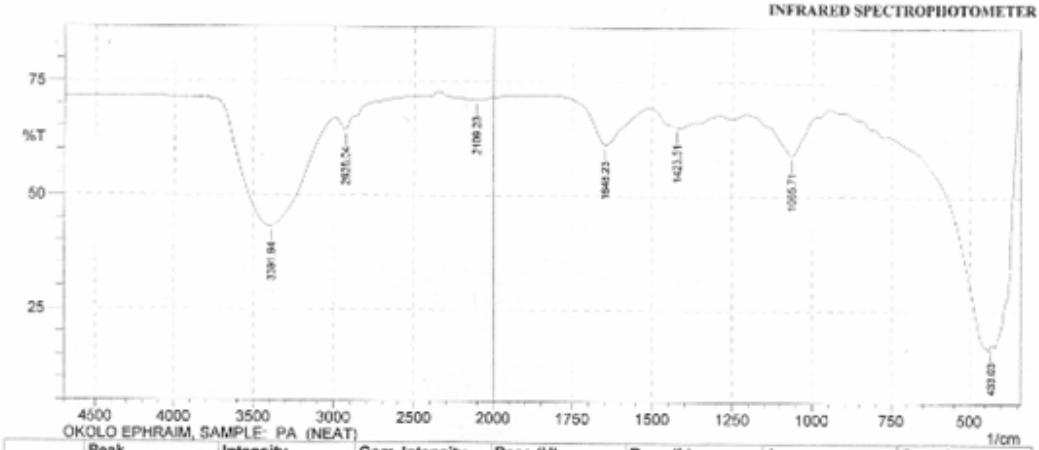


Figure 2 UV spectrum of PA

SHIMADZU

FTIR ANALYSIS RESULT NARICT,ZARIA

FTIR- 8400S FOURIER TRANSFORM INFRARED SPECTROPHOTOMETER



OKOLO EPHRAIM, SAMPLE: PA (NEAT)

	Peak	Intensity	Corr. Intensity	Base (H)	Base (L)	Area	Corr. Area
1	433.03	17.83	0.09	434	431.1	2.16	0.01
2	1065.71	59.1	8.78	1206.51	986.62	42.25	5.34
3	1423.51	85.21	0.04	1439.91	1422.55	3.19	0
4	1648.23	61.39	0.13	1708.02	1647.26	11.4	0.12
5	2109.23	71.1	0	2110.19	2088.01	3.28	0
6	2928.04	64.26	2.97	2990.73	2874.03	21.16	1.03
7	3391.94	43.2	25.44	3693.81	2990.73	192.05	76.24

Comment;
OKOLO EPHRAIM, SAMPLE: PA (NEAT)

User; Administrator

No. of Scans;
Date/Time; 5/13/2013 12:00:12 PM
Resolution;

Figure 3 IR spectrum of PA

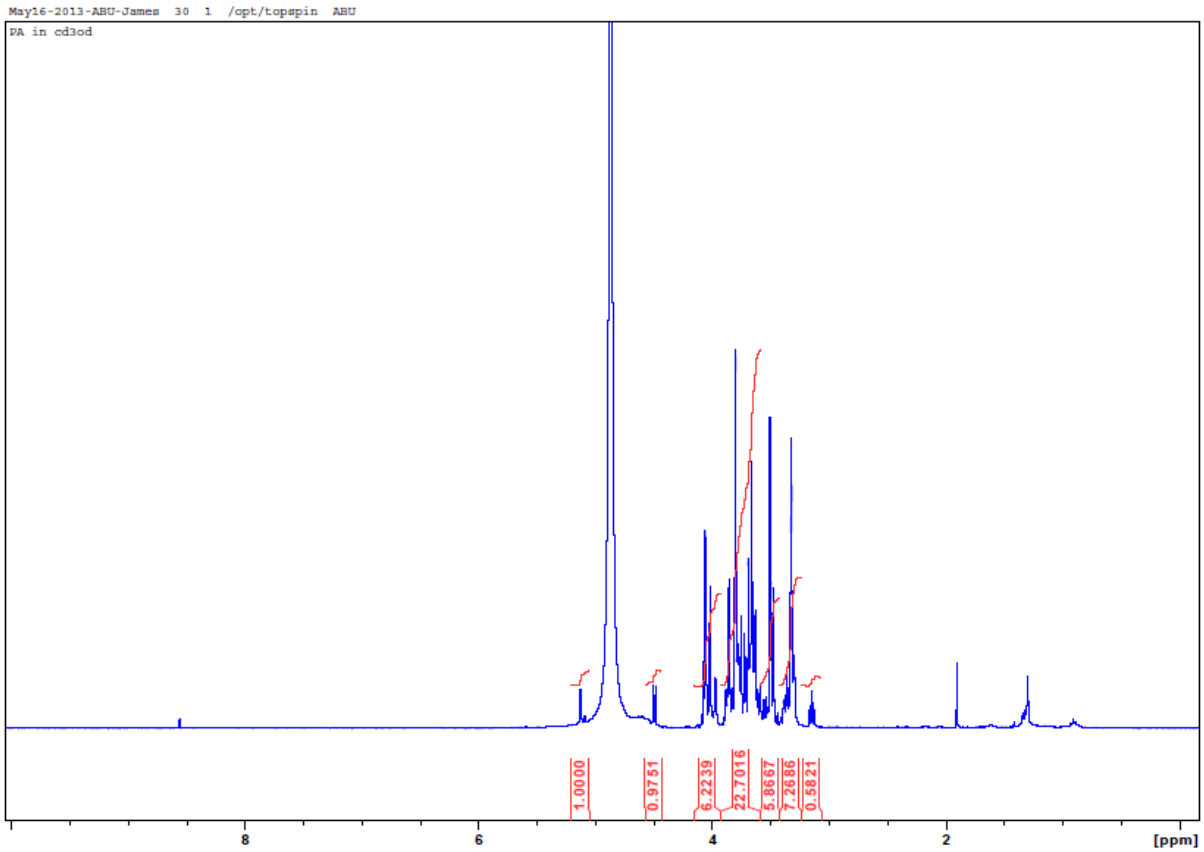


Figure 4 Proton NMR spectrum of PA

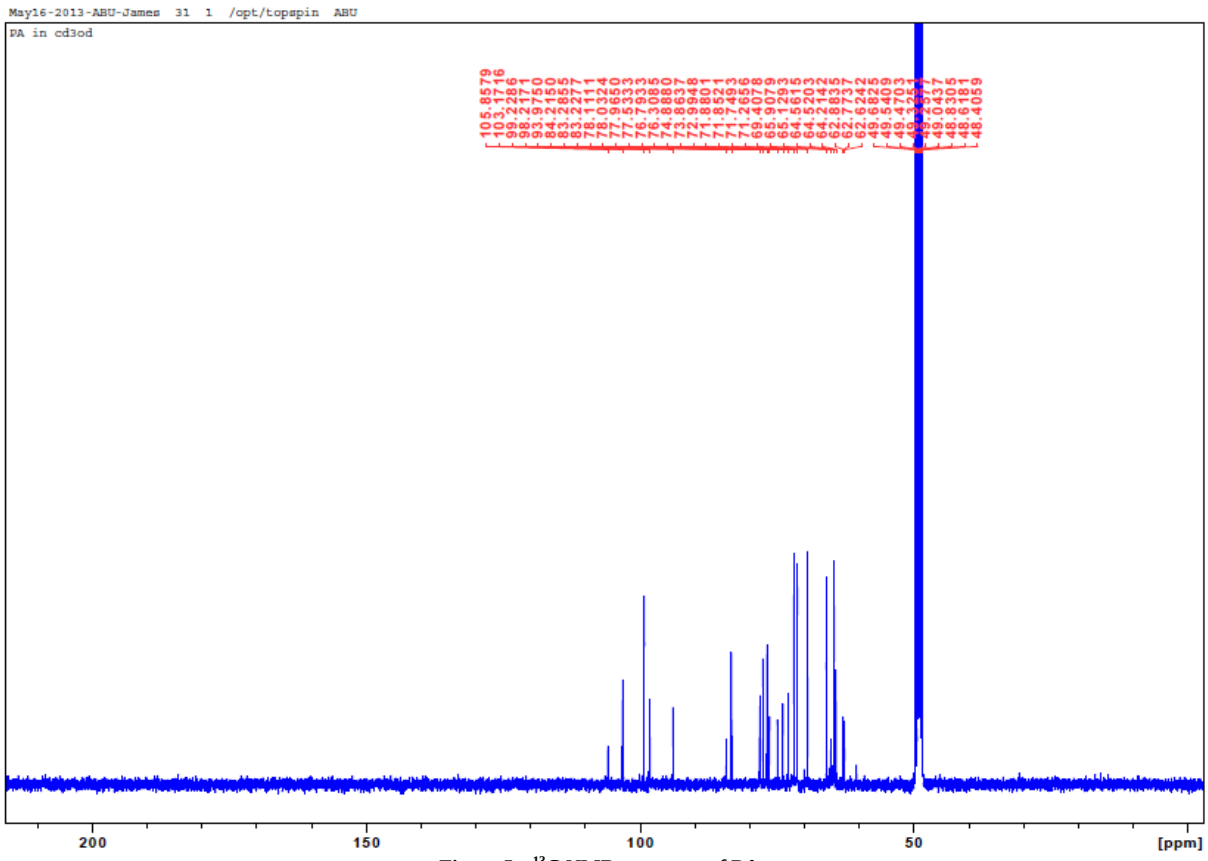


Figure 5 ^{13}C NMR spectrum of PA

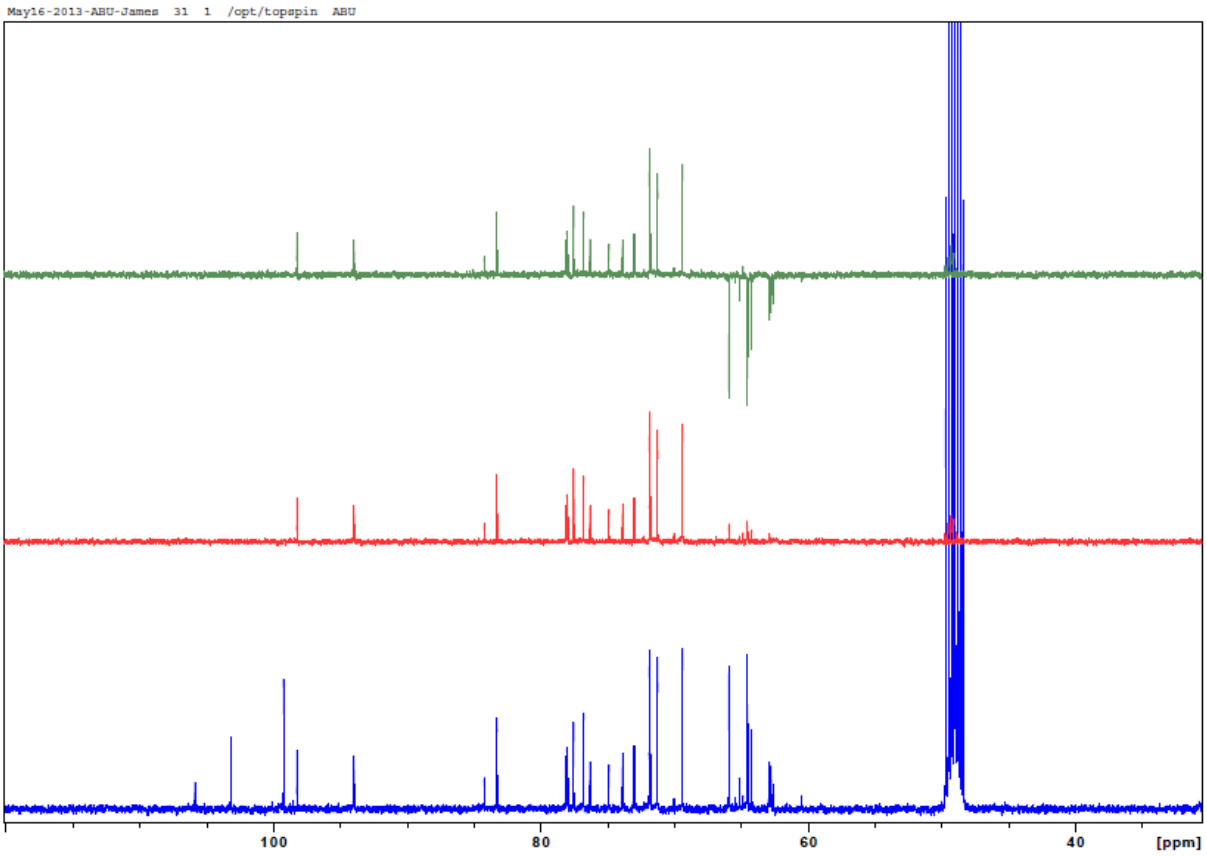


Figure 6 DEPT NMR spectrum of PA

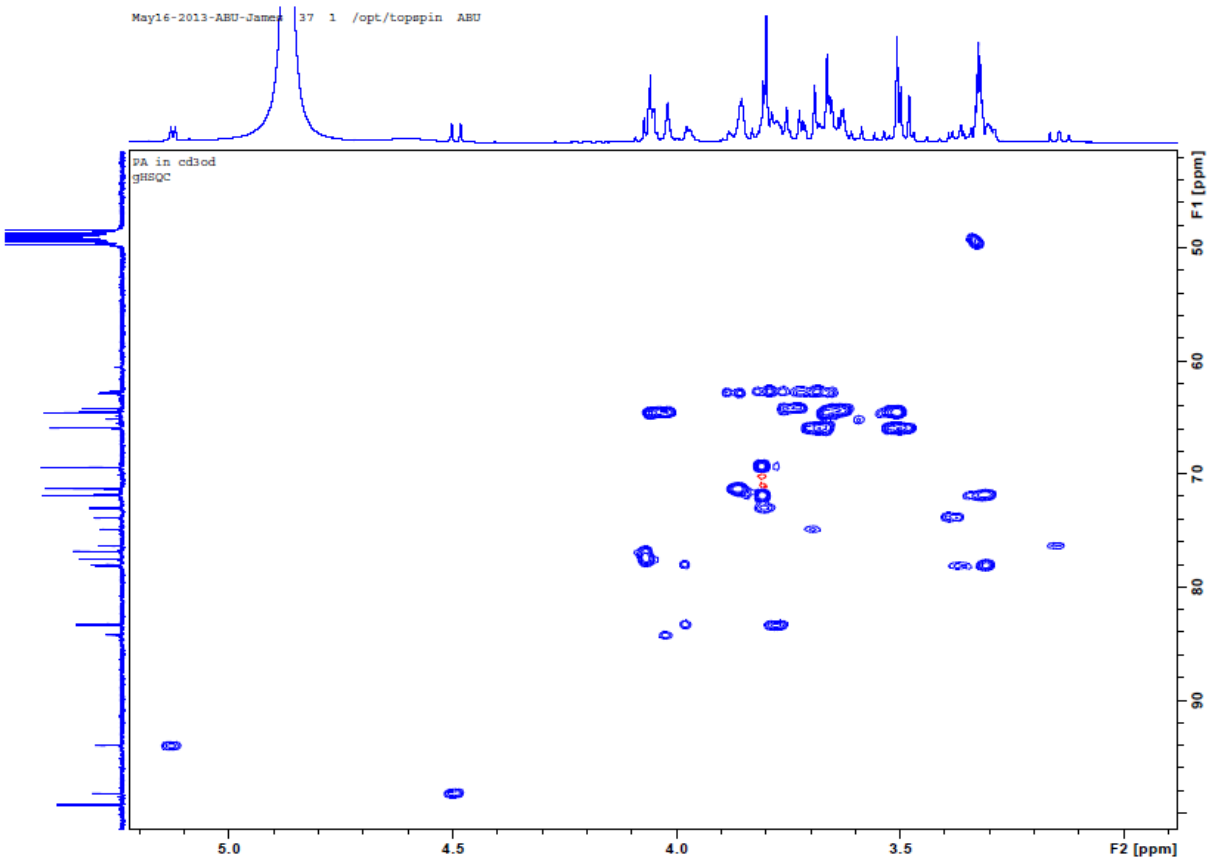


Figure 7 HSQC NMR spectrum of PA

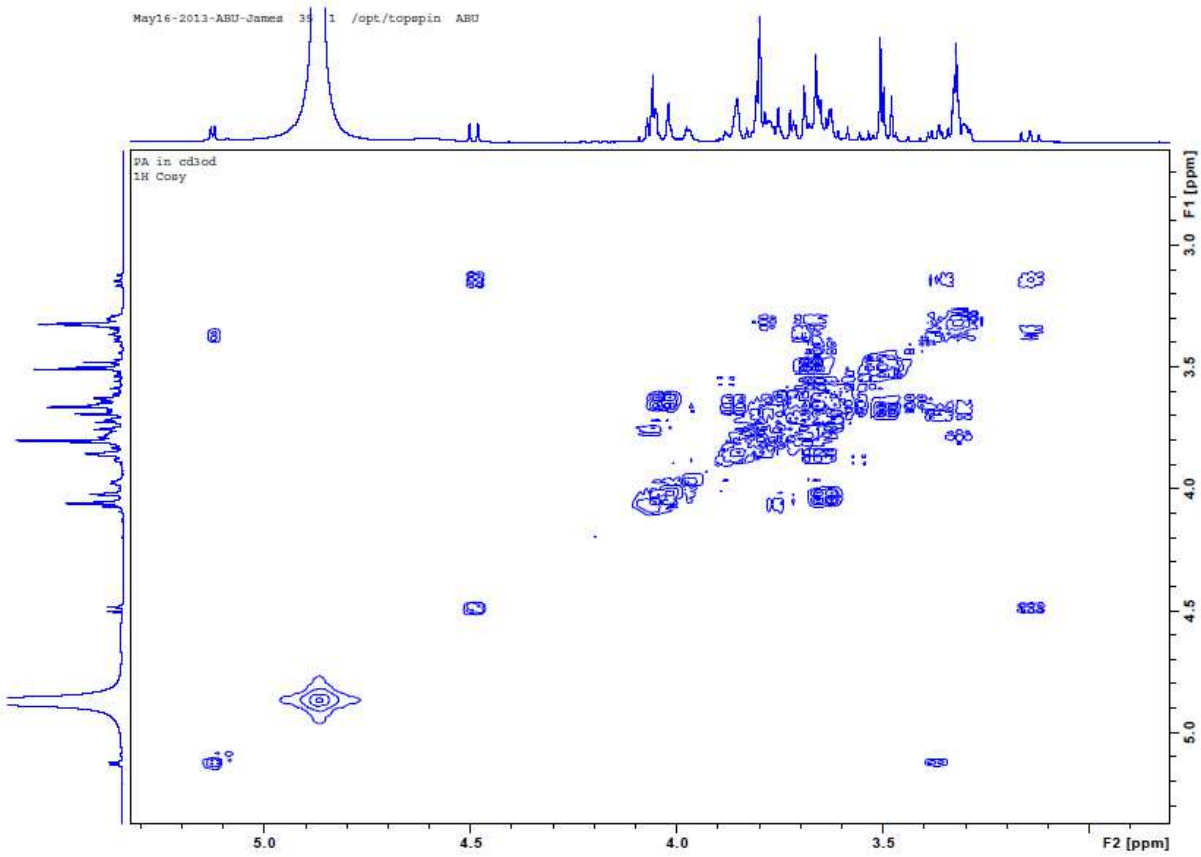


Figure 8 COSY NMR spectrum of PA

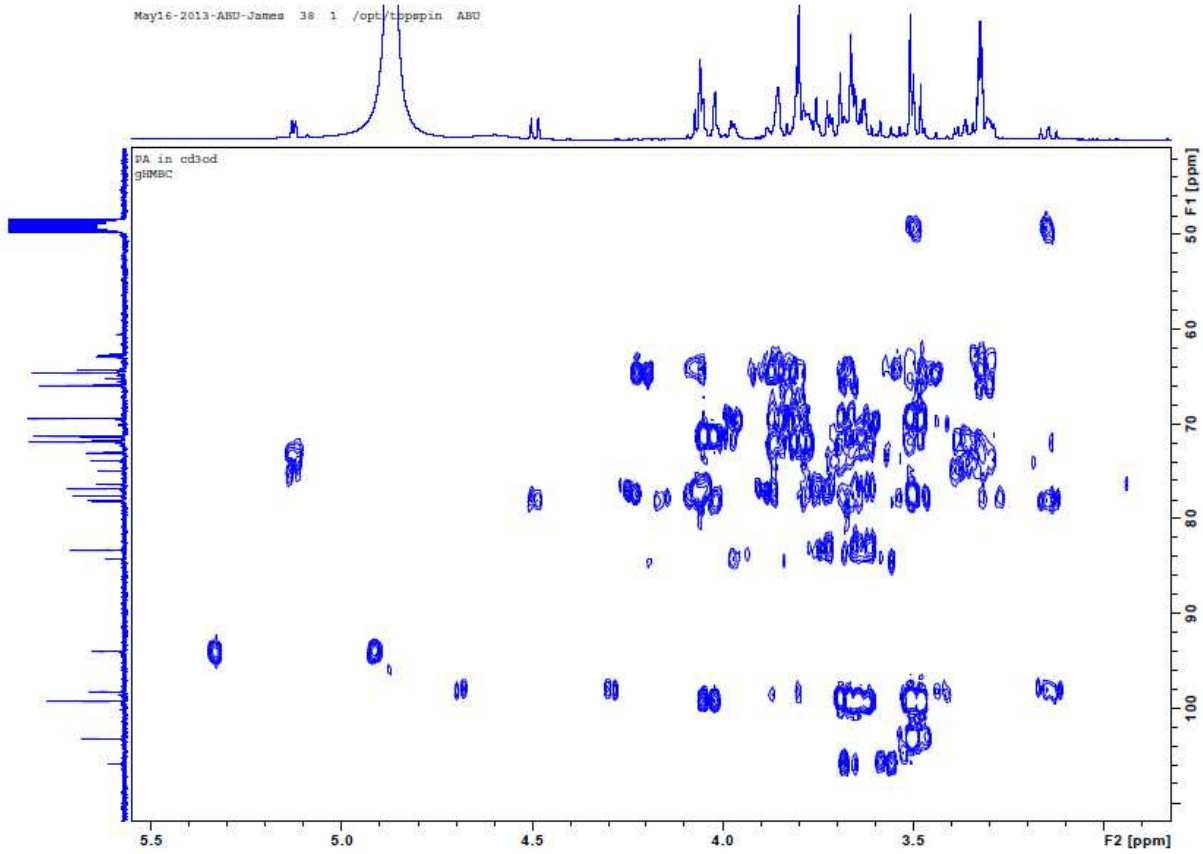


Figure 9 HMBC NMR spectrum of PA

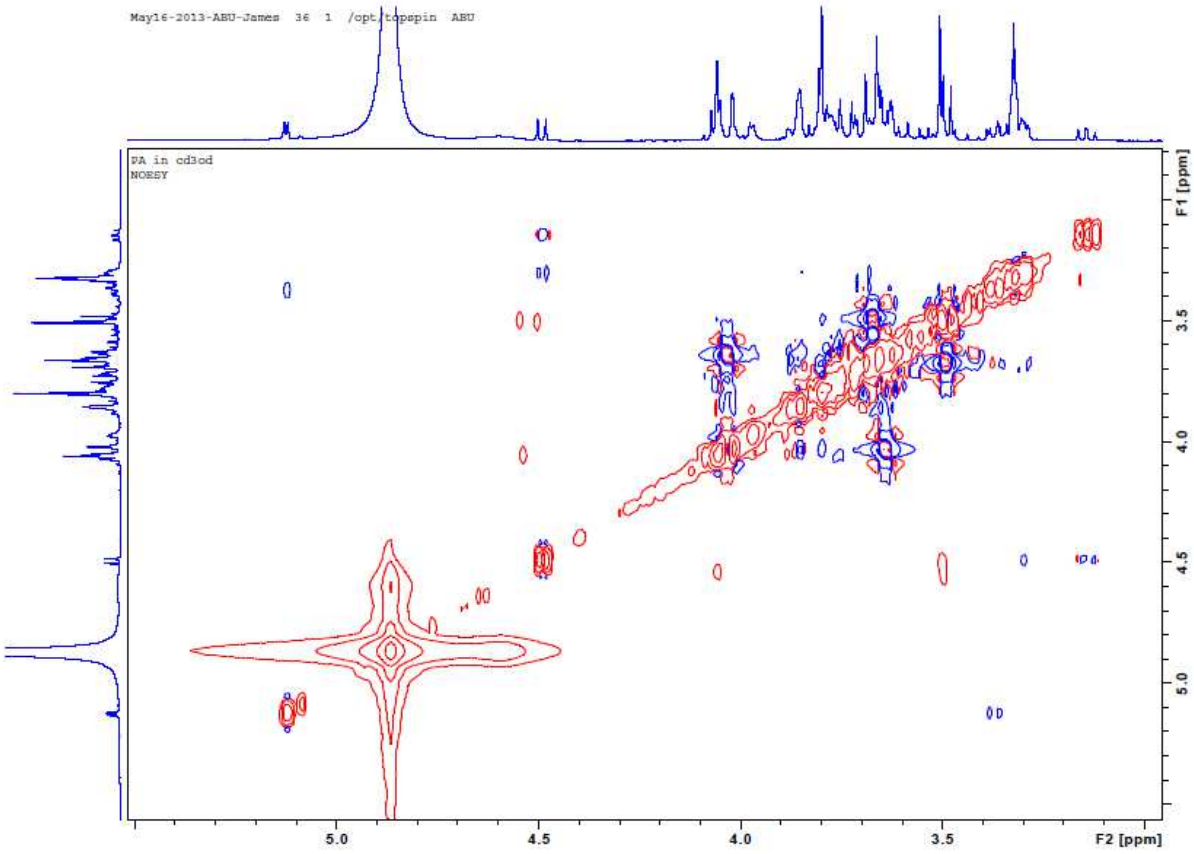


Figure 10 NOESY NMR spectrum of PA

CONCLUSION

Phyllanthus muellerianus is distributed in secondary forests from Guinea-Bissau, Mali, West Cameroons and other parts of tropical Africa. It is a shrub or climber, deciduous and occasionally arborescent. It rarely develops a large stem. [12]

The n-butanol fraction of the methanol extract of the root bark of this plant is sugar-containing and this led to the isolation of a new pentasaccharide. Considering the fact that galactose, glucose, levulose (fructose) and saccharose (sucrose) have been isolated from *Phyllanthus sellowianus*, [8] sugars could be considered to be chemotaxonomic markers of the *Phyllanthus* genus after an extensive study of the various species within the genus.

Acknowledgement
We register our sincere appreciation with Mallam Mohammed Sani Yahaya and U.S. Gallah. Mallam Sani gave vital technical advice in the course of the isolation while U.S. Gallah identified the plant.

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