Isolation of Pheophytin A and β-amyrin from *Newbouldia laevis* (P. Beauv) Leaf Extract

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Abstract. Pulverized leaves samples of *Newbouldia laevis* were extracted by cold maceration using methanol. The extract was concentrated in vacuo to yield a reddish brown solid of 120.191 g. The crude methanol extract was partitioned into n-hexane 0.1 g, dichloromethane 2.5 g, ethyl acetate 4.6 g, and methanol 10.0 g, fractions via coarse chromatography. Methanol fraction gave the highest yield and was subjected to further purification using repeated column chromatography to yield pure components, namely NLM24 (R_f 0.48), EAc:n-hex:MeOH (4:5:1) and NLM19 (R_f0.47), EAc:n-hex:MeOH, respectively. These pure fractions were subjected to ^{1}H NMR, ^{13}C, COSY, HSQC and HMBC spectroscopy. Pheophytin A and β-amyrin were proposed as the structures of the isolated compounds. Even though the pure fractions were not used for the analgesic activity, the literature reveals that pheophytin A & β-amyrin are potent analgesics.

Keywords: analgesic; *Newbouldia laevis;* pheophytin A; β-amyrin.

INTRODUCTION

Newbouldia laevis (P. Beauv.) is a common plant that is widely used in African traditional medicine [1], and its efficacy against specific health problems such as ulcers, pain, inflammation and microbial infections [2] has been reported and gained wider acceptance. In Nigeria, particularly in the South East, the plant is often used to construct barns for storage of farm produce such as yam, fences around houses and marking of boundaries. In Enugu State, specifically Amede Eha-Amufu and Amankanu, the plant is locally known as ojilishi and is often used to treat wounds.

Pheophytin A and β-amyrin have been naturally isolated from plants such as *Brachystelma togoense* Schltr, and *Protium heptaphyllum* [3, 4]. Pheophytin A was reported to possess numerous biological activities such as anti-cancer, antifungal and anti-inflammatory [3], while β-amyrin was found to have antihyperglycemic and hypolipidemic effects [4].

While studying the analgesic activity of *Newbouldia laevis* leaf extracts in white whisker albino rats, the current research isolated pheophytin A and β-amyrin from *Newbouldia laevis*.

METHODS

Materials for Extraction

A solvent distillation machine (PS/1598) is used to distil the solvents, and big glass containers are used for cold maceration.

Precoated microscopic slides were used for spotting; capillary tubes were used for finding; hot air oven ADARSH was used for charring and colour development. Long Big column (60 cm) & 7.0 diameters used for elution of different components; silica gel 60 (70–230 mesh ASTM) used for column chromatography; silica gel 60 (230–400 mesh ASTM) used for flash chromatography.

Spectrophotometer NMR-Bruker AV3-500 MHZ was used for the structure elucidation of isolated pure compounds.

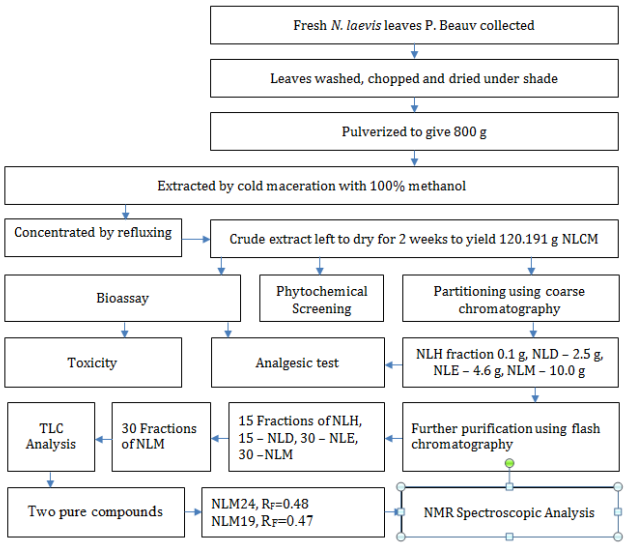
The reagents that were used are listed in Table 1.

Table 1 – Chemicals and Reagents

Reagent	Boiling point (°C)	Purity (%)	Suppliers
Ethyl acetate	77.1	Purity > 80	Sigma Aldrich
10 % Tetraoxosulphate (vi) acid	101	Purity > 90	M&B
Distilled water	100	Purity ≥ 100	Ecochem
Methanol	64.7	Purity < 100	Pubchem
Ammonia	-33.34	Purity ≥ 99.98	Pubchem
n-hexane	68.7	Purity 95–99	Sigma Aldrich
Hydrochloric acid	-85.05	Purity 35–38	Sigma Aldrich
Dil. Iron II chloride	1023	Purity > 98	Sigma Aldrich
(Pirovic acid) 2-oxopropanoic acid	165	Purity > 98	Sigma Aldrich
Acetic acid	118	Purity ≥ 99.9	Sigma Aldrich
Silica	2230	Purity ≥ 99.99	EMD
Aspirin	140	Purity > 34.07	CSUN
Dichloromethane	39.6	Purity ≥ 99.9	Sigma-Aldrich

Summary of Experimental Procedure

Partitioning and Isolation of Chemical Components from Crude Extract. About 600 mg of the crude extract was used to pack the column to partition it into four different fractions. The detailed partitioning and isolation are shown in the flowchart below (Figure 1).



Notes: NLH – *Newbouldia laevis* n-hexane extract, NLD – *Newbouldia laevis* dichloromethane extract, NLE – *Newbouldia laevis* ethyl acetate extract, NLM – *Newbouldia laevis* methanol extract

Figure 1 – Summary of Experimental procedure

Flowchart of Purification using Flash Chromatography is as follows 10.0 g NLM → Flash column chromatography → Solvent mixtures (n-hex:EAc), ml → TLC Analysis → Medium (EAc:n-hex), ml → Further TLC analysis → NLM24, NLM19.

Collection and preparation of plant material

The leaves of the plant *Newbouldia laevis* were collected at Amede Eha-Amufu, Enugu State, on 8 November 2019. A Forester confirmed the leaves at the College of Natural Resources and Environmental Management, Michael Okpara University of Agriculture, Umudike, Abia State. The Habarum Number was identified as Daramola FHI 35500.

The leaves were properly washed and air dried. It was further grounded into powder, weighed, and found to be 800 g. It was soaked with distilled methanol, and after two weeks, it was filtered, and the filtrate was refluxed. Thus the solvent was recovered. The crude methanol extract was then kept to air dry. After one week, it was weighed and found to be 120.191 g. It was thus labelled MCNL.

Extraction. After the fresh leaves of *N. laevis* were collected and identified, it was washed, chopped and dried under shade. The dried leaves were thus pulverized to give 800 g. The 800 g was put in a big glass container, and 100 % of methanol was poured into the container to the brim. The container was thus covered and kept. After two weeks, the sample was filtered, the filtrate was refluxed, and the crude extract was kept to dry.

The residue was resoaked again in 100 % methanol, and one week later, it was filtered, and the filtrate was refluxed; thus, the crude extract was left to air dry. The dried crude extract was weighed to give 120.191 g. It was therefore labelled *Newbouldia laevis* crude methanol (NLCM).

This method of extraction is called cold maceration. The NLCM obtained was used for bioassay, phytochemical screening and fractionation.

Fractionation. The NLCM 120.191 g was thus partitioned via coarse chromatography to give different fractions as NLH fraction 0.1 g, NLD – 2.5 g, NLE – 4.6 g, and NLM – 10.0 g.

The abbreviations above will be explained later.

Column chromatography. The following procedure was used for the column chromatography: the large column (60 & 7.0 diameter) was hung on a retort stand, and the queue was rinsed with n-hexane. Cotton wool was soaked in the solvent to be used and pushed down the bottom of the column using a steel rod. A mixture of silica gel and poured inside the column. The extract of about (600 mg) was mixed very well with a small silica gel, and the mixture of the crude extract plus the n-hexane and silica gel (slurry) was poured inside the column immediately. About 100 ml of n-hexane was used to wash down the column's sides and fill it up. The solvent system introduced into the column was (n-hexl:EAC 90/10 ml). The labelled vials bottles were used to collect the eluate. This collection continued for the subsequent mixtures of the solvent system (n-hexl:EAC), ml: 80/20, 70/30, 60/40, 50/50, 40/60, 30/70, 20/80, 10/90, 0/100.

Methanol 100 ml, a more polar solvent, was used to wash off the more polar components remaining in the column.

Thin layer chromatography. This technique was used to isolate pure compounds from any fractions collected from column chromatography. Each fraction collected was spotted on a precoated Thin layer Chromatography (TLC) plate with a capillary tube. About four 250 ml beakers were used to develop the spot as it travels from the origin through the solvent front. An Aluminum foil was used to cover the beaker each before the spots travelled through the solvent front. The solvent mixture adopted for a good separation are: 8:2 ml, 7:3 ml (EAc:n-Hex) and 4:5:1 ml (EAc:n-hex:CH_3OH).

A spot was made on the plate, developed in the solvent front. The dish was brought out from the beaker, 10 % H_2SO_4 was sprayed on the scale, and charred inside a hot oven at 50 °C for colour visualization. The retention factor for each spot was calculated using the relation (1):

$$R_f = \frac{Distance\ moved\ by\ spot}{Distance\ moved\ by\ the\ solvent\ front} \quad (1)$$

At the end of the TLC, similar samples with the same spot were pooled together. Thus NLM24 & NLM19 had single marks each. This means that the fractions are pure; therefore, they were packaged and sent for spectral analysis.

Spectroscopic analysis. Spectroscopy studies the interactions between particles such as electrons, protons and ions, as well as their interaction with other particles as a function of their collision energy. To be more precise, spectroscopy is the study of absorption and emission of light and other radiation by matter, as related to the dependence of these processes on the wavelength of the radiation. For the research, the following are the instrument, samples obtained and the laboratory where the experiment was done.

Table 2 – Equipment, samples obtained and the laboratory where the experiments were done

Equipment	Name of Laboratory	Samples Obtained
Long big column 60 cm and 7.0 diameter	John Igolis' Chemistry laboratory, Markurdi (Benue State)	NLH 1-15 NLD 1-15 NLE 1-30 NLM 1-30
Flash column 30 cm and 3.5 diameter	John Igolis' Chemistry laboratory, Markurdi (Benue State)	NLM19 and NLM24
NMR-Bruker AV3 (400 MHZ) Spectrophotometer	Chemistry Laboratory University of Glasgow, Scotland, UK	1H NMR (Pheophytin A) from NLM24 and β-amyrin from NLM19
NMR-Bruker AV3 (100 MHZ) Spectrophotometer	Chemistry Laboratory University of Glasgow, Scotland, UK	2D NMR (Pheophytin A) from NLM24 and β-amyrin from NLM19

RESULTS AND DISCUSSION

The extracts NLM, NLE, and NLDCM obtained were used to conduct chemical analysis viz: phytochemical screening, column chromatography, thin layer chromatography, and NMR spectroscopy.

Table 3 – The extracts NLM, NLE and NLDCM with their yields and appearance

Extract	Yield (%)	Appearance
NLM	10.0	Reddish brown
NLE	4.6	Greenish yellow
NLDCM	2.5	Dark green
NLH	0.1	Yellow

Table 4 – Result of Column chromatography of *Newboudia laevis* Leaves extract

Vials label	The volume of solvent mixture used for elution (ml)		Colour of fraction
	Hexane	Ethyl acetate	
	100	0	Colourless
	90	10	Colourless
NLHI	80	20	Yellow
NLH2	80	20	Light Yellow
NLH3	80	20	Yellow
NLH3	80	20	Grey
NLH4	80	20	Blue
NLH5	80	20	Deep Blue
NLH6	80	20	Deep blue
NLH7	80	20	Yellow
NLH8	80	20	Yellow
NLH9	80	20	Light green
NLH10	80	20	Light green
NLH11	80	20	Light green
NLH12	80	20	Dark green
NLH13	80	20	Dark green
NLH13	80	20	Green
NLH14	80	20	Light yellow
NLH15	80	20	Light yellow
NLD1	80	20	Light yellow
NLD2	60	40	Greenish Yellow
NLD3	60	40	Greenish Yellow
NLD4	60	40	Light yellow
NLD5	60	40	Light yellow
NLD6	60	40	Golden Yellow
NLD7	60	40	Golden Yellow
NLD8	60	40	Yellow
NLD9	60	40	Deep yellow
NLD10	60	40	Deep Yellow
NLD11	50	50	Black
NLD12	50	50	Black
NLD13	50	50	Dark brown
NLD14	50	50	Dark brown
NLD15	50	50	Dark brown
NLE1	50	50	Black
NLE2	50	50	Black
NLE3	50	50	Black
NLE4	50	50	Dark brown
NLE5	40	60	Greenish yellow
NLE6	40	60	Greenish yellow
NLE7	40	60	Greenish yellow
NLE8	40	60	Yellow
NLE9	40	60	Yellow
NLE10	40	60	Golden yellow
NLE11	40	60	Golden yellow
NLE12	40	60	Light yellow
NLE13	40	60	Yellow
NLE14	30	70	Light green
NLE15	30	70	Light green
NLE16	30	70	Light green
NLE17	30	70	Greenish yellow
NLE18	30	70	Greenish yellow
NLE19	30	70	Light green
NLE20	30	70	Light green
NLE21	30	70	Light green
NLE22	30	70	Light green
NLE23	20	80	Black
NLE24	20	80	Blue
NLE25	20	80	Yellow
NLE26	20	80	Light blue
NLE27	20	80	Light blue
NLE28	20	80	Brown
NLE29	20	80	Reddish brown
NLE30	20	80	Black
NLM1	20	80	Dark red
NLM2	10	90	Brown

Vials label	The volume of solvent mixture used for elution (ml)		Colour of fraction
NLM3	10	90	Brown
NLM4	10	90	Black
NLM5	10	90	Yellow
NLM6	10	90	Yellow
NLM7	10	90	Light blue
NLM8	10	90	Blue
NLM9	10	90	Deep blue
NLM10	10	90	Deep blue
NLM11	0	100	Reddish brown
NLM12	Methanol 100 %		Brown
NLM13			Brown
NLM14			Brown
NLM15			Reddish brown
NLM16			Reddish brown
NLM17			Brown
NLM18			Brown
NLM19			White
NLM20			Green
NLM21			Green
NLM22			Yellow
NLM23			Yellow
NLM24			Brown
NLM25			Brown
NLM26			Reddish brown
NLM27			Reddish brown
NLM28			Reddish brown
NLM29			Brown
NLM30			Brown

Notes: NLH – *Newbouldia Laevis* hexane, NLD – *Newbouldia Laevis* dichloromethane, NLE – *Newbouldia Laevis* ethyl acetate, NLM – *Newbouldia Laevis* methanol.

Column chromatography was done using about 10.0 g of methanol extract. The vials label, volume of the solvent mixture used for elution (ml) and colour of fractions when visualized with the naked eye are shown in Table 4 above.

With the solvent mixture of Hexane/Ethyl acetate (100/0, 90/10), respectively, the fractions appeared colourless. On changing the solvent mixture to Hexane/Ethyl acetate (100/20), about 15 bits (5 ml each) of NLH were collected. Their corresponding colours are shown in Table 4 above.

When the solvent mixture was changed to Hexane/Ethyl acetate (60/40), ten fractions (5 ml each) of NLD we collected and another five fractions (5 ml each) of NLD were collected when the solvent mixture was changed to Hexane/Ethyl acetate (50/50). Their corresponding colours are shown in the table above. Still, on the solvent mixture of Hexane/Ethyl acetate (50/50) and changing the solvent mixture (40/60, 30/70 and 20/80), 30 fraction (5 ml each) of NLE were collected. The colours corresponding to each of them are seen in Table 4 above.

Changing the solvent mixture further to Hexane/Ethyl acetate (10/90, 0/100) and finally washing down with 100 ml methanol, 30 fractions (5 ml each) of NLM were collected, and the colour of each particle can be seen in the table above. The whole bits collected were kept to dry, and further TLC was carried out on each. Afterwards, fractions with the same RF values were pooled together. The colour of each bit signifies the possible organic compound present in it.

Thin layer chromatography results. The thin layer chromatography on the fractions obtained from column chromatography above in vials bottle labelled NLH 1-15, NLD 1-15, NLE 1-30, and NLM 1-30 and developed using a solvent mixture as EAC:n-hexane:MeOH (4:5:1) shows that only NLM24 and NLM19 gave single spot with $R_f$values of 0.48 and 0.47, respectively. The two pure fractions were thus packaged for NMR spectra analysis.

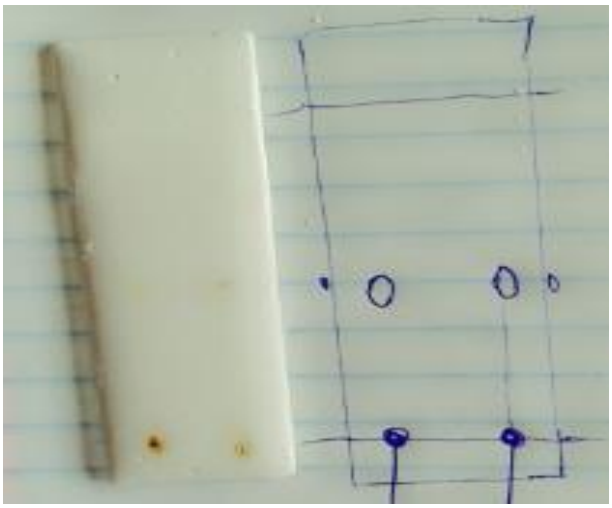


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Figure 2 – TLC chromatogram for NLM24 and NLM19 using this solvent system EAc:n-hex (8:2, 7:3, 4:5:1 ml)

Spectral analysis results show in Table 5.

Table 5 – ^{1}H (400 MHZ) and ^{13}CNMR (100 MHZ) data of NLM24 and Literature data in $CDCL_3$

Position	NLM24		Literature data [10]		2D NMR		
	^{1}H (δppm)	^{13}C (δppm)	^{1}H (δppm)	^{13}C (δppm)	COSY.	HMBC(3J)	(2J)
1	-	142.1	-	141.22	-	-	-
2	-	131.7	-	131.44	-	C-1, C-3	C-2
2^1	3.43 (s)	12.1	3.44(S)	12.13	-	C-1, C-3	C-2
3	-	136.5	-	136.06	H-3^1, H-4	-	-
3^1	8.03 (dd)	129.1	8.02(dd, 11.9; 17.8)	129.00	H-3^2, 4-3	C-2, C-4	C-3^2
3^2	6.20	122.8	6.34(dd, 1.3; 17.8)	122.78	-	C-3	C-3
	6.30		6.17(dd, 1.3; 11.9)				
4	-	136.5	-	136.55	H-3, H-5	-	-
5	9.48 (1H, s)	97.9	9.56 (s)	99.66	H-4	C-7	-
6	-	155.3	-	131.52	-	-	-
7	-	136.5	-	145.60	-	-	-
7^1	3.22(s)	11.2	3.28(s)	11.32	H-8	C-6, C-8	C-7
8	-	145.2	-	149.72	H-8^1, H-7^1	-	-
8^1	3.62 (q)	19.7	3.75 (q, 7.6)	19.73	-	-	C-8, C-8^2
8^2	1.72 (t)	17.4	1.72(t, 7.6)	19.66	-	C-8	-
9	-	149.9	-	142.87	-	-	-
10	9.50 (1H, s)	104.2	9.76 (s)	104.15	-	C-12, C-8	C-11
11	-	137.8	-	130.27	-	-	-
12	-	129.1	-	141.22	-	-	-
12^1	3.74 (s)	12.1	3.90 (s)	12.46	-	C-11, C-13	C-12
13	-	129.1	-	101.96	-	-	-
13^1	-	190.4	-	150.02	-	-	-
13^2	6.34	64.9	-	161.04	-	-	-
13^3	-	172.5	-	171.5	-	-	-
13^4	3.91 (s)	51.9	3.74 (s)	54.16	-	-	-
14	-	149.9	-	111.34	-	-	-
15	-	104.5	-	100.45	-	-	-
16	-	162.5	-	166.33	-	-	-
17	4.16 (m)	51.9	5.14 (m)	53.70	-	-	-
17^1	2.35 (m)	31.9	2.81 (m)	24.77	-	C-18	-
17^2	2.17 (m)	31.9	2.34 (m)	32.13	-	-	C-17
			2.16 (m)				-
17^3	-	173.1	-	173.32	-	-	-
18	4.48 (m)	50.4	4.44 (m)	50.16	-	-	-
18^1	1.92 (d)	22.7	1.63(d)	22.72	-	C-19	C-18
19	-	172.49	-	170.90	-	-	-
20	8.64 (1H, s)	93.6	8.70(s)	93.89	-	C-18, C-2	C-1
P1	4.48 (2H, m)	61.58	4.44(m)	61.49			
P2	5.25 (1H, t)	117.94	5.14(t, 7.6)	117.74			
P3	-	-	-	-			
P^{1}3	1.58 (3H, s)	22.71	1.57(s)	16.23			
P4	1.75 (2H, m)	39.37	1.81	39.86			
P5-P14	1.4-0.9 ppm	19.73	1.61	25.04			
P6	1.09 (4H, m)	36.67					
P7	1.72 (2H, s)	32.77					
P^{1}7	0.80 (3H, d)	22.62	0.77(b,6.6)	19.63			
P8	1.25 (3H, m)	36.67					
P9	1.14 (2H, m)	22.67					
P10	1.72 (4H, m)	36.67					
P 11	1.72 (2H, s)	31.94	1.31	31.66			
P^{1}11	0.99 (2H, d)	19.73	0.80(d,6.6)	19.57			
P12	1.72 (4H, d)	36.67					
P13	1.25 (3H, d)	39.37	1.26	25.04			
P15	1.25 ($3CH_2$)	31.94	1.29	31.95			
P^{1}15	0.86 (CH_3, d)	27.97	0.85(d, 6.6)	22.62			
P16	0.78 (CH_3, d)	19.42	0.85(d, 6.6)	22.62			

*Spectroscopic analysis of isolated compounds***.** The ^{1}H-NMR spectrum of the compound showed the presence of ten methyls, thirteen methylene, eleven methine, and two ester protons. The three singlet signals seen at 9.50, 9.48 and 8.64 ppm are characteristic of H-10, H-5 and H-20 protons, respectively. This indicates the porphyrin unit of olefinic methine (=CH) protons bridging the pyrrole ring. Also, the signal is at 3.74 ppm (3H, s-12^{1}). 3.22 ppm (1H, s-7^{1}) and 3.43 ppm (3H, s-2^{1}) correspond to substituents (comprising four methyl and one ethyl group) attached to the pyrrole ring of the porphyrin unit. Other identified signals at 8.03 ppm include; a triplet at 3.62 ppm (2H, m-8^{1}), four isolated CH_3-Hs at H-13^{4}, H-12^{1}, H-2^{1} and H-7^{1}as well as signals at 8.03 ppm and 6.20 ppm are characteristics of olefinic protons. The signals at 4.48 ppm (2H, m-P_1) and 5.26 ppm (1H, t-P_2) are of ester and methylene protons of the phytyl group, confirming the esterified and also the presence of phytol group in the structure, the signals at 1.58 ppm (3H, s-P^{1}3), 0.80 ppm (3H, d),0.99 ppm (3H, d) were assigned to the four methyl substituents at P^{1}3, P^{1}7, P^{1}11 and P^{1}15, respectively. The signals at 0.86 ppm (3H, d P16) are characteristic of (CH_3-P16) protons

The multiplets at 1.92, 1.09, 1.25, 1.72, and 1.72 ppm correspond to the methylene protons indicated at P4, P6, P8, P10 and P12, respectively. The remaining signals at 1.58 and 1.25 ppm were assigned to the three methylene protons labelled P4, P9 and P13, respectively. There were no signals observed at P3.

The singlet at 7.26 ppm in the ^{1}H-NMR spectrum of NLM24 is characteristic of the $CDCL_3$ solvent. This signal was due to some impurity in the deuterated chloroform used.

13C NMR (100 MHZ $CDCL_3$) of NLM24. APT (Attached proton test) was used to distinguish the carbon types (multiplicities). CH_3/CH is shown in the positive phase CH_2/-C- is shown in the negative phase. The solvent $CDCL_3$ resonances visible in low field aromatic carbon atoms were absent.

The Nitrogen resonances were not seen. They were on the opposing side.

The 13C NMR Spectrum showed 55 carbons, with a carbonyl at σ173.1 (C-17^{3}) in NLM24, suggesting an esterified position. One oxymethylene (σc61.58), Ten methyl carbons at σ129.1 (C-3^{1}), σ97.9 (C-5), σ104.2 (C-10), σ64.9 (C-13^{2}), σ51.9 (C-17),σ50.4 (C-18), σ93.6 (C-20) & σ117.94 (P-2), σ32.77 (P-7) and σ31.94 (P-11).

Eight methine C-carbons, 7 in the pheophorbide and 1 in the phytyl side chain. Four methylenes in the pheophorbide at σ19.7 (8^{1}), σ31.9 (17^{1}), σ31.9 (17^{2}) and σ122.8 (3^{2}). Ten methylene in the phytyl side chain. Also, methyl carbons were observed, 6 in pheophorbide and 5 in phytyl side chain.

The signal at C-13^{1} (σ190.4) is the carbonyl of the cleaved E-ring.

The carbonyl at 17^{1}&17^{3} is carbonyl of esters resonating σ172.5 & σ173.1 because they are all ester carbonyl.

Signals at 104.20 ppm, 97.90 ppm and 93.63 ppm were observed in the negative phase corresponding to the olefinic methine (=CH) carbons of σ104.2 (C-10), σ97.9 (C-5) and σ93.6 (C-20), which indicates a porphyrin moiety. Also, the signals at 51.90 ppm and 50.40 ppm observed in the positive phase corresponded to σ51.9 (C-17) and σ50.4 (C-18) methine carbons of the porphyrin moiety, while the signals at 61.58 ppm (COOCH, P^{1}) corresponds to the oxymethylene carbon which confirmed the esterification of the porphyrin ring by phytyl group. More so, the signal at 117.78(C P^{2})is characteristic of the olefinic carbon of the phytol group. The triplet at 77 ppm in the ^{13}C spectrum was due to the solvent (Deuterated chloroform) signal.

The ^{1}H-^{1}H-COSY NMR Spectrum of NLM24 showed some singlets at 9.63, 9.50 and 8.64 ppm corresponding to H-10, H-5 and H-20, respectively.

Signals were observed at 3.74 ppm (3H, d 18^{1}) and 1.72 ppm (3H, t8^{2}), resulting from four methyls and one ethyl group bonded to the pyrrole ring of the porphyrin unit. Also, the ^{1}H-^{1}H correlation signals at 4.48 ppm (2H, m-P_1) and 5.26 ppm (1H, t-P_2) were assigned to the ester and olefinic protons of the phytyl group and correlated in the ^{13}C Spectrum of NLM24 with carbon signals at 61.58 ppm (2H, m-P_1) and 117.94 ppm (=CH-P_2). This confirms the esterification of the porphyrin moiety at C-17^{3} by phytol.

The HMBC Spectrum of NLM24 showed that the triplet at 8.03 ppm (H-3^{1}) displayed a wide range correlation to the olefinic methine (=CH) carbon at 122.8 ppm (C-3^{2}) Via ^{3}J and ^{2}J coupling. In comparison, the doublet at 6.20 ppm (H-3^{2}) showed a ^{3}J coupling to the olefinic (CH_2) carbon

at 129.10 ppm (C-3^1), establishing the attachment of (-CH=CH_2) group to C-3. The methyl (CH_3-8^2) at 172 ppm showed a ^{3}J coupling to the methylene at 19.70 ppm (C-8^1). They confirmed the attachment of (-CH_2CH_3) ethyl group to C-8. The methine protons at 4.16 ppm (H-17) and 4.48 ppm (H-18) also correlated to C-17 through ^{3}J and ^{2}J coupling, respectively. The methyl protons at 1.92 ppm (H-18^1) connected to C-17 at 51.90 ppm and C-18 at 50.40 ppm through ^{3}J and ^{2}J collar with the methine carbon (C-18).

Hence, the oxymethylene protons at 4.48 ppm (H-P_1) correlated to the (olefinic) carbon at 117.94 ppm (C-P_2), establishing the presence of the phytyl group in the structure (NLM24).

Isolation of pheophytin A from *Newbouldial aevis* leaf is hereby reported for the first time. Pheophytin A is an Mg-free analogue of chlorophyll formed by replacing the Mg^{2+} in the chlorophyll molecules with (2H). NLM24 is brownish. It is practically insoluble in water but soluble in ethanol, diethyl ether, chloroalkanes and hydrocarbons.

The isolated compound pheophytin A has a lot of pharmacological importance, such as Antimicrobial [2], Antioxidant [5], Free radical scavenger [6], Anti-inflammatory [7], and Cancer Chemotherapy [8, 9].

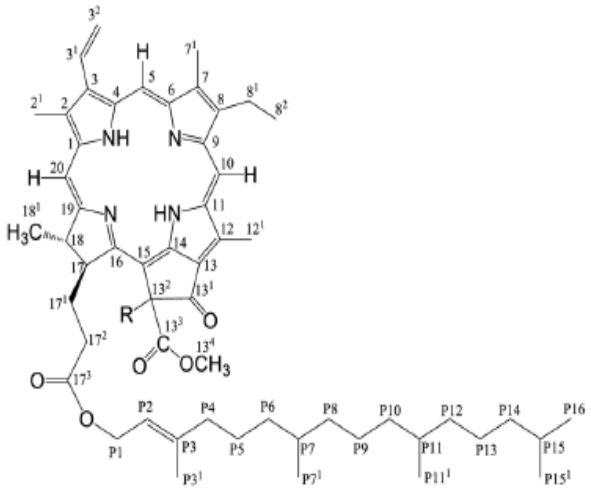

Figure 3– Structure of Pheophytin A

The signals at 0.78 ppm (s, Me-23), 0.80 ppm (s, Me-24), 0.93 ppm (s, Me-25), 0.94 ppm (s, Me-26), 0.95 ppm (s, Me-27), (s, Me-30) are characteristics of methyl protons. The rest of the signals were for methylene (Figure 4).

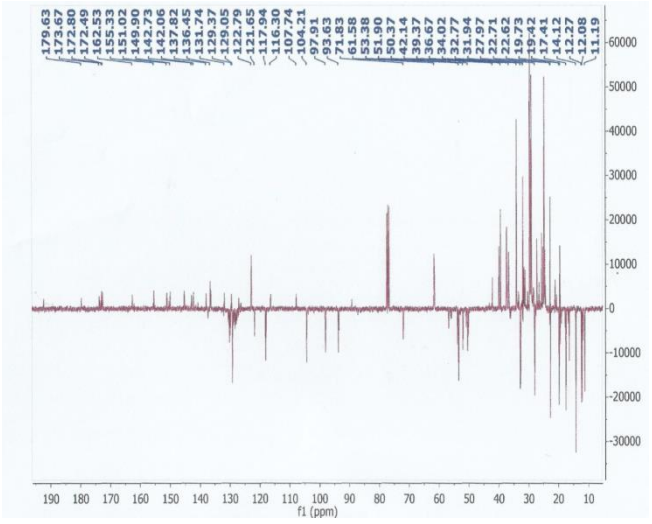

Figure 4a – ^{1}H-NMR spectrum for NLM24 (Pheophytin A)

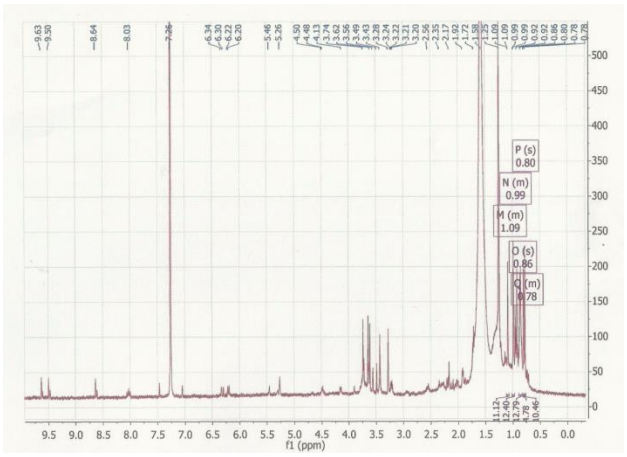

Figure 4b – ^{13}C spectrum for NLM24 (Pheophytin A)

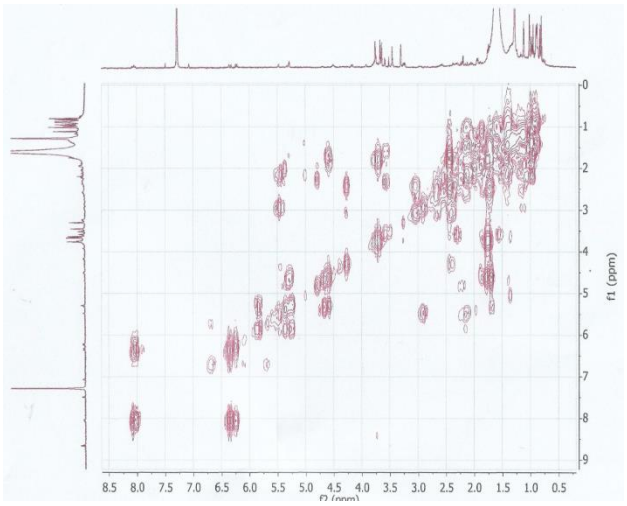

Figure 4c – COSY NMR spectrum for NLM24 (Pheophytin A)

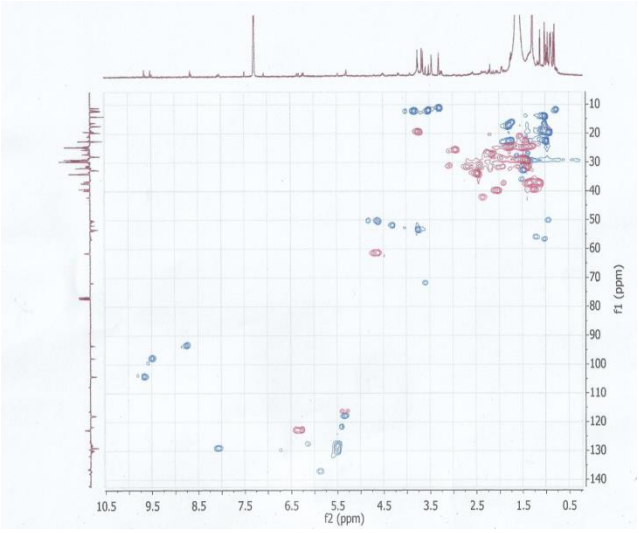

Figure 4d – HSQC NMR Spectrum for NLM24 (Pheophytin A)

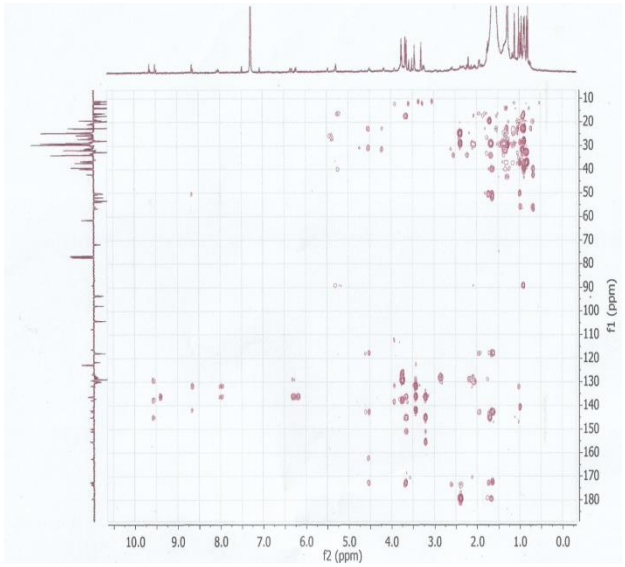

Figure 4e – HMBC NMR Spectrum for NLM24 (Pheophytin A)

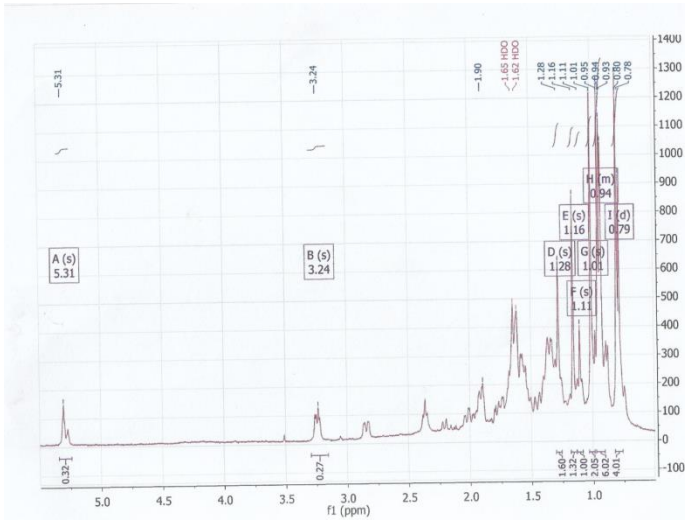

Figure 4f – 1H NMR Spectrum for NLM19 (β-amyrin)

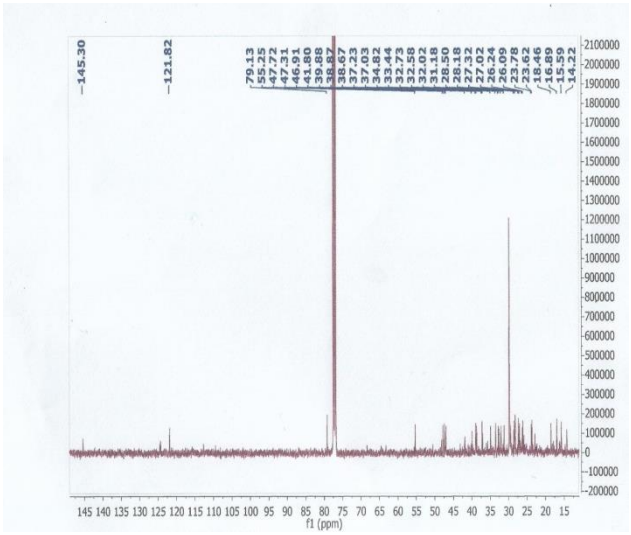

Figure 4g – 13C NMR Spectrum for NLM19 (β-amyrin)

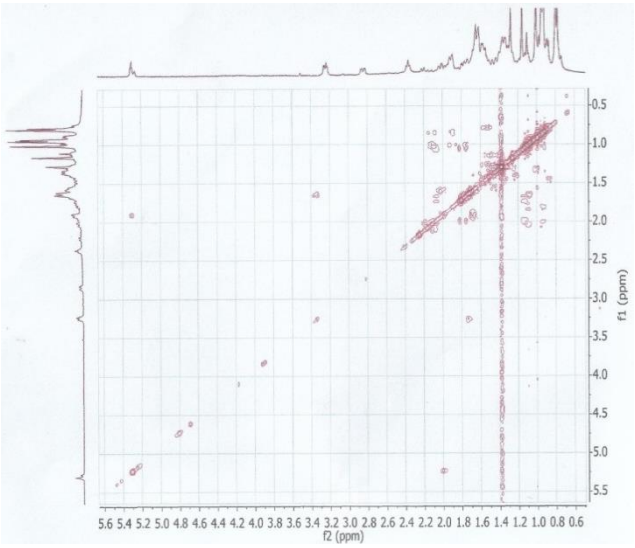

Figure 4h – COSY NMR Spectrum for NLM19 (β-amyrin)

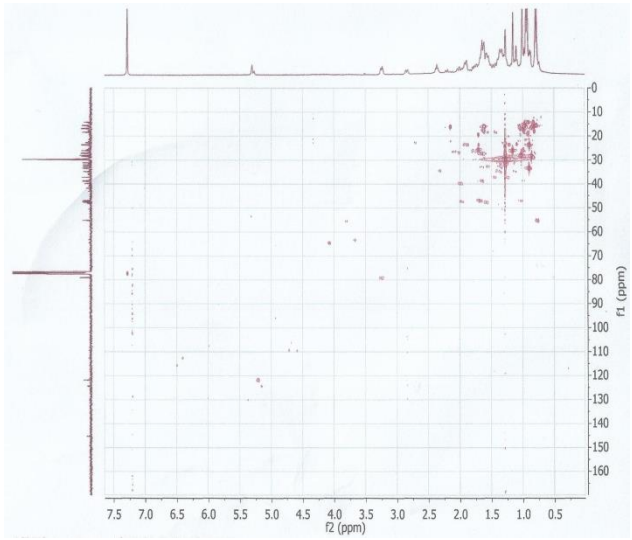

Figure 4i – HSQC NMR Spectrum for NLM19 (β-amyrin)

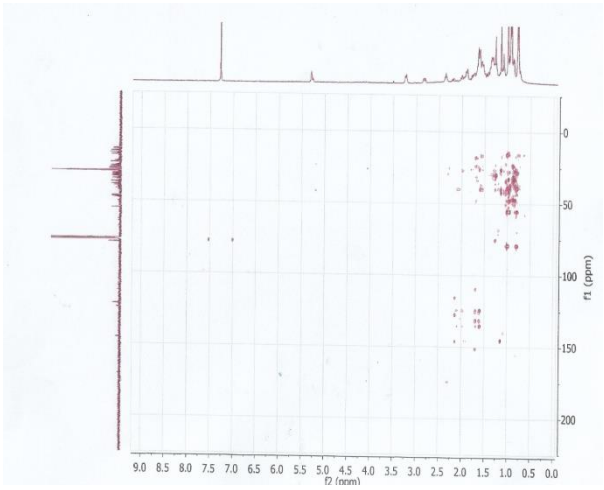

Figure 4j – HMBC NMR Spectrum for NLM19 (β-amyrin)

Characterization of NLM19 as β-amyrin. The ^{l}H NMR for NLM19 (Table 6) showed the presence of eight methyl singlets, one olefin proton at δ5.31 (J=3.5 Hz), and an oxygenated proton at δ3.24 (J=4.4, 11.5). All of them suggest an oleanane type of triterpenoid. The triplet diversity is due to coupling with the H-H protons at 1.62, 1.65 and 1.90 ppm. An oxymethine proton (H-3) at δH.3.24 (dd, J=11.5) was also approximately observed and integrated for two protons.

Table 6 – ^{1}H (500 MHZ) and ^{13}C(125 MHZ) NMR Data of NLM19 and Literature Data in $CDCl_3$

Position	NLM19		Literature data [10]		2D NMR		
	^{1}H(δppm)	^{13}C(δppm)	^{1}H(δppm)	^{13}C(δppm)	COSY	HMBC (3J)	2J
1	1.55/1.48	38.87	1.55(Hb-1) 1.49(Ha-1)	38.8	H-2	C-3, C-25	-
2	1.52/1.56	27.32	1.52(Hb-2) 1.55(Ha-2)	27.4	H-1, H-3	-	C-3
3	3.24	79.13	3.20dd (4.4, 11.5)	79.2	H-2	-	-
4	-	38.67	-	39.0	-	-	-
5	-	55.25	0.71	55.4	H-6	-	-
6	18.5	1.53(Hb-6), 1.30(Ha-6)	18.6	H-5, H-7	-	-	-
7	-	32.73	-	32.9	H-6	-	-
8	-	39.88	-	40.2	-	-	-
9	-	47.31	1.95	47.4	-	C-5	-
10	-	37.3	-	37.2	-	-	-
11	1.90	23.62	1.84	23.8	H-12	-	-
12	5.31	121.82	5.16t (3.5)	121.9	-	-	-
13	-	145.30	-	145.4	-	-	-
14	-	41.80	-	41.9	-	-	-
15	-	26.24	-	26.4	H-16	-	-
16	-	27.02	-	27.1	H-15	-	-
17	-	32.58	-	32.7	-	-	-
18	1.90	47.72	1.89	47.8	H-19	-	-
19	1.65	47.32	1.59	47.0	H-18	-	-
20	-	31.18	-	31.3	-	-	-
21	1.62	37.03	1.66	37.4	H-22	-	-
22	-	34.82	34.9	H-21	-	-	-
23	0.78 (s)	15.59	0.77s	15.7	-	C-3, C-5, C-24	C-4
24	0.95 (s)	28.50	0.98s	28.3	-	C-3, C-5, C-23, C-5	C-4
25	0.93 (s)	15.59	0.92s	15.8	-	C-5	-
26	0.94 (s)	16.89	0.94s	17.0	-	-	-
27	1.12 (s)	26.09	1.11s	26.2	-	C-8, C-13, C-15	C-14
28	0.90 (s)	28.50	0.81s	28.6	-	C-30	-
29	0.80 (s)	33.44	0.85s	33.6	-	C-30	
30	0.80 (s)	23.78	0.85s	23.9	-	-	-

The ^{13}C NMR spectrum showed a total of 30 carbons, with two olefinic methine carbons at δ121.8 (C-12) and δ145.3 (C-13) in NLM19 suggesting an oleanane triterpene:

- one oxygenated carbon (δc79.1);

-eight methyl carbons at δ15.59 (C-23), δ 28.50 (C-24), δ 15.59 (C-25), δ16.89 (C-26), δ26.09 (C-27), δ28.50 (C-28), δ33.44 (C-29) and δ23.78 (C-30);

- three methine carbon at δ55.25 (C-5), δ47.31 (C-9) and δ47.72 (C-18);

- six quatenery carbons at δ38.67 (C-4), δ37.3 (C-10), δ39.88 (C-8), δ41.80 (C-14), δ32.58 (C-17), δ145.30 (C-13), δ31.18 (C-20);

- ten methylene carbons at δ38.87 (C-1), δ 27.32 (C-2), δ1.53 (Hb-6), 1.30 (Ha-6) (C-6), δ32.73 (C-7), δ23.62 (C-11), δ26.24 (C-15), δ27.02 (C-16), δ47.32 (C-19), δ27.32 (C-2), δ34.82 (C-22).

The signals observed at 121.82 and 145.30 ppm correspond to the olefinic methine (=CH) carbons of C-12 and C-13, indicating unsaturation in the oleanane skeletal structure. The signal at 79.13 ppm corresponding to C-3 indicates an oxygenated oleanane triterpene. Signals observed at 38.67, 37.3, 39.88, 41.80, 32.58, 145.30 and 31.18 ppm are characteristics of the quaternary carbons of triterpenes. The triplet at 38.87 ppm in the ^{13}C NMR spectrum was the solvent (chloroform) signal. The remaining assignments are shown in Table 6.

The ^{1}H-^{1}H – COSY NMR spectrum of NLM19 showed some singlets at 0.78, 0.95, 0.93, 0.94, 1.12, 0.90, 0.80 and 0.80 ppm corresponded to H-23, H-24, H-25, H-26, H-27, H-28, H-29, and H-30, respectively. The signals observed at 5.16t (3.5), 3.20 dd (4.4, 11.5) resulted from the olefinic methine (=CH) carbons and the oxygenated group bonded to the oleanane structure. Also, the ^{1}H-^{1}H correlation signals at 1.55 (Hb-I), 1.49 (Ha-I), 1.52 (Hb-2) and 1.55 (Ha-2) result from two methylenes bonded to H-1 and H-2, respectively.

The HMBC spectrum of NLM19 in Table 6 showed the methylene protons at 1.55/1.48 ppm (H-1) and 1.52/1.56 ppm (H-2) correlated to C-1 and C-2, respectively, through ^{3}J and ^{2}J coupling. The methyl protons at 0.78 ppm (H-23), 0.95 ppm (H-24), 0.93 ppm (H-24), 0.93 ppm (H-25), 1.12 ppm (H-27), 0.90 ppm (H-28) and 0.80 ppm (H-29) correlated to C-4, C-10, C-8, C-17 and C-20 through ^{3}J and ^{2}J wide range coupling, respectively. Other correlations were not observed, as shown in Table 6.

It is from the above information as well as comparison with the literature data that led to the characterization of NLM19 as β-amyrin. The results were in good agreement with previous reports from [10]. Isolation of β-amyrin from *Newbouldia laevis* leaf is hereby reported for the first time. The amyrins are three closely related natural chemical compounds of triterpenes. Amyrins can exist as α-amyrin, β-amyrin and δ-amyrin [11].

The isolated compound "β-amyrin" from NLM19 is a pentacyclic triterpenoid, an oleanane substituted at the three beta-position by a hydroxyl group with a double bond between carbon positions 12 and 13.

β-amyrin was first isolated in 1968 by corey and gross. β-amyrin possesses a white colour and is primarily white solid upon isolation. The compound "β-amyrin" from NLM19 is soluble in ethanol and dimethyl formamide (DMF).

β-amyrin has a lot of pharmacological importance such as: Anti-inflammatory [12], Anti spasmodic activity [13], Pain-killer [14], Antinociceptive [1], Antiarthritic [15].

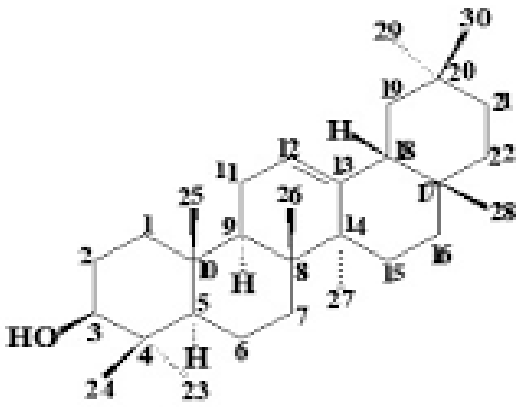

Figure 5 – Structure of β-amyrin

CONCLUSIONS

The NMR (^{1}H, ^{13}C, COSY, HSQC and HMBC) spectral analyses and characterization revealed the presence of pheophytin A, in NLM24 and β-amyrin in NLM19 fractions of leaf extracts of *Newbouldia laevis.*

This result shows that *N. laevis* contains bioactive components that can be used for the treatment of various health problems such as pain, inflamation, oedema, rheumatism, arthritis and heartburn.

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