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 (Rf 0.48), EAc:n-hex:MeOH (4:5:1) and NLM19 (Rf 0.47), EAc:n-hex:MeOH, respectively. These pure fractions were subjected to ¹H NMR, ¹³C, 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

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-hex: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-hex: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 Aluminium 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:CH₃OH).

A spot was made on the plate, developed in the solvent front. The dish was brought out from the beaker, 10 % H₂SO₄ 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):

\[
R_f = \frac{Distance \ moved \ by \ spot}{Distance \ moved \ by \ the \ solvent \ front}
\]

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.

**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 *New-boudia laevis* Leaves extract

| Vials label | The volume of solvent mixture used for elution (ml) | Colour of fraction |
|-------------|----------------------------------------------------|--------------------|
| Vials label | Hexane | Ethyl acetate | Colour of fraction |
| 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              |
| 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 | Brown
NLM4 | 10 | Black
NLM5 | 10 | Yellow
NLM6 | 10 | Yellow
NLM7 | 10 | Light blue
NLM8 | 10 | Blue
NLM9 | 10 | Deep blue
NLM10 | 10 | Deep blue
NLM11 | 0 | 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.

When the solvent mixture was changed to Hexane/Ethyl acetate (60/40), ten fractions (5 ml each) of NLE were 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 Rf values of 0.48 and 0.47, respectively. The two pure fractions were thus packaged for NMR spectra analysis.

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

*Spectral analysis results* show in Table 5.
| Position | NLM24 | Literature data [10] | 2D NMR |
|----------|--------|----------------------|--------|
|          | ¹H (δ ppm) | ¹C (δ ppm) | ¹H (δ ppm) | ¹C (δ ppm) | COSY. | HMBC (3J) | (2J) |
| 1        | -       | 142.1       | -          | 141.22      | -     | -         | -    |
| 2        | -       | 131.7       | -          | 131.44      | -     | C-1, C-3  | C-2  |
| 3        | 3.43 (s) | 12.1        | 3.44(S)    | 12.13       | -     | C-1, C-3  | C-2  |
| 3¹       | -       | 136.5       | -          | 136.06      | -     | -         | -    |
| 3²       | 803 (dd) | 129.1       | 8.02(dd, 11.9; 17.8) | 129.90     | H-3¹, H-4 | -    |
| 3³       | 62.0    | 122.8       | 6.34(dd, 1.3; 17.8) | 122.78     | -     | C-3       | C-3  |
| 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      | -     | -         | -    |
| 7¹       | 3.22(s) | 11.2        | 3.28(s)    | 11.32       | H-8   | C-6, C-8  | C-7  |
| 8        | -       | 145.2       | -          | 149.72      | H-8¹, H-7¹ | -   |
| 8¹       | 3.62 (q) | 19.7        | 3.75 (q, 7.6) | 19.73       | -     | C-8, C-8² | C-7  |
| 8²       | 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      | -     | -         | -    |
| 12¹      | 3.74 (s) | 12.1        | 3.90 (s)   | 12.46       | -     | C-11, C-13| C-12 |
| 13       | -       | 129.1       | -          | 101.96      | -     | -         | -    |
| 13¹      | -       | 190.4       | -          | 150.02      | -     | -         | -    |
| 13²      | 6.34    | 64.9        | -          | 161.04      | -     | -         | -    |
| 13³      | -       | 172.5       | -          | 171.5       | -     | -         | -    |
| 13⁴      | 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       | -     | -         | -    |
| 17¹      | 2.35 (m) | 31.9        | 2.81 (m)   | 24.77       | C-18  | -         | -    |
| 17²      | 2.17 (m) | 31.9        | 2.34 (m)   | 32.13       | -     | -         | C-17 |
|          |          |             |            | 2.16 (m)    | -     | -         | -    |
| 17³      | -       | 173.1       | -          | 173.32      | -     | -         | -    |
| 18       | 4.48 (m) | 50.4        | 4.44 (m)   | 50.16       | -     | -         | -    |
| 18¹      | 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       | -       | -          | -          | -           | -     | -         | -    |
| P³       | 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      | -          | -           | -     | -         | -    |
| P7¹      | 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      | -          | -           | -     | -         | -    |
| P11      | 1.72 (2H, s) | 31.94      | 1.31       | 31.66       | -     | -         | -    |
| P¹¹      | 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 (3CH₃) | 31.94      | 1.29       | 31.95       | -     | -         | -    |
| P¹⁵      | 0.86 (CH₃, d) | 27.97      | 0.85(d,6.6) | 22.62       | -     | -         | -    |
| P16      | 0.78 (CH₃, d) | 19.42      | 0.85(d,6.6) | 22.62       | -     | -         | -    |

Table 5: ¹H (400 MHz) and ¹³C NMR (100 MHz) data of NLM24 and Literature data in CDCl₃.
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'), 3.22 ppm (1H, s-7') and 3.43 ppm (3H, s-2') 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-\(^2\)), four isolated CH\(_3\)-Hs at H-13\(^3\), 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 phytly group, confirming the esterified and also the presence of phytol group in the structure, the signals at 1.58 ppm (3H, s-P\(^3\)), 0.80 ppm (3H, d), 0.99 ppm (3H, d) were assigned to the four methyl substituents at P\(^3\), P\(^7\), P\(^11\) and P\(^15\), 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 \(\sigma 173.1\) (C-17) in NLM24, suggesting an esterified position. One oxymethylene (\(\sigma 61.58\)). Ten methyl carbons at \(\sigma 129.1\) (C-3\(^1\)), \(\sigma 97.9\) (C-5), \(\sigma 104.2\) (C-10), \(\sigma 64.9\) (C-13\(^2\)), \(\sigma 51.9\) (C-17), \(\sigma 50.4\) (C-18), \(\sigma 93.6\) (C-20) & \(\sigma 117.94\) (P-2), \(\sigma 32.77\) (P-7) and \(\sigma 31.94\) (P-11).

Eight methine C-carbons, 7 in the pheophorbide and 1 in the phytly side chain. Four methylenes in the pheophorbide at \(\sigma 197.8\) (8\(^1\)), \(\sigma 319\) (17\(^1\)), \(\sigma 31.9\) (17\(^2\)) and \(\sigma 122.8\) (3\(^2\)). Ten methylenes in the phytly side chain. Also, methyl carbons were observed, 6 in pheophorbide and 5 in phytly side chain.

The signal at C-13\(^1\) (\(\sigma 190.4\)) is the carbonyl of the cleaved E-ring.

The carbonyl at 17\(^1\)&17\(^3\) is carbonyl of esters resonating \(\sigma 172.5\) & \(\sigma 173.1\) 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 \(\sigma 104.2\) (C-10), \(\sigma 97.9\) (C-5) and \(\sigma 93.6\) (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 \(\sigma 51.9\) (C-17) and \(\sigma 50.4\) (C-18) methine carbons of the porphyrin moiety, while the signals at 61.58 ppm (COOCH-\(\text{P1}\)) corresponds to the oxymethylene carbon which confirmed the esterification of the porphyrin ring by phytly group. More so, the signal at 117.78 (C \(\text{P2}\)) 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 pyrroly 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 phytly 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-P2). This confirms the esterification of the porphyrin moiety at C-17\(^3\) by phytly.

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\(_2\)CH\(_3\)) 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 phytol group in the structure (NLM24).

Isolation of pheophytin A from *Newbouldia aenis* 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].

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 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)
Characterization of NLM19 as β-amyrin. The $^1$H NMR for NLM19 (Table 6) showed the presence of eight methyl singlets, one olefin proton at 5.31 (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 quaternary 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 (Ha-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, respective-

ly. 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].

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-amyрин from Newbouldia laevis 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, inflammation, oedema, rheumatism, arthritis and heart-burn.
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