SUPPLEMENTARY MATERIAL

Bioguided Fractionation of Hypoglycaemic Component in Methanol Extract of Vernonia amygdalina: an in vivo Study

Stanley I.R. Okoduwa a,b*, Isma’ila A. Umar a, Dorcas B. James a, Hajiya M. Inuwa a, James D. Habila c, Alessandro Venditti d

a Department of Biochemistry, Ahmadu Bello University, Zaria, Nigeria
b Directorate of Research and Development, Nigerian Institute of Leather and Science Technology, Zaria, Nigeria
c Department of Chemistry, Ahmadu Bello University, Zaria, Nigeria
d Department of Chemistry, “Sapienza” University of Rome, Rome, Italy

*Correspondence: Email: siroplc@gmail.com

Abstract

Nine components (C1-C9) were isolated from chloroform fraction of fractionated methanol extracts of Vernonia amygdalina leaves (FMEVA) by column chromatography. All the components C1 to C9 were purified and screened for hypoglycaemic activities in type-2 diabetic rats. The most potent hypoglycaemic component was elucidated on the basis of extensive spectroscopic (1D-, 2D-NMR, GC-MS, FTIR) data analysis. The Component C5 was found to be the most potent hypoglycaemic in reducing blood glucose by 12.55 ± 3.55% at 4 h post-oral administration, when compared to the positive (18.07 ± 1.20%) and negative (-1.99 ± 0.43%) controls. The spectroscopic data analysis reveals that the isolated compound has a structure consistent with 11β,13-dihydrovernolide. The isolated compound is part of the hypoglycaemic components present in V. amygdalina leaves that is responsible for the anti-diabetic activities. Further research is needed in the development of this compound or its derivatives for pharmaceutical use.

Keywords: Anti-diabetic; hyperglycaemia; hypoglycaemic; Vernonia amygdalina; Type-2 diabetes
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Experimental

Plant material
Fresh leaves of *V. amygdalina* Delile (VA) plant were harvested in the month of May, 2015 from local farm in Samaru, Zaria, Kaduna State, Nigeria (Located on latitude: 11°9´55.3´´ longitude 7°39´5.84´´). Samples of the leaves were identified and authenticated at the herbarium unit of the Department of Biological Sciences, Ahmadu Bello University, Zaria Nigeria and a voucher specimen number 1166 was deposited.

Experimental animals
Wistar Albino rats weighing 150 - 200 g were used for the research. They were acquired from the animal house of the Department of Pharmacology, Ahmadu Bello University, Zaria Nigeria. The rats were kept in well aerated cages where bedding was replaced daily, at room temperature and normal (12 hrs) night/dark light cycle. They were allowed to acclimatize for two weeks prior to experimentation. During this period, they were all provided with the same commercially available rat pellets (normal diet feed) and tap water *ad libitum*. The Institutional Animal Research Ethics Committee reviewed and certified the experimental protocol in conformity with guidelines that are in compliance with National and International Laws and Guidelines for Care and Use of Laboratory Animals in Biomedical Research.

Treatment and extraction of the plants crude extracts
The leaves of VA were sorted out to obtain only fresh samples and washed with distilled water without squeezing to remove debris. Samples of the VA leaves were dried for seven days in the shade at room temperature to constant weight. The dried samples were crushed into fine particles. The powdered samples were collected and extracted using cold maceration method described previously (Okoduwa et al., 2016). The dried crude extract was kept at 4°C in a refrigerator until required.

Fractionation of the plants crude extracts
The dried crude extract (100 g) was suspended in 500 ml distilled water and then fractionated using organic solvents in an increasing order of polarity (*n*-hexane, chloroform, ethyl acetate, *n*-butanol and water). Each of the fractions obtained from the methanol extracts was screened *in vivo* for anti-diabetic activities using T2D rat model in our previous report (Okoduwa *et al.*, 2017b).
**Chromatographic analysis**

Based on our previous *in vivo* antidiabetic study (Okoduwa et al., 2017b) the chloroform portion was subjected to column chromatography to separate the components of the fraction. Silica gel was used in packing the column while varying solvent combinations of increasing polarity were used as the mobile phase.

**Analytical thin layer chromatography (TLC), pooling and purification:**

The concentrated sub-fractions derived from column fractionation were spotted on pre-coated (silica gel 60 F$_{254}$) aluminium plates in a small chromatographic tank to separate the different components based on their relative mobilities in solvent systems and colour reactions with ultra-violet light. The eluted column fractionated sub-fractions with similar profile on the basis of their TLC pattern were combined and subjected to further column chromatographic purification to obtained nine purified components. The purified components (C1, C2, …C9) were concentrated and evaporated using rotary evaporator and dried under vacuum then kept at 4°C in the refrigerator until needed for further hypoglycaemic activity examination.

**Induction and Confirmation of Type 2 Diabetes**

The fortified diet-fed streptozotocin-treated (FDF-STZ) rats model of T2D was adopted (Okoduwa et al., 2017a). Pre-confirmation was done three days after STZ induction. Animals with FBG ≥ 200 mg/dl were considered diabetic subject to a further confirmation at day 10. At day 10 following the pre-confirmation, animals with non-fasting blood glucose (NFBG) ≥ 300 mg/dL were confirmed diabetic and incorporated in the study as diabetic animals.

**Grouping of Experimental Animals**

- **Group C1:** Diabetic rats treated with purified component C1
- **Group C2:** Diabetic rats treated with purified component C2
- **Group C3:** Diabetic rats treated with purified component C3
- **Group C4:** Diabetic rats treated with purified component C4
- **Group C5:** Diabetic rats treated with purified component C5
- **Group C6:** Diabetic rats treated with purified component C6
- **Group C7:** Diabetic rats treated with purified component C7
- **Group C8:** Diabetic rats treated with purified component C8
- **Group C9:** Diabetic rats treated with purified component C9
- **Group DC:** Diabetic control: diabetic rats treated with vehicle alone
- **Group PC:** Positive control: diabetic rats treated with standard drug (metformin 500 mg/kg b.w.).
The purified components were administered at a dose of 10 mg/kg b.w. by oral intubation to the diabetic rats.

**Nuclear Magnetic Resonance spectroscopy (NMR analysis)**

Samples of purified components were dissolved in CDCl$_3$, the NMR data were recorded at 31°C using an Agilent-NMR-vnmrs400 instrument (Germany) operating at 400 and 100 MHz for proton and carbon (decoupled $^{13}$C and DEPT), respectively,

**Fourier transform infrared spectroscopy (FTIR) analysis**

Samples of purified components were dissolved in CDCl$_3$, the FTIR data were recorded at 31 °C using Agilent-FTIR, (Cary 630 FTIR, Germany) at the Multiuser Science Research Laboratory, ABU Zaria Nigeria.

**Gas chromatography-mass spectroscopy (GC-MS) analysis**

Samples of purified components C5 were analysed using GC-MS instrument, Agilent Technologies, 7890B GC System, USA coupled to Agilent Technologies 5977A MSD, System, USA. Helium was used as the carrier gas at 1.2 ml/min. The MS operating conditions were: ionization voltage 70 eV, ion source 230°C.

**Statistical Analysis**

All statistical analyses were conducted using the computer software, Statistical Package for the Social Sciences (SPSS Cary, NC, USA) version 20.0. The results are expressed as mean ± S.D. The data were analyzed by one-way analysis of variance (ANOVA) and post hoc test. Differences between purified components and animal groups were compared using Duncan Multiple Range Test (DMRT). Values of $p<0.05$ were considered significant.

**Identification of the Most Potent Hypoglycaemic Component**

Presented here are the 1D $^1$H NMR (Supplementary material Figure S1), 1D $^{13}$C NMR (Supplementary material Figure S2), distortionless enhancement by polarization transfer (DEPT) 1D $^{13}$DEPT (Supplementary material Figure S3). The proton spectrum gives little information for the structure elucidation due to the low resolution of the spectrum and the overlapping of several resonances. The observation of resonances in the DEPT and the decoupled $^{13}$C-NMR indicated that the component in
fraction C5 showed 19 carbon resonances accounting for seven methines (CH), five methylenes (CH₂), two methyls (CH₃) and five quaternary carbons (C). In the HSQC spectrum it was possible to observe two AB spin systems due to the presence of the respective proton-carbon resonances. The less deshielded one, which showed a direct H-C correlation with carbon at 64.3 ppm was assigned to H-15 (4.50 and 3.61 ppm, for H_a-15 and H_b-15, respectively). While the second AB system was assigned to H-19 (6.08 and 5.62 ppm for H_a-19 and H_b-19, respectively) which showed a direct H-C cross peak with the carbon signal resonating at 127.44 ppm. These evidences are consistent with the presence of a terminal olefinic methylene in αβ-position with respect to a carboxylic function. It was also possible to recognize the correlation between the methin proton in H-14 (4.50 ppm) with the corresponding carbon at 99.4 ppm and this confirmed the presence of a gem-dioxygenated carbon group at this position. The HMBC experiment showed several diagnostic correlations for the structure elucidation. It was visible correlation between the geminal olefinic protons in 19 position (6.08 and 5.62 ppm) and C16 (167.96 ppm), C17 (135.62 ppm) and C18 (18.28 ppm); the further correlations between H-18 (1.97 ppm) and C16 (167.96 ppm), C17 (135.62 ppm) and C19 (127.44 ppm). Therefore, these signals correlations are consistent with the presence of a methacrylic acid moiety. The protons in H-15 (4.50 and 3.61 ppm) showed long range correlations with the adjacent carbons C3 (33.56 ppm), C4 (142.85 ppm) and C5 (129.23 ppm), thus confirming the presence of an allylic oxygenate function may be determined. The proton: in H-15 correlates also with the near C14 (99.47 ppm) a gem-dioxygenated carbon. Lastly, H-13 (1.54 ppm) showed long range couplings with carbons at C7 (57.11 ppm), C11 (39.93 ppm) and C12 (177.54 ppm) positions. This gives evidence of the presence of a methyl-substituted pentacyclic lactone ring in the structure. The signal of H-8 resulted to be overlapped with one of the protons belonging to H-19 (5.62 ppm) and the direct correlation with carbon at 71.5 ppm was clearly visible in the HSQC spectrum. The H-8 proton showed also a long range correlation (HMBC) with the quaternary carbon at 167.96 ppm (C16). This confirmed the methacrylate ester functionalization at this position. All these experimental evidences are in accordance with the structure of a substituted (methacrylate) sesquiterpene lactone such as those of 11β,13-dihydrovernolide (Figure S4). The experimental NMR data resulted to be consistent with those reported in literature (Rabe et al, 2002) (Supplementary material Table S3). Diagnostic HMBC correlations are depicted in Supplementary material Figure S5.

In the EI-MS spectrum of component C5 are recognizable signals relative to three fragments: one at m/z 292.2 (1100 rel. ab.) [M-C_3H_3O_2]^+ due to the cleavage and lost of the pentacyclic lactone ring, the second one at m/z 279.1 (900 rel. ab.) [M-C_4H_5O_2]^+ derived from the cleavage of the ester bond and release of the methacrylate moiety and the third one at m/z 251.2 (2500 rel. ab.) [M-C_4H_5O-CO_2]^+.
derived from the cleavage of the ester bond, release of the methacrylate moiety, opening of the lactone ring and decarboxylation, together with the \( m/z \) value at 364.1 (150 rel. ab.) and relative to the pseudo molecular ion (Supplementary material Figure S6) [M]\(^+\) (C\(_{19}\)H\(_{24}\)O\(_7\)). These data are consistent with the fragmentation pattern of 11\( \beta \),13-dihydrovernolide and give an additional evidence to confirm the proposed structure.

**Figures**

**Figure S1:** \(^1\)H-NMR Spectra for Purified Component C-5 from Column Fractionated Chloroform Fraction of *V. amygdalina* Methanol Leaf Extract
Figure S2: $^{13}$C-NMR Spectra for Purified Component C-5 from Column Fractionated Chloroform Fraction of *V. amygdalina* Methanol Leaf Extract
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**Figure S6:** Observed fragmentation pathway for 11β,13-dihydrovernolide.
Figure S7: FTIR Spectra for Purified Component C-5 from Column Fractionated Chloroform Fraction of V. amygdalina Methanol Leaf Extract.
### Table S1: Change (%) in Fasting Blood Glucose in Type-2 Diabetic Rats After Treatment with Purified Components from Column Fractionated Chloroform Fraction of *V. amygdalina* Methanol Leaf Extract (10 mg/kg bw)

| VACF purified components | 0.5 h   | 1 h     | 2 h     | 3 h     | 4 h     |
|--------------------------|---------|---------|---------|---------|---------|
| C1                       | 0.07±0.42<sup>cd</sup> | 0.84±0.48<sup>a</sup> | 1.30±0.37<sup>a</sup> | 2.10±0.64<sup>a</sup> | 3.75±1.13<sup>ab</sup> |
| C2                       | 0.59±0.23<sup>ab</sup> | 2.40±1.97<sup>a</sup> | 6.79±1.72<sup>bcde</sup> | 8.96±1.96<sup>cd</sup> | 9.50±1.75<sup>cd</sup> |
| C3                       | 0.22±0.27<sup>bcde</sup> | 2.20±1.78<sup>a</sup> | 3.77±0.86<sup>ab</sup> | 6.15±3.48<sup>bc</sup> | 5.79±3.42<sup>abc</sup> |
| C4                       | 0.91±0.15<sup>a</sup> | 1.50±0.12<sup>a</sup> | 2.42±0.38<sup>ab</sup> | 4.63±0.87<sup>ab</sup> | 4.74±0.58<sup>ab</sup> |
| C5                       | 0.20±0.21<sup>de</sup> | 5.76±3.10<sup>b</sup> | 8.63±2.67<sup>cd</sup> | 11.52±2.98<sup>d</sup> | 12.55±3.55<sup>d</sup> |
| C6                       | 0.17±0.21<sup>bcde</sup> | 0.55±0.17<sup>a</sup> | 1.87±2.01<sup>a</sup> | 2.94±1.71<sup>ab</sup> | 3.10±2.55<sup>ab</sup> |
| C7                       | 1.79±0.25<sup>f</sup> | 5.41±2.79<sup>b</sup> | 6.24±3.95<sup>bcd</sup> | 6.77±3.94<sup>bc</sup> | 6.96±4.41<sup>bc</sup> |
| C8                       | 0.49±0.30<sup>e</sup> | 1.42±0.28<sup>a</sup> | 2.62±1.04<sup>a</sup> | 3.40±1.21<sup>ab</sup> | 3.58±1.68<sup>ab</sup> |
| C9                       | 0.32±0.18<sup>bc</sup> | 1.32±0.55<sup>a</sup> | 2.48±0.74<sup>a</sup> | 3.09±0.41<sup>ab</sup> | 3.33±0.64<sup>ab</sup> |
| DC                       | 0.31±0.10<sup>bc</sup> | 1.00±0.70<sup>a</sup> | 1.15±0.73<sup>a</sup> | 1.90±0.25<sup>a</sup> | 2.11±0.35<sup>a</sup> |
| PC                       | 3.09±0.32<sup>e</sup> | 7.41±1.04<sup>b</sup> | 9.50±0.08<sup>d</sup> | 17.48±0.92<sup>e</sup> | 17.80±0.96<sup>e</sup> |

Data are presented as mean ± SD of 3 animals per group. Values with different superscript down the column indicate significant difference (*p*<0.05). VACF: Chloroform fraction of *V. amygdalina* methanol Leaf extract; PC: Positive control; DC: Diabetic control; C1, C2, … and C9: Purified components from column fractionated VACF.
Table S2: Decrease (%) in Fasting Blood Glucose in Type-2 Diabetic Rats after Treatment with Different Doses of Purified Component C5 from Column Fractionated Chloroform Fraction of *V. amygdalina* Methanol Leaf Extract

| Group | 0.5 h | 1 h    | 2 h    | 3 h   | 4 h    |
|-------|-------|--------|--------|-------|--------|
| G5    | 0.51 ± 0.21<sup>a</sup> | 1.06 ± 1.21<sup>a</sup> | 2.35 ± 1.29<sup>a</sup> | 3.96 ± 0.24<sup>b</sup> | 4.10 ± 1.57<sup>b</sup> |
| G10   | 0.70 ± 0.27<sup>a,b</sup> | 4.92 ± 2.23<sup>b</sup> | 7.84 ± 1.58<sup>b</sup> | 10.55 ± 1.19<sup>c</sup> | 11.97 ± 1.64<sup>c</sup> |
| G20   | 1.23 ± 0.24<sup>b</sup> | 5.32 ± 2.27<sup>b</sup> | 9.38 ± 1.60<sup>b,c</sup> | 13.41 ± 2.75<sup>d</sup> | 13.62 ± 1.44<sup>c</sup> |
| DC    | 0.28 ± 0.23<sup>a</sup> | 0.84 ± 0.29<sup>a</sup> | 1.02 ± 0.13<sup>a</sup> | 1.27 ± 0.16<sup>a</sup> | 1.98 ± 0.12<sup>a</sup> |
| PC    | 2.81 ± 0.82<sup>c</sup> | 6.72 ± 2.53<sup>b</sup> | 10.40 ± 1.20<sup>c</sup> | 15.21 ± 2.48<sup>d</sup> | 18.33 ± 1.53<sup>d</sup> |

Data are presented as mean ± SD of 5 animals per group. Values with different superscript down the column indicate significant difference (*p*<0.05). PC: Positive control; DC: Diabetic control; G<sub>5</sub>, G<sub>10</sub>, … and G<sub>20</sub> are diabetic rat groups treated with three different doses (5, 10 and 20 mg/kg b.w.) respectively of Purified Components C5 from Chloroform fraction of *V. amygdalina* methanol Leaf extract.
**Table S3**: $^{13}$C- NMR data for Purified Component C5 from *V. amygdalina* Methanol Leaf Extract

| Position | $^{13}$C-NMR, 125 MHz, $\delta$ | $^{13}$C-NMR, HSQC, 100 MHz, $\delta$ | DEPT | HMBC |
|----------|---------------------------------|---------------------------------|-------|------|
| 1        | 66.32                           | 66.32                           | CH    |      |
| 2        | 22.74                           | 22.70                           | CH$_2$|      |
| 3        | 33.56                           | 33.56                           | CH$_2$|      |
| 4        | 142.85                          | 142.94                          | C     |      |
| 5        | 129.23                          | 129.25                          | CH    | C15  |
| 6        | 77.31                           | 78.51                           | CH    | C4, C8, C11 |
| 7        | 57.11                           | 57.06                           | CH    |      |
| 8        | 71.50                           | 71.50                           | CH    | C16  |
| 9        | 40.79                           | 40.78                           | CH$_2$| C14  |
| 10       | 58.89                           | 59.02                           | C     |      |
| 11       | 39.93                           | 40.78                           | CH    | C12  |
| 12       | 177.54                          | 177.87                          | C     |      |
| 13       | 16.72                           | 16.75                           | CH$_3$| C7, C11, C12 |
| 14       | 99.47                           | 99.39                           | CH    |      |
| 15       | 64.30                           | 64.29                           | CH$_2$| C3, C4, C5,C14 |
| 16       | 167.96                          | 167.93                          | C     |      |
| 17       | 135.62                          | 135.63                          | C     |      |
| 18       | 18.28                           | 18.39                           | CH$_3$| C16, C17, C19, |
| 19       | 127.44                          | 127.58                          | CH$_2$| C16, C17, C18, |

*Rabe et al, 2002*
Table S4: Functional Groups Identified Using FTIR Spectra Analysis for Purified Component C-5 from Column Fractionated Chloroform Fraction of *V. amygdalina* Methanol Leaf Extract. Wavenumbers (cm$^{-1}$)

|   | Absorbance | Absorption Ranges | Functional Group |
|---|------------|-------------------|------------------|
| 1 | 3354.60    | 3100-3500         | O-H              |
| 2 | 2926.00    | 2500-3300         | C-H              |
| 3 | 1777.90    | 1670-1820         | C=O              |
| 4 | 1707.10    | 1700-1725         | C=O              |
| 5 | 1446.2     | 1440-1480         | =C=H$_2$         |
| 6 | 969.1      | 960-990           | =C=H$_2$         |
| 7 | 808.8      | 790-840           | -C=CH-           |

Functional Group region: 4000 cm$^{-1}$ - 1400 cm$^{-1}$; Finger print region: 1400 cm$^{-1}$ - 400 cm$^{-1}$
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