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Bioguided Fractionation of Hypoglycaemic Component in Methanol Extract of *Vernonia amygdalina:* An *in vivo* study

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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

1. Introduction

Type-2 diabetes is the specific and common form of diabetes mellitus that is of major dilemma to everyone in the world today, particularly in the way it deteriorates the quality of human life. It accounts for about 90% of all reported cases of diabetes and affects everyone irrespective of age (WHO, 2017, Okoduwa et al., 2015a), gender and/or socioeconomic status (Okoduwa et al., 2015b). Despite the current development of therapeutic agents, there is no effective treatment without side effects (Gheibi et al 2017). Quite a lot of drugs (such as sulfonylurea, biguanide, pioglitazone and glucosidase inhibitors) currently exist for the treatment of diabetes mellitus. But,

the use of these drugs are restricted by their pharmacokinetics properties, secondary failure rates and an accompanying side effects, such as lactic acidosis, diarrhoea and liver problem (Tariq et al., 2016, Ezuruike and Prieto, 2014). This has necessitated the scientific search for a new class of compounds of relatively less toxic natural resources to overcome diabetic problem.

Vernonia amygdalina Delile (VA) is commonly known as bitter leaf in English language due to its bitter taste. African familiar names for VA include "chusar-doki" (Hausa), "onugbu" (Igbo), "etidot" (Efik, Ijaw and Ibibio), "ewuro" (Yoruba), "oriwo" (Edo) and "ndoleh" (Cameroon) (Toyang and Verpoorte, 2013). *V. amygdalina* belong to the family Asteraceae (Compositae) and genus *Vernonia*. Traditional healers use VA leaf extract to treat diabetes mellitus (Asase and Yohonu, 2016; Ezuruike and Prieto, 2014) and as anti-malarial, anti-helminth, digestive tonic, appetizer and for treatment of wounds (Okpe et al., 2016; Ijeh, 2011). Several works have been done in the past on VA to verify its folkloric uses (Okolie et al 2008; Toyang and Verpoorte 2013). Earlier study by the authors on the fractionated extracts of the plant on a unique rat model of T2D (fortified diet-fed streptozotocin-treated (FDF-STZ) rat model of T2D) have established some significant antidiabetic properties (Okoduwa et al., 2017b). Furthermore, the toxicity of the plant extract has been studied and reported in previous research by the authors (Okoduwa et al 2017b). Therefore, the present work is an effort geared towards the bioassay-guided chromatographic analysis of the most active fraction from methanol extracts of VA with a view to isolating and identifying the most effective constituents responsible for the hypoglycaemic properties observed in the leaves of the plant.

2. Experimental

2.1 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.

2.2 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 (protocol number: AREC/EA074) 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.

2.3 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.

2.4 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).

2.5 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.

2.6 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 ^{0}C in the refrigerator until needed for further hypoglycaemic activity examination.

2.7 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.

2.8 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.

2.9 Nuclear Magnetic Resonance spectroscopy (NMR analysis

Samples of purified components were dissolved in $CDCl_3$, the NMR data were recorded at 31ºC using an Agilent-NMR-vnmrs400 instrument (Germany) operating at 400 and 100 MHz for proton and carbon (decoupled ^{13}C and DEPT), respectively,

2.10 Fourier transform infrared spectroscopy (FTIR) analysis

Samples of purified components were dissolved in $CDCl_3$, the FTIR data were recorded at 31 ºC using Agilent-FTIR, (Cary 630 FTIR, Germany) at the Multiuser Science Research Laboratory, ABU Zaria Nigeria.

2.11 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 230^0C.

2.12 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.

3. Results and discussion

Identification of the Most Potent Hypoglycaemic Component

Presented here are the 1D ^1H 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 (CH_2), two methyls (CH_3) 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.) $[M\text{-}C_3H_4O_2]^+$ due to the cleavage and lost of the pentacyclic lactone ring, the second one at *m/z* 279.1 (900 rel. ab.) $[M\text{-}C_4H_5O_2]^+$ 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.) $[M\text{-}C_4H_5O\text{-}CO_2]^+$ 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 11β,13-dihydrovernolide and give an additional evidence to confirm the proposed structure.

The Supplementary material Table S1 shows the change (%) in fasting blood glucose after oral administration of the purified components from VACF. It was observed that the purified components C5 was most active in reducing the blood glucose of diabetic rats by 12.55 % when compared to the untreated diabetic (DC) and metformin treated diabetic group (PC) with -1.99 % and 18.07 % respectively. The decrease (%) in fasting blood glucose after oral administration of the purified component C5 at three different doses (5, 10 and 20 mg/kg b.w.) respectively are presented in the Supplementary material Table S2.

The result of the spectroscopic analysis of the most active component of C5 was confirmed to be 11β,13-dihydrovernolide (see Supplementary materials Figures S1-S7 and Tables S3-S4). The compound (11β,13-dihydrovernolide) was shown in this investigation to have hypoglycaemic effect (Table S1). The compound has been isolated previously from related specie called *V. colorata,* were it was reported to exhibit little antibacterial action in respect to vernolide and vernodalin (Rabe *et al*., 2002). The FTIR spectra broad band at 3324cm^{-1} was conspicuous for the hydroxyl group and 2832.8cm^{-1} for =C-H group. The saturation of the double bond at 11-13 positions may have reduced the activity as an antibacterial observed by Rabe *et al* (2002). Although, the activity of a molecule may be related to its 3D-conformation, in the structure of 11β,13-dihydrovernolide there are one oxygen bridge between C-14 and C-15 which confer rigidity to the whole structure. Moreover, there is present also an epoxide (oxirane) function which is an extremely reactive group. This functionality may be responsible for some of its activity as observed in this study. Furthermore, a biologically active compound may have more than one specific activity. Only a few

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compounds show specific activity and this statement is valid also for drugs currently used in therapeutic purposes. For instance, the side effect of a drug is due to the drug itself which is administered to cure or treat a specific disease. The same is applicable for natural products which may be active toward several biological targets. A typical example is the case of daphnetin, a simple coumarin contained in *Daphne* spp. (Venditti et al., 2017). Dapnetin resulted to be an active anti-inflammatory (Li *et al.*, 2017), anti-cancer (Kumar *et al.*, 2016) and anti-diabetic and modulator of apoptosis pathways (Vinayagam and Xu, 2017). Also the polyphenols offer an example of the multi-target action of natural compounds. In fact, besides the well known antioxidant activity, they may exert in *in vivo* systems a very different bioactivity, such as the antiobesity effect showed by the polyphenol rich extract from *Citrus sinensis* (L.) Osbeck var. *moro* in a clinical study (Cardile et al., 2015). Another example is that of syringin which showed a dose-dependent effect on sleep induction in mice (Cui *et al.*, 2015) but exert also an anti-feedant properties against stored products insect pests (Cis *et al.*, 2006). The possibility that natural compounds could be active towards more than one cellular target does not have to be a surprise because the secondary metabolites have developed and selected in a sort of evolutionary process together with the plant species that biosynthesize and use them to protect itself from predators.

4. Conclusions

The most potent bioactive compound responsible for the hypoglycaemic activities of *V. amygdalina* was identified. This is the very first of its kind in available published literatures. The compound 11β,13-dihydrovernolide isolated from *V. amygdalina* leaves resulted to exert hypoglycaemic activity.

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Conflict of interests

The authors declare that there is no conflict of interests.

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