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Isolation and characterization of 2-hydroxy-3-[4-hydroxyphenyl]-2-propenoic acid and 4-bromophenol from anti-diabetic extract of the root bark of *Uvaria afzelii*

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ABSTRACT

The anti-diabetic activity of the methanolic extract of *Uvaria afzelii* (Annonaceae) root bark was investigated in normoglycaemic, glucose and alloxan-induced diabetic rats using glibenclamide (5 mg/kg) and normal saline as positive and negative controls respectively. The acute oral toxicity test of the extract in rats using Lorke's method showed LD_{50} of 693 mg/kg. The results showed that the extract at the tested doses (100, 200 and 400 mg/kg), gave a statistically lower hypoglycaemic activity ($p < 0.05$) than glibenclamide (5 mg/kg) in normoglycaemic rats. In glucose-loaded hyperglycaemic rats, the extract elicited a dose dependent activity with 400 mg/kg showing comparable activity ($p > 0.05$) with glibenclamide (5 mg/kg) at 1 h. The extract (400 m/kg) caused 75% blood glucose level reduction in a 14 day treatment in alloxan-induced diabetic rats that was significantly higher than the 58% of glibenclamide (5 mg/kg). The ethyl acetate fraction (200 and 400 mg/kg) gave a comparable activity with glibenclamide (5 mg/kg) at all-time points in glucose loaded rats while other fractions lacked appreciable activity. The ethyl acetate fraction afforded two compounds namely; 2-hydroxy-3-(4-hydroxyphenyl) acrylic acid (**1**) and 4-bromophenol (**2**). The structures of these compounds were elucidated and characterized by Infrared spectroscopy (IR), 1D- and 2D-Nuclear Magnetic Resonance spectroscopy (NMR) and Mass spectrometry (MS).

1. Introduction

Diabetes mellitus (DM) is a metabolic disorder characterized by the presence of chronic hyperglycaemia resulting from a deficiency of insulin secretion, abnormalities of insulin action on target tissues, or combination of the two (Tedong, 2006; Zheng et al., 2007; Adebajo et al., 2013a, 2013b). About 382 million people were estimated to be diabetic globally in 2013 and this number was estimated to increase to 592 million by 2035. Type 2 DM made up about 90% of the reported cases (Vos et al., 2012; American Diabetes Association, 2013). The serious macro and micro vascular complications of DM such as coronary artery disease, stroke, diabetic nephropathy, neuropathy, retinopathy and adverse effects of the available synthetic hypoglycaemic agents have led to increased investigations of ethno-medicinal plants for anti-diabetic activity (Olayiwola et al., 2004; Rang et al., 2007; Adebajo et al., 2013a, 2013b).

Uvaria afzelii Scot Elliot (Annonaceae) is a shrub that grows widely in West Africa especially in the South and Eastern part of Nigeria where it is known as "gbogbonishe" (Yoruba) and "Umimi ofia" (Igbo) (Odugbemi, 2008). It is used traditionally in the treatment of cough, vaginal tumour, breast aches, swollen hands and feet, diabetes as well as leucorrhoea and gonorrhoea (Burkil, 1985; Odugbemi, 2008; Kayode et al., 2011). The anti-tubercular, antimicrobial, (Lawal et al., 2011), hepatoprotective, (Ofeimun et al., 2013), bactericidal, anti-helminthic and anti-parasitic (Okoli, 2004; Okpekon et al., 2004; Lawal et al., 2011) activities have been reported. However its anti-diabetic activity has not been scientifically validated, hence this present work.

Abbreviations: DM, diabetes mellitus; LD_{50}, lethal dose that killed 50% of the rats; bg, blood glucose; FBG, fasting blood glucose; T_o, 0 h; TLC, thin layer chromatography; ^1H NMR, proton nuclear magnetic resonance spectroscopy; ^{13}C NMR, carbon-13 nuclear magnetic resonance spectroscopy; DEPT135, distortionless enhancement by polarization transfer; HMQC, heteronuclear multiple quantum coherence; HMBC, heteronuclear multiple bond coherence; 1D-NMR, one-dimensional nuclear magnetic resonance spectroscopy; 2D-NMR, two-dimensional nuclear magnetic resonance spectroscopy; s, singlet; d, doublet; dd, doublet of doublets; MS, mass spectrometry; HREIMS, high resolution electron impact mass spectrometer; UV, ultra violet; KBr, potassium bromide; FTIR, Fourier transform infrared instrument; Hz, hertz; CD_3COCD_3, deuteriated acetone; hex, n-hexane; DCM, dichloromethane; EtoAC, ethyl acetate; MeOH, methanol.

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2. Methodology

2.1. General

NMR spectra were measured on Bruker DMX Avance 300 instrument using CD_3OD as solvent and internal standard. HREIMS spectra were determined on a Waters GCT-Premier spectrometer. IR was run on Shimadzu FTIR-8400S using KBr. For TLC, pre-coated silica gel $60F_{254}$ plates were used and compounds were detected under Ultraviolet (UV) lamp (254 and 366 nm) and further visualized by spraying with vanillin-sulfuric acid solution. CareSens TM N Glucometer (model PGA 1E3028 REV3 (i-SENS, Inc., Korea) with CareSens TM test strips (i-SENS, Inc., Korea). Alloxan monohydrate (Sigma-Aldrich Co. LLC, U.S.A.).

2.2. Plant material

The roots were collected from the zoological garden of Obafemi Awolowo University, Ile-Ife, Nigeria. The plant was identified and authenticated by Mr. Ademoriyo and a voucher specimen (IFE16941) was deposited at the Herbarium of the Department of Botany, Obafemi Awolowo University, Ile-Ife.

2.3. Extraction and partitioning

The roots of the plant were peeled and the barks oven dried at about 40 °C, powdered and 1.5 kg of the powder was extracted exhaustively with methanol. The methanolic solution was evaporated at about 40 °C under reduced pressure using rotator evaporator to obtain an extract (142.9 g, **A**) which was taken up in 400 mL of methanol/water (1:1). The aqueous methanol solution was partitioned with n-hexane (4 × 400 mL), dichloromethane (5 × 400 mL) and ethyl acetate (5 × 400 mL) to obtain their corresponding n-hexane (13.4 g, $\mathbf{B_1}$), dichloromethane (1.6 g, $\mathbf{B_2}$), ethyl acetate (3.7 g, $\mathbf{B_3}$) and aqueous methanol (123.3 g, $\mathbf{B_4}$) fractions.

2.4. Bioassays

2.4.1. Animals

Healthy albino rats (150–270 g) of both sexes bred under standard conditions (temp. 27 ± 3 °C, relative humidity 65%) at the animal house, Department of Pharmacology, Faculty of Pharmacy, O.A.U., Ile-Ife, Nigeria were used for the experiment. They were fed on a standard pellet diet (Bendel Feeds, Nigeria) and water was given *ad libitum*.

2.4.2. Hypoglycaemic effect of the extract

Five groups of five normoglycaemic rats each were fasted for 24 h and orally administered with either 1% Tween 80 (negative control), extract (100, 200, 400 mg/kg), or glibenclamide (5 mg/kg), (positive control) (Adebajo et al., 2013a, 2013b). A drop of blood taken from the tip of the tail of each rat at 0.0, 0.5, 1.0, 2.0 and 4.0 h was dropped onto a glucometer strip and the blood glucose (bg) level read off directly. The blood glucose levels at 0.0 h (T_o) were taken as 100% while those at other times were expresses as percentage of these values (Adebajo et al., 2013a).

2.4.3. Effect of the extract in alloxan-induced rats

Diabetic rats were obtained by intraperitoneal injection of alloxan monohydrate (150 mg/kg) dissolved in normal saline and 1% Tween 80 according to (Ojezele and Abatan, 2011). Rats with fasting blood glucose (FBG) level ≥ 11.0 mmol/L (200 mg/dL) 6 days after alloxanisation were considered diabetic and were divided into three groups of five rats each. They were administered daily for 14 days with 1% Tween 80/extract/glibenclamide (5 mg/kg). Their FBG levels were determined on days 1, 4, 7, 10 and 14 as given in Section 2.4.2.

2.4.4. Effects of extract and partition fractions in glucose-loaded rats

A glucose tolerance test was performed by oral administration of glucose (10 g/kg) to 24 h fasted rats. Hyperglycaemic rats with blood glucose level ≥ 7.0 mmol/L (126 mg/dL) after 0.5 h (time point 0, T_o) were divided into five groups of five rats each and administered orally with 1% Tween 80, extract (100, 200, 400 mg/kg) and glibenclamide (5 mg/kg). Their blood glucose levels were determined at 0, 0.5, 1, 2 and 4 h after administration as demonstrated in Section 2.4.2. All values were normalised to blood glucose at T_o taken as 100% (Adebajo et al., 2013a, 2013b). The partition fractions ($\mathbf{B_1}$–$\mathbf{B_4}$) were similarly tested for their hyperglycaemia-lowering activity.

2.4.5. Statistical analysis

Data were expressed as the mean ± SEM for the number (N) of animals in the group. Analysis of variance (ANOVA) was used, followed by Dunnett and Bonferroni t-test *post hoc* comparisons to determine the source of significant differences for all determinations. $p < 0.05$ was considered to be significant.

2.5. Isolation of compounds and their spectroscopic data

The ethyl acetate fraction, $\mathbf{B_3}$ (3.7 g), was dissolved in a suitable organic solvent and the solution was adsorbed on silica gel (3.8 g) and left to dry. The adsorbed fraction was packed into glass column dry and gradient elution from n-hexane (hex) through dichloromethane (DCM), ethyl acetate (EtoAC) to methanol (MeOH) was carried out. About 5 mL of eluent were collected into each test tube, the 157 fractions collected were bulked based on their TLC profile to obtain 20 bulked fractions.

An impure solid (36.0 mg) was collected from the fraction eluted with DCM-EtoAc 60:40. Repeated preparative thin layer chromatography (PTLC) of this solid using DCM-EtoAc (1:4, 50 mL) as solvent system yielded compound **1** (22 mg). In the fraction eluted with DCM-EtoAc 40:60, another impure constituent (210.0 mg) was recovered. This was further subjected to column chromatography with gradient elution using hex, DCM, EtoAc and MeOH. An impure solid (79.9 mg) was eluted with 100% DCM. It was further purified by PTLC using DCM-EtoAc (1:4, 50 mL) as solvent system to yield compound **2** (9 mg).

2.5.1. 2-Hydroxy-3-[4-hydroxyphenyl]-2-propenoic acid (*1*)

Brown powder; IR (KBr): υ_{max} (cm^{-1}) 3370, 3231, 1717, 1682, 1601, 1528, 1292. UV (MeOD), λ_{max} (nm) 314, 236. ^1H and ^{13}C NMR: Table 1. MS (EI, 70 eV): m/z (%), 180.0429 $[M]^+$ (100), 181.0456 (9.7). HREIMS: m/z $[M]^+$ calcd for $C_9H_8O_4$, 180.0423; found 180.0429

2.5.2. 4-Bromophenol (*2*)

White powder. ^1H and ^{13}C NMR: Table 2. MS (EI, 70 eV): m/z (100), 171.9430 $[M]^+$ (100). HREIMS; m/z $[M]^+$ calcd for C_6H_5BrO, 171.9425; found 171.9430

Table 1
^1H and ^{13}C NMR data for compound **1** in MeOD (multiplicities and J values are given in Hz in the parenthesis).

Position	δ_H	δc	HMBC[a]
1	–	168.9	
2	–	144.7	
3	7.47 (s)	116.3	1, 2, 4, 5
4	–	121.8	
5 & 9	7.46 (dd, 1.5 & 7.2)	122.5[b]	
6 & 8	6.81 (dd, 1.5 & 7.2)	114.4[b]	4, 5, 7
7	–	150.1	

[a] The numbers are the carbons to which the proton had a correlation.
[b] The intensities of these signals were doubled and represent two carbon atoms each.

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