

ANTIHYPERGLYCEMIC PROFILE OF ERINIDINE ISOLATED FROM *HUNTERIA UMBELLATA* SEEDAdeneye Adejuwon Adewale^{1,2*}, Crooks Peter Anthony², Fadhel-Albayati Zaine², Miller Anne-Frances³, Zito S. Williams⁴, Adeyemi Olufunmilayo Olaide⁵, Agbaje Esther Oluwatoyin⁵

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E-mail: adeneye2001@yahoo.com, aad228@uky.edu**Abstract**

Water decoction made from the seed of *Hunteria umbellata* is widely used in the traditional management of diabetes mellitus by Nigerian herbalists, particularly, in the southwest region of the country. Recently, a new bisindole alkaloid, erinidine, was isolated but its antihyperglycemic profile remains largely un-investigated scientifically. This forms the basis for the current study which is primarily designed at investigating the antihyperglycemic profile of erinidine and other fractions in both *in vitro* and *in vivo* models of diabetes mellitus. In the present study, erinidine was isolated and purified using the earlier described methods and its antihyperglycemic potentials tested in *in vitro* models such as dipeptidylpeptidase (IV), glycogen phosphorylase, HIT-T15 cell insulin secretion, glucose uptake activity, aldose reductase assays and α -glucosidase inhibition assay testings. In addition, 50 mg/kg of erinidine and that of other fractions were evaluated in *in vivo* models of normal and chemically-induced hyperglycemic rats. Results showed that erinidine was a light yellow, amorphous solid with UV (CHCl₃) λ_{max} 256 nm, HRESIMS m/z 382.1881 [(M+H)⁺] (calculated for C₂₂H₂₆N₄O₂, 382.1876) and melting point of 230 °C. The *in vitro* study showed the antihyperglycemic action of erinidine to be weakly mediated via α -glucosidase inhibition mechanism as the results for other *in vitro* tests such as dipeptidylpeptidase (IV), glycogen phosphorylase, HIT-T15 cell insulin secretion, glucose uptake activity and aldose reductase assays were all negative. However, the *in vivo* results showed 50 mg/kg erinidine given *per os* to normal and alloxan-induced hyperglycemic rats to significantly ($p < 0.05$, $p < 0.001$) attenuate an increase in their post-absorptive blood glucose concentrations after 3 g/kg glucose loading in the rats, suggesting its antihyperglycemic mechanism to be via α -glucosidase inhibition. This result, although, further corroborated the *in vitro* findings but also suggests that erinidine needs to be bio-transformed *in vivo* for its inhibitory activity on intestinal glucose absorption to become evident. Thus, the present study suggests erinidine to be the possible antihyperglycemic agent in *Hunteria umbellata* seed extract mediating its antihyperglycemic action via intestinal glucose uptake inhibition.

Key words: *Hunteria umbellata*, Erinidine, Hyperglycemia, Alpha-glucosidase inhibition**List of Abbreviations**

ACTH – adrenocorticotropic hormone; ASW – automatic switchable; CHCl₃ – chloroform; ¹²CNMR – carbon nuclear magnetic resonance; DCM – dichloromethane; DM – diabetes mellitus; DMEM – Dulbecco's Modified Eagle medium; DMSO – dimethylsulfoxide; DPBS – Dulbecco's phosphate buffer solution; DPP-IV – dipeptidylpeptidase-IV; ELISA – enzyme-linked immunosorbent assay; Gpa – glycogen phosphorylase; HBSS – Hank's balanced salt solution; HCl – hydrochloric acid; HIT-T15 – Insulin-secreting hamster insulinoma; HU_{Af} – crude alkaloid fraction from the aqueous seed extract of *Hunteria umbellata*; HU_n – neutral compounds from the aqueous seed extract of *Hunteria umbellata*; ¹HNMR – proton nuclear magnetic resonance; HRESIMS – high resolution electron impact ionization mass spectrometer; IIE ID – Institute of International Education Grant Identification Number; INS-1 – rat (*Rattus norvegicus*) insulinoma cell line; i.p. – intraperitoneal; KBr – potassium bromide; LC-MS – liquid chromatography-mass spectrometry; MeOH – methanol; MIN6 – stably transformed insulinoma cell lines; NADPH – reduced nicotinamide adenine dinucleotide phosphate; Na₂CO₃ – sodium bicarbonate; PBS – phosphate buffer solution; PGLUM – phosphoglucomutase; pNPG – p-nitrophenyl-D-glucopyranoside; p.o. – per oral; RIN β -cells – permanent rat insulinoma beta-cells; UK IACUC – University of Kentucky Institutional Animal Care and Use Committee; 3T3-L1 cell – cell lines derived from 3T3 cell with adipocyte-like phenotype; β -TC-6 cell – murine SV-40 T-antigen-transformed pancreatic beta cell

Introduction

Diabetes mellitus (DM) is a metabolic disorder characterized by chronic hyperglycemia and glucose intolerance, either due to insulin deficiency or impaired effectiveness of insulin's action, or to a combination of both factors (Deutschländer, 2009). The global prevalence of DM was estimated at 2.8% in 2000 (171 million people) and this is expected to rise to 9.9% (552 million people) of adult population by the year 2030 if no urgent efforts are directed at stemming the tide of this global public health burden (Whiting et al., 2011). It is also predicted that by 2030, India, China and the United States will have the largest number of diabetes sufferers (Wild et al., 2004). However, the burden of the disease is high, not only because life-long treatment is necessary but also due to the prohibitive cost and unavailability of orthodox/conventional treatment options in rural areas. In the effective management of DM, combination therapy is often employed, particularly, in the management of type 2 diabetes mellitus (Gerich, 2001). In 2007, World Health Organization reported that one of the strategies to be adopted in combating/stemming the tide of the disease is to support research in community interventions, including traditional and herbal medicine, for which reason the search for new anti-diabetic agents continues (World Health Organization, 2007). One of such herbal plants used in the local management of diabetes mellitus among the Yoruba herbalists in Southwest Nigeria is *Hunteria umbellata*.

Hunteria umbellata (K. Schum.) Hallier f. (locally known as "Abeere") (family: Apocynaceae) is tropical rainforest tree which is popularly used for the local management of diabetes mellitus and obesity (Adegoke and Alo, 1986; Falodun et al., 2006; Adeneye and Adeyemi, 2009a). In African folk medicine, water decoction made from the dry seeds of *Hunteria umbellata* is employed in the local management of diabetes and obesity (Adegoke and Alo, 1986; Adeneye and Adeyemi, 2009a; Igbe et al., 2009). The crude aqueous extract of the plant have been shown to possess antihyperglycemic (Adeneye and Adeyemi, 2009a, 2009b; Igbe et al., 2009), anti-obesity and antihyperlipidemic (Adeneye et al., 2010a), anti-inflammatory and antioxidant (Adeneye et al., 2011) with the crude alkaloid fraction found to be its principal antihyperglycemic, anti-inflammatory and antioxidant agent (Adeneye et al., 2011; Adeneye et al., 2012a). The aqueous seed extract of the plant has also been reported to be relatively safe when administered orally (Ibeh et al., 2007; Adeneye et al., 2010b).

Recently we isolated a new bisindole alkaloid, erinidine (Figure 1), from the crude aqueous seed and alkaloid extracts of *Hunteria umbellata* (Adeneye et al., 2012b). Literature search indicated that till date, there is no scientific report on the screening of antihyperglycemic potential of erinidine. Thus, this forms the basis for the current study which screens erinidine and, indeed other fractions, for their possible antihyperglycemic potentials in both *in vitro* and *in vivo* models of diabetes mellitus. However, the choice of the therapeutic dose (50 mg/kg) of erinidine and other fractions screened was based on results of preliminary test earlier conducted. For the purpose of clarity, of the fractions of *Hunteria umbellata* extract investigated, more emphasis was placed on erinidine, particularly, in the *in vitro* studies, erinidine being the only pure and new fractions obtained.

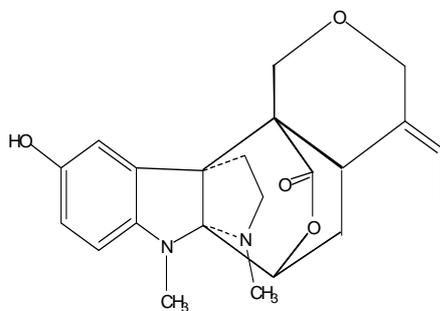


Figure 1: Chemical structure of erinidine from *Hunteria umbellata* seed

Materials and methods

Plant materials

Eight fresh mature fruits of *Hunteria umbellata* were collected from the deciduous forest of Odorasanyin District of Ijebu-Igbo in Ogun State, Nigeria, in the month of June 2010. From these, 3 kg of fresh seeds was harvested, rinsed in tap water and air-dried at room temperature (25 ± 1 °C) for 1 month, protected from direct heat and sunlight. Plant identification and deposit of voucher specimen were done as earlier described by Adeneye and Adeyemi (2009a). Two kilogram of the dried seed was pulverized into fine powder using Laboratory Hammer Mill at the Department of Plant Sciences, College of Agriculture, University of Kentucky, U.S.A. The powdered sample was kept in a thick water- and air-proof transparent white polythene bag and stored in the refrigerator at 4 °C.

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Aqueous extraction process

60 g of the pulverized *Hunteria umbellata* seed was completely cold extracted in 1L of distilled water after dissolving it in distilled water and kept in the refrigerator for 72 hours. The solution was continuously stirred using magnetic stirrer for 6 hours after which it was filtered using Celite (Analytical filter-aid)-packed filter funnel. The deep brown filtrate was completely dried *in vacuo* using a freeze dryer (LABCONCO®FreeZone 18 Liter Console Freeze Dry Systems, Labconco Corporation, Kansas City, MO, USA) to give a deep brown, sweet-smelling fluffy residue. This procedure was repeated for 6 more times and the residues were pooled into a water- and air- proof container and kept in the freezer to prevent decomposition of the extract.

Isolation of erinidine

10 g of *Hunteria* seed water extract (HU) in 10 ml of distilled water (pH = 4.2) was repeatedly titrated with 50 ml of 5% aqueous HCl solution (Aldrich-Sigma, St. Louis, MO, U.S.A.) to acidify the solution to a pH of 2. The acidify solution was extracted with ethyl acetate (150 ml x 3) to remove the neutral compounds (HU_n) in the acidify HU solution. The acidify HU solution was then carefully basified with 5% sodium bicarbonate (Na₂CO₃) (Aldrich-Sigma, St. Louis, MO, U.S.A.) solution to a pH of 10. Using a 5 L separating funnel, the mixture was repeatedly extracted with small portions of ethyl acetate (Aldrich-Sigma, St. Louis, MO, U.S.A.) (150 ml x 3) until the last extract was colourless and the basic solution gave negative tests with alkaloid detecting (Dragendorff's) reagents. The ethyl acetate extract obtained was evaporated to complete dryness using rotary evaporator coupled with a water bath (BUCHI Rotavapor Model R-215, BUCHI Labortechnik AG, Flawil, Switzerland) preset at 40 °C to give the alkaloid fraction (HU_{Af}) (a yellow-brown solid residue) weighing 0.81 g (yield: 8.10%). HU_n solution was also completely dried off using this same procedure described for HU_{Af} giving a chocolate-colour fine powder weighing 0.378 g (yield: 3.78%). This procedure was repeated five times. The HU_n and HU_{Af} were separately pooled into tight-capped containers which were stored in the refrigerator until required for experiment. 350 mg of HU_{Af} was column purified on silica gel flash column into 40 fractions using DCM (Sigma Chemical Co., St. Louis, MO, USA) and MeOH (Sigma Chemical Co., St. Louis, MO, USA) solvent system (5:1) to obtain fractions 16-25 which were of similar chromatographic pattern were pooled and completely dried *in vacuo* using rotor evaporator, yielding a pale yellow powder (erinidine). This procedure was repeated for 10 more times.

Instrumentation

The melting point of alkaloid fractions 16-25 (erinidine) was determined using Fisher-Johns Melting Point Apparatus (Thermo Fisher Scientific, Park Lane Drive, Pittsburgh, U.S.A.). IR spectra were recorded on a Shimadzu FT IR 8300 spectrophotometer (Vmax in cm⁻¹, using KBr pellets) while its LC-MS studies were done on Agilent 6200 Series Time-of-Flight LC-MS Systems (Agilent Technologies, Elmhurst, Illinois, U.S.A.). Erinidine was re-dissolved in dry deuterated chloroform (Cambridge Isotope Labs DLM-7) and its ¹H NMR spectra were collected at 600 MHz for ¹H on a Varian-Agilent INOVA spectrometer equipped with a 5 mm bioprobe at 30 °C. ¹³C spectra were collected at 100 MHz for ¹³C on a Varian-Agilent INOVA Spectrometer equipped with a 5 mm ASW probe (automatic switchable) at 30 °C and 9 kHz ¹H decoupling. Chemical shifts are reported in parts per million (ppm) relative to TMS in deuterated CHCl₃ solution. High resolution electron impact ionization mass spectra (HRESIMS) were recorded at 25 eV on a JEOL JMS-700T MStation (magnetic sector instrument) at a resolution of greater than 10000. Samples were introduced via heatable direct probe inlet and Perfluorokerosene (pfk) was used to produce reference masses.

In vitro antihyperglycemic studies of erinidine

Alpha glucosidase assay

Alpha-glucosidase from Baker's yeast (Type I) and p-nitrophenyl-D-glucopyranoside (pNPG) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Other reagents were of analytical grade and were used without further purification. 2 µL of 1–2.5 U/mL of alpha-glucosidase {from Baker's yeast (Type I)} was premixed with 20 µL of erinidine at varying concentrations and incubated for 5 min at 37 °C. 20 µL of 1 mM pNPG (Sigma Chemical Co., St. Louis, MO, USA) in 50 mM of phosphate buffer was added to initiate the reaction, and the mixture was further incubated at 37 °C for 20 min. The reaction was terminated by the addition of 50 µL of 1M Na₂CO₃, and the final volume was made up to 150 µL. Alpha glucosidase activity was determined spectrophotometrically at 405 nm on a Biorad 550 microplate reader (Hercules, California, U.S.A.) by measuring the quantity of p-nitrophenol released from pNPG. The assay was performed in triplicate with appropriate blanks and controls.

Dipeptidylpeptidase-IV (DPP-IV) assay

DPP-IV, Gly-Pro-4-Nitroanilide, Diprotin A (positive control) and Trizma base were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). The 96 well plates were purchased from VWR International Inc. The 96 well plate reader was purchased from Bio-Rad Laboratories, Inc. (Richmond, CA, USA). 6.5 mg of erinidine was weighed and dissolved in 13 ml 0.1 M Tris:DMSO (1:1) at pH 8.0 to produce a 0.5 mg/ml solution. Serial dilutions of the sample were made in PBS (0.250, 0.125, 0.063, 0.031 and 0.008 mg/ml). The enzyme solution was prepared to a concentration of 0.055 unit/mL (pH = 8.0) in 0.1 M

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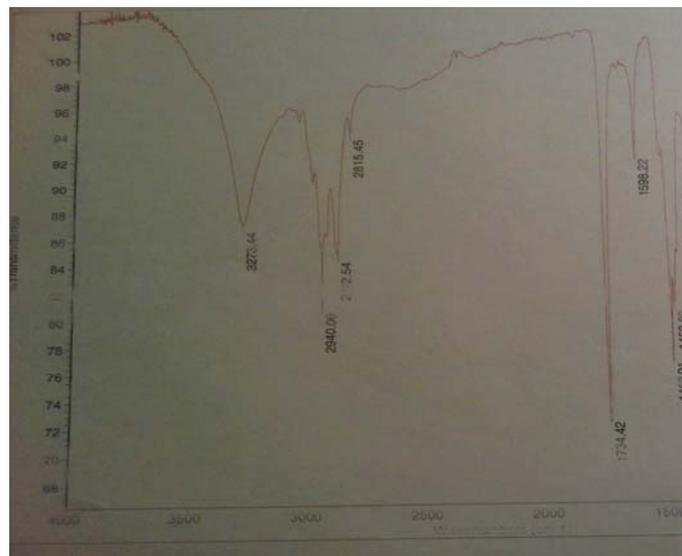


Figure 2: Infrared (IR) spectrum of erinidine of *Hunteria umbellata* seed alkaloids showing the ν_{\max} at 3273, 2940, 2872 and 1734 cm^{-1}

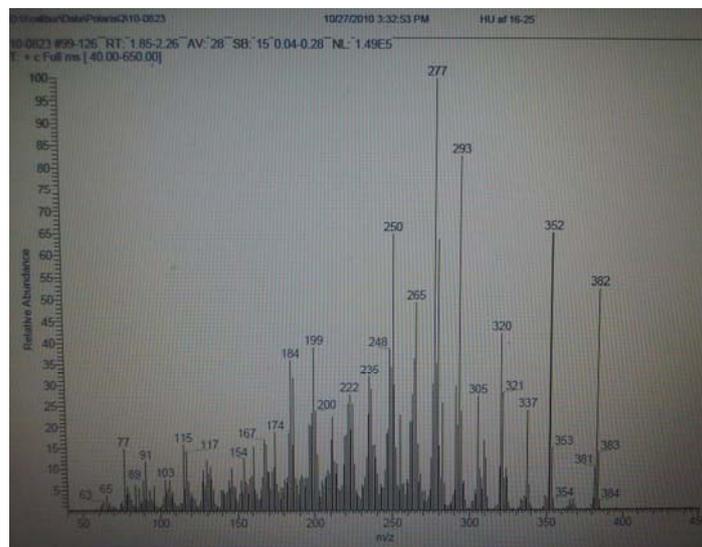


Figure 3: Full mass spectrum of erinidine of *Hunteria umbellata* seed

Tris buffer. One unit will produce 1 μmol of *p*-nitroaniline from Gly-Pro-*p*-nitroanilide per min in 0.1 M Tris buffer (pH = 8.0) at 37 °C. A 1 mM stock solution of Gly-Pro-*p*-nitroanilide (M.W. 328.75; Sigma Aldrich chemicals, St. Louis, MO, U.S.A.) was prepared at pH 8.0 with 0.1 M Tris buffer. The assay was run by mixing 15 μL of 0.1 M Tris buffer, 25 μL of enzyme solution and 10 μL of control or erinidine samples. Then the mixture was incubated at 37 °C for 5 min. After that 50 μL of Gly-Pro-*p*-nitroanilide substrate was pipetted into the appropriate wells to commence the reaction between enzyme and

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substrate. The reaction mixture was incubated at 37 °C for 15 min. The reaction was terminated by adding 25 µL of 25% acetic acid to the appropriate wells. The absorbance of the resulting end product was measured at 405 nm. The assay was performed in triplicate with appropriate blanks and controls.

Glycogen phosphorylase assay

6.5 mg of erinidine was weighed and dissolved in 6.5 ml of PBS, and the sample was not soluble (1 mg/ml). 6.5 ml of DMSO was added and the sample was soluble (0.5 mg/ml). Then two times and four times dilutions of the sample were made in PBS to get 0.250 mg/ml, 0.125 mg/ml of erinidine sample. The cuvette containing 2.7 ml of cocktail, 0.1 ml of glucose-6-phosphate dehydrogenase (1 unit), 0.1 ml of Phosphoglucomutase (PGLUM) (1 unit), 0.1 ml of DMSO (25% or 12.5%), and 0.1 ml of glycogen phosphorylase *a* (Gpa), to a final volume of 3.1 ml, was read against a reference cuvette containing 0.1 ml of diluent instead of Gpa. This serves as the negative control. The sample cuvette containing 2.7 ml of cocktail, 0.1 ml of glucose-6-phosphate dehydrogenase (1 unit), 0.1 ml of PGLUM (1 unit), 0.1 ml of erinidine solution was dissolved in 25% or 12.5% DMSO (0.250 mg/ml or 0.125 mg/ml), and 0.1 ml of glycogen phosphorylase *a* (Gpa), to a final volume of 3.1 ml, was read against a reference cuvette containing 0.1 ml of diluent instead of Gpa. This is used to test the inhibitory activity of erinidine sample on Gpa. The enzymatic reaction was started by the addition of the Gpa and absorbance was recorded at 340 nm for 10 min at 60 s interval. Glycogen phosphorylase activity was expressed as change in units/ml/min of enzyme and erinidine inhibitory activity also takes into consideration of the negative control.

Insulin secretion in cultures of HIT-T15 cells

HIT-T15 cells and tissue labware which were used for this study were purchased from VWR (West Chester, PA, USA). All other reagents used were of analytical grade and purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 5.0 mg of erinidine was weighed and dissolved in 5.0 ml dimethyl sulfoxide (DMSO) to a concentration of 1 mg/ml. and then diluted in Hank's balanced salt solution (HBSS) to obtain 0.25 mg/ml and 0.125 mg/ml samples. 25% and 12.5% DMSO in HBSS were used as blank. Samples were filtered through 0.45 µm sterile filters. HIT-T15 cells were seeded into collagen-coated sterile 96-well plates at a density of 7.5×10^4 cells per well. The HIT-T15 cells were cultured in 200 µl in 300 cm³ vented cap tissue culture flasks using Hams F-12K modified nutrient mixture supplemented with 10 % dialyzed horse serum (Life Technologies, Rockville, MD, USA) and 2.5 % bovine fetal serum (Life Technologies, Rockville, MD, USA). The cells in the plate were incubated and maintained at 37 °C for 24 hr in an atmosphere of humidified air and CO₂ (95:5) to allow for cell attachment and recovery. The plate with attached cells was removed from the incubator. The spent medium was aspirated and replaced with 200 µl of HBSS, which was removed and replaced with fresh 100 µl of HBSS. The cells were incubated for 30 min at 37 °C. Then 100 µl of blank and sample were added to wells after HBSS was removed from wells. The plate was incubated for 1 hr and resulting insulin concentrations in individual wells were quantified using insulin enzyme-linked immunosorbent assay (ELISA) kit (American Laboratory Products Co., Windham, NH, USA) according to the manufacturer's recommendations. The potential loss of cells due to multiple liquid aspiration/dispensing cycles was evaluated by counting the cells in randomly selected wells following completion of the incubation. The effect of erinidine on HIT-T15 cell membrane permeability and integrity was assessed by the Tryptan Blue method. Upon completion of inhibition, a diluted solution of Tryptan Blue was added to the selected wells. Stained and colorless cells were counted under a microscope, the objective of which was fitted with a counting field frame (Rotshteyn and Zito, 2004).

Glucose uptake activity assay

Glucose uptake activity was analyzed by measuring the uptake of 2-deoxy-D-[3H] glucose. The confluent 3T3-L1 adipocytes grown in 24-well plates were washed three times with serum-free Dulbecco's Modified Eagle Medium (DMEM) and incubated with 1 ml of the same medium at 37 °C for 2 hr. The cells were washed 3 times with Dulbecco's phosphate buffer saline (DPBS) and incubated with 0.8 ml DPBS at 37 °C for 30 min. 0.1 ml DPBS (control), Insulin (10 µM) and test compound were then added and adipocytes were incubated at 37 °C for 10 min. Glucose uptake was initiated by the addition of 0.1 ml of 10 µCi/ml 2-deoxy-D-[³H] glucose in DPBS, and 2-deoxy-D-[³H] glucose was 1 µCi/ml as final concentrations. After 10 min, glucose uptake was terminated by washing the cells 3 times with cold phosphate buffer saline (PBS). The cells were lysed with 0.7 ml of 1 % Triton X-100 at 37 °C for 60 min, shaking every 20 min to make sure the cells were fully lysed. The radioactivity retained by the cell lysate was determined by a scintillation counter with a scintillation fluid, ScintiSafe 30% cocktail.

Aldose reductase assay

6.5 mg of erinidine was weighed and dissolved in 13 ml of 50 mM PBS:DMSO (1:1) at pH of 6.8 to produce a 0.5 mg/ml solution. Serial dilutions of the sample were made in PBS (0.250, 0.125, 0.063, 0.031 and 0.008 mg/ml). Six rat lenses were pooled and 10% homogenate was prepared in 0.1M phosphate buffer saline (pH=7.4). After centrifugation at 5000 g for 10 min, the supernatant was collected and kept in ice to determine aldose reductase activity. The assay procedure adopted is summarized below as follows: A sample cuvette containing 0.7 ml of phosphate buffer (0.1 M), 0.1 ml of NADPH (25×10^{-5} M), 0.1 ml of lens supernatant, 0.1 ml of DL-Glyceraldehyde (5×10^{-4} M) to a final volume of 1 ml was read against a reference cuvette containing all components except the substrate. The enzymatic reaction was started by the addition of the substrate and

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absorbance was recorded at 340 nm for 7 min at 30 s interval. Aldose reductase activity was expressed as change in absorbance/min/mg of protein.

***In vivo* antihyperglycemic studies**

Experimental Animals

A total of one hundred and twenty young adult male Wistar rats (weight range: 210-250 g) were obtained in 3 batches after ethical approval (UK IACUC no.: 2010-077) has been obtained from the Institutional Ethical Committee on the Use of Experimental Animals in University of Kentucky, U.S.A. The rats were acclimatized for 7 days, fed on standard rat chow and tap water *ad libitum*. The rats were housed in a standard rat cages in the Animal Facility of the College of Pharmacy, University of Kentucky, and maintained at standard laboratory conditions (12/12 hr light-dark periodicity, temperature: 23-26 °C) as prescribed by the United States National Institute for Health (1985). Two days prior to commencement of the experiment, rats were randomly divided into 6 groups of 6 rats per treatment group such that the weight differences within and between treatment groups do not exceed $\pm 20\%$ of the average weight of the rat population, respectively.

Oral glucose tolerance test in normal rats

Rats were fasted overnight for 12-14 hr prior to commencement of the animal experiment. Their baseline fasting blood glucose values were obtained before oral treatment with the drugs or β -D (+) glucose (97% anhydrous (Sigma Chemical Company, St. Louis, MO, USA) dissolved in 10 ml/kg distilled water-Tween 80 (9:1). Group I rats, which served as the untreated control, were orally pretreated with 10 ml/kg 10% Tween 80-distilled water 1 hr before treatment with another 10 ml/kg *per os* 10% Tween 80-distilled water. Group II rats served as the model control and were pretreated with 10 ml/kg *per os* 10% Tween 80-distilled water 1 hr before oral treatment with 3 g/kg D-glucose. Groups III - VI rats which served as the treatment groups were orally pretreated with 50 mg/kg HU, HU_{AF}, HU_n and erinidine, respectively, for 1 hr before the oral administration of 3 g/kg D-glucose, as described by Sepici et al. (2004) and Adeneye and Adeyemi (2009a). The choice of 10% Tween 80-distilled water as the vehicle was made because the test compounds-HU_{AF}, HU_n and erinidine were incompletely soluble in distilled water alone.

Oral glucose tolerance test in alloxan-induced hyperglycemic rats

Following a 24-hr fast, rats were made hyperglycemic by injecting each rat with a single intraperitoneal dose of 120 mg/kg of alloxan monohydrate (Sigma Chemical Company, St. Louis, U.S.A.) dissolved in 3 mM of freshly prepared cold citrate buffer (pH = 4.5). The baseline fasting blood glucose was first determined before alloxan treatment. Six hours after alloxan injection, rats were orally infused with 20% Dextrose (Unique Pharmaceuticals, Sango-Otta, Ogun State, Nigeria) at an oral dose of 10 ml/kg so as to prevent the onset of fatal hypoglycemia which often accompanies administration of alloxan as a result of acute massive pancreatic release of insulin (Prince and Menon, 2001). Gradual onset of hyperglycemia was confirmed on the 3rd day post-induction but a steady hyperglycemic state was achieved by the 5th day post-alloxan treatment. By the 5th day, rats with fasting blood glucose of equal or greater than 200 mg/dl were considered hyperglycemic. Six normal and thirty-six alloxan-induced hyperglycemic rats were randomly allocated into 7 treatment groups as follows:

Group I consists of normoglycemic rats which were orally treated with 10 ml/kg 10% Tween 80-distilled water 1 hr before treatment with another 10 ml/kg *per os* 10% Tween 80-distilled water;

Group II consists of alloxan-induced hyperglycemic rats orally treated with 10 ml/kg 10% Tween 80-distilled water 1 hr before treatment with another 10 ml/kg *per os* 10% Tween 80-distilled water;

Group III consists of alloxan-induced hyperglycemic rats orally pre-treated with 10 ml/kg 10% Tween 80-distilled water 1 hr before treatment 3 g/kg D-glucose dissolved in 10% Tween 80-distilled water;

Groups IV-VII consist of alloxan-induced hyperglycemic rats that were orally pre-treated with 50 mg/kg HU, HU_{AF}, HU_n and erinidine, respectively, for 1 hr before the oral administration of 3 g/kg D-glucose, as described by Sepici et al. (2004) and Adeneye and Adeyemi (2009a). The blood glucose levels were determined at 30 min interval over the period of 4 hours.

Adrenaline-induced hyperglycemia test

This phase of animal studies also involved the use of 36 normoglycemic adult male Sprague-Dawley rats. Rats were fasted overnight for 12-14 hr prior to commencement of this phase of animal experiment. The rats' baseline fasting blood glucose values were obtained before oral treatment with the extracts or intraperitoneal injection of 50 μ g/kg of adrenaline (Sigma Chemical Company, St. Louis, MO, U.S.A.) dissolved in 1 ml/kg 10% Tween 80-distilled water. Group I rats, which served as the untreated control, were orally pretreated with 10 ml/kg 10% distilled water-Tween 80 1 hr before intraperitoneal injection of 1 ml/kg of 10% Tween 80-distilled water. Group II rats served as the model control and were pretreated with 10 ml/kg *per os* 10% Tween 80-distilled water 1 hr before intraperitoneal injection of 50 μ g/kg adrenaline. Groups III - VI rats which served as the treatment groups were orally pretreated with 50 mg/kg HU, HU_{AF}, HU_n and erinidine, respectively, for 1 hr before intraperitoneal injection of 50 μ g/kg adrenaline, as described by Alada et al. (2001).

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Blood glucose measurements

Following administration of hyperglycemia-induced agents, whole blood glucose levels were collected at 0 min, 30 min, 60 min, 90 min, 120 min, 150 min, 180 min, 210 min and 240 min by tail tipping method at between 08:00 and 12:00 hr. The glucose levels were measured by glucose oxidase method of Trinder (1969) using [®]TheraSense FreeStyle Flash Glucose Meter (Model: T-H010-64110, TheraSense Inc., Alameda, California, U.S.A.). The blood glucose meter was calibrated and validated at the beginning of, midway into and at the end of the experiment.

Statistical analysis

Results were presented as mean \pm S.E.M. of six observations. Statistical analysis was done using two-way analysis of variance followed by post-hoc test, Student-Newman-Keuls test on SYSTAT 10.6. Statistical significance were considered at $p < 0.05$ and $p < 0.001$

Results

Extraction and chromatographic studies on erinidine

The alkaloid fractions 16-25 (erinidine) was a light yellow, amorphous solid with UV (CHCl_3) λ_{max} 256 nm; IR (KBr) ν_{max} 3273, 2940, 2872 and 1734 cm^{-1} (Figure 2), HRESIMS m/z 382.1881 $[(M+H)^+]$ (calculated for $\text{C}_{22}\text{H}_{26}\text{N}_4\text{O}_2$, 382.1876), melting point of 230 $^{\circ}\text{C}$, full mass spectroscopy (Figure 3) and LC/MS pattern (Figure 4).

In vitro antihyperglycemic studies of erinidine

Alpha glucosidase assay

The inhibition of α -glucosidase by treatment with 0.016-0.25 mg/ml of erinidine is given in Table 1. The % inhibition of erinidine on α -glucosidase ranges from 0.25 % (for 0.016 mg/ml) to 28.17 % (for 0.25 mg/ml) and these inhibitory effects are considered non-significant (Table 1).

Table 1: Inhibitory effect of 0.008-0.25 mg/ml of erinidine on α -glucosidase

Erinidine concentration (mg/ml)	Percentage (%) inhibition
0.250	28.170
0.125	8.370
0.063	8.240
0.031	2.910
0.016	2.280
0.008	0.250

Table 2: Inhibitory effect of 0.008-0.25 mg/ml of erinidine on DPP-IV assay

Erinidine concentration (mg/ml)	Percentage (%) Inhibition
0.250	3.170
0.125	1.370
0.063	0.000
0.031	0.000
0.016	0.000
0.008	0.000
Diprotin A (control)	85.000

DPP-IV assay

Inhibition of DPP-IV by treatment with 0.008-0.25 mg/ml of erinidine is given in Table 2. The inhibitory values obtained for erinidine range from 0.00 % (for 0.008 mg/ml) to 3.17 % (for 0.25 mg/ml) concentration which is considered as a non-significant inhibitory effect on the DPP-IV enzyme when compared to that of the standard drug, Diprotin A (Table 2).

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Table 3: Inhibitory effect of 0.125 and 0.250 mg/ml of erinidine on glycogen phosphorylase

Erinidine concentration (mg/ml)	Percentage (%) Inhibition on
0.250	0.000
0.125	0.000

Table 4: Insulin secretory effect of 0.125 and 0.25 mg/ml of erinidine on HIT-T15 cells

Erinidine concentration (mg/dl)	Insulin concentration (ng/ml)
0.125	0.070
0.250	0.000

Table 5: Inhibitory effect of 0.008-0.125 mg/ml of erinidine on aldose reductase activity

Erinidine concentration (mg/dl)	% inhibition on aldose reductase activity
0.125	0.000
0.062	0.000
0.031	0.000
0.016	0.000
0.008	0.000
0.1 mg/ml of Epilrastat (control)	87.000

Glucose uptake activity assay

Similar non-significant effect ($p>0.05$) with erinidine was recorded in the glucose uptake test when compared to the control (DPBS) (Figure 6 and Table 3).

Effect of erinidine on insulin secretion in cultures of HIT-T15 cells

In order to evaluate the secretory potential of erinidine on HIT-T15 cells, the cell cultures were incubated with varying amounts of erinidine and measured the amount of insulin secreted. Results showed that treatment with 0.125 and 0.25 mg/ml of erinidine did not cause any significant ($p>0.05$) increase in insulin secretion when compared to the buffer control (Figure 5 and Table 4). In addition, there was no detrimental effect on cell viability or membrane integrity by the varying concentrations of erinidine used as determined by the Trypan Blue exclusion method. The cell viability was at least 95 % in all experimental groups including control.

Aldose reductase assay

In the aldose reductase assay, five different concentrations ranging from 0.125 mg/ml to 0.008 mg/ml were tried and none of the concentrations used was effective in the assay (Table 5). However, treatment with 0.1 mg/ml of Epilrastat (standard control) resulted in 87.00 % inhibition of aldose reductase enzyme (Table 5).

In vivo antihyperglycemic studies

Oral glucose tolerance test in normal rats

Single oral administration of 3 g/kg of glucose dissolved in 10% Tween 20-distilled water resulted into a significant ($p<0.05$) postprandial hyperglycemia within 30 min postprandial, reaching its peak at 90 min and gradually decreases from 180 min and subsequently returned to about normal value from 150 min to 240 min postprandial (Table 6). Oral pretreatment with 50 mg/kg of HU, HU_{Af} and erinidine significantly ($p<0.05$) attenuated a rise in postprandial hyperglycemia within 60 min of glucose administration and blood glucose levels significantly ($p<0.05$, $p<0.001$) and time-dependently decreasing from 90 min to 240 min postprandial to the baseline values at 0 min (Table 6). Results also showed that although erinidine significantly attenuated a rise in

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postprandial blood glucose levels and ultimately induced hypoglycemia, these biological effects were lower than that recorded for HU_{Af}. However, HU_n neither prevented postprandial glucose rise within 60 min of its administration nor subsequently lower postprandial blood glucose levels from 120 min to 240 min (Table 6).

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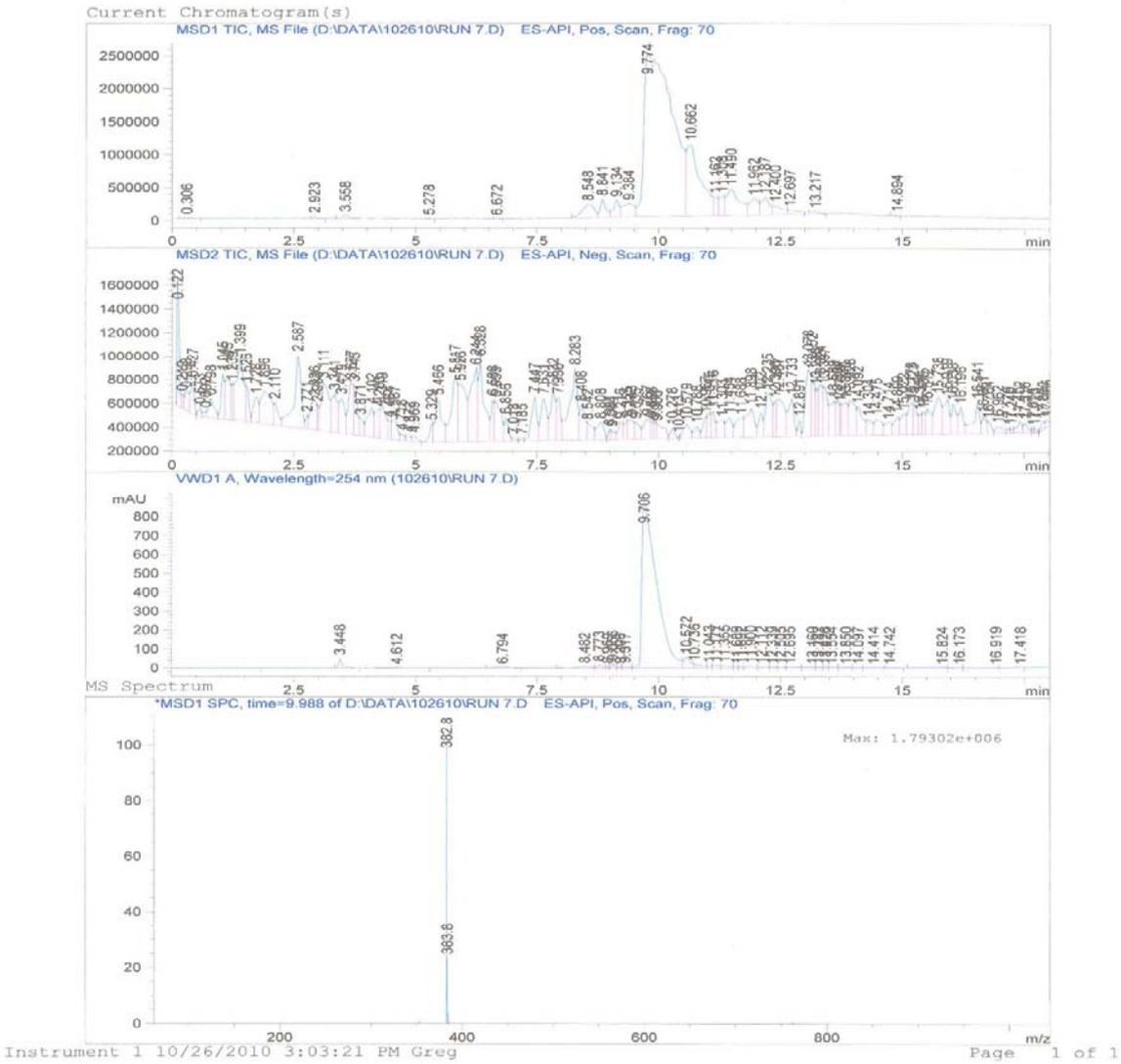


Figure 4: LC-MS fingerprint of erinidine of *Hunteria umbellata* seed extract

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Table 6: Effect of 50 mg/kg of HU, HU_{Af}, HU_n and erinidine on blood glucose (BG) levels in glucose-induced hyperglycemic rats

Time (min)	Blood glucose levels (mg/dl) of treatment groups					
	I	II	III	IV	V	VI
0	53.0 ± 1.3	54.3 ± 3.1	54.7 ± 3.1	57.7 ± 2.2	56.3 ± 1.1	55.7 ± 2.2
30	69.7 ± 4.4	93.3 ± 8.4 ^a	65.7 ± 2.4 ^b	65.0 ± 2.7 ^b	90.7 ± 4.4 ^a	64.7 ± 1.1 ^b
60	66.0 ± 4.0	97.7 ± 2.4 ^a	64.3 ± 2.2 ^b	67.3 ± 1.8 ^b	82.7 ± 4.9 ^a	70.7 ± 1.8 ^b
90	64 ± 2.7	89.7 ± 3.1 ^a	61.0 ± 2.0 ^b	62.7 ± 3.6 ^b	81.7 ± 3.6 ^a	74.3 ± 3.1
120	59.7 ± 6.4	77.0 ± 6.7 ^a	58.7 ± 2.2 ^b	59.7 ± 2.4 ^b	83.0 ± 2.7 ^a	67.3 ± 3.8 ^b
150	60.7 ± 5.8	81.3 ± 19.1 ^a	55.7 ± 1.1 ^b	57.7 ± 2.8 ^c	70.0 ± 6.7	60.3 ± 3.1 ^b
180	60.3 ± 4.9	69.7 ± 4.4 ^b	54.3 ± 2.9 ^b	56.7 ± 4.9 ^c	74.3 ± 5.1	59.3 ± 4.9 ^b
210	56.3 ± 4.4	65.3 ± 2.2 ^b	51.3 ± 2.2 ^b	50.7 ± 3.6 ^c	73.0 ± 8.7	54.3 ± 5.6 ^c
240	55.0 ± 6.7	61.7 ± 4.2 ^b	49.3 ± 3.1 ^b	49.0 ± 5.3 ^c	76.3 ± 9.1	48.7 ± 5.1 ^c

^a represents a significant increase at $p < 0.05$ when compared to the basal value at 0 min while ^b and ^c represent significant decreases at $p < 0.05$ and $p < 0.001$ when compared to 30 min and 60 min values for Group II.

Group I: 10 ml/kg of 10% Tween 80-DW

Group II: 10 ml/kg of 10% Tween 80-DW + 3 g/kg of D-Glucose in 10% Tween 80-DW

Group III: 50 mg/kg HU in 10% Tween 80-DW + 3 g/kg of D-Glucose in 10% Tween 80-DW

Group IV: 50 mg/kg of HU_{Af} in 10% Tween 80-DW + 3 g/kg of D-Glucose in 10% Tween 80-DW

Group V: 50 mg/kg of HU_n in 10% Tween 80-DW + 3 g/kg of D-Glucose in 10% Tween 80-DW

Group VI: 50 mg/kg of erinidine in 10% Tween 80-DW + 3 g/kg of D-Glucose in 10% Tween 80-DW

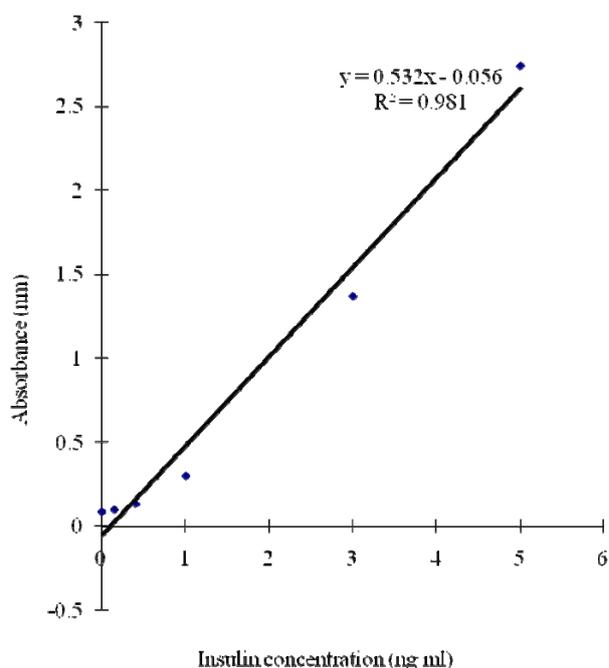


Figure 5: Standard curve for the insulin secretion by the HIT-T15 cells

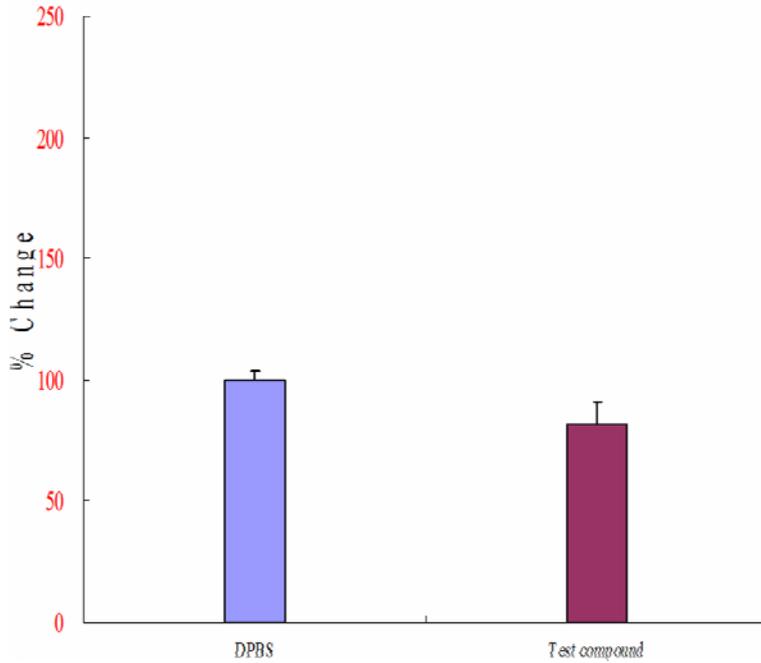


Figure 6: Glucose uptake ratio of erinidine (test compound) relative to that of the control buffer solution showing no significant difference from each other.

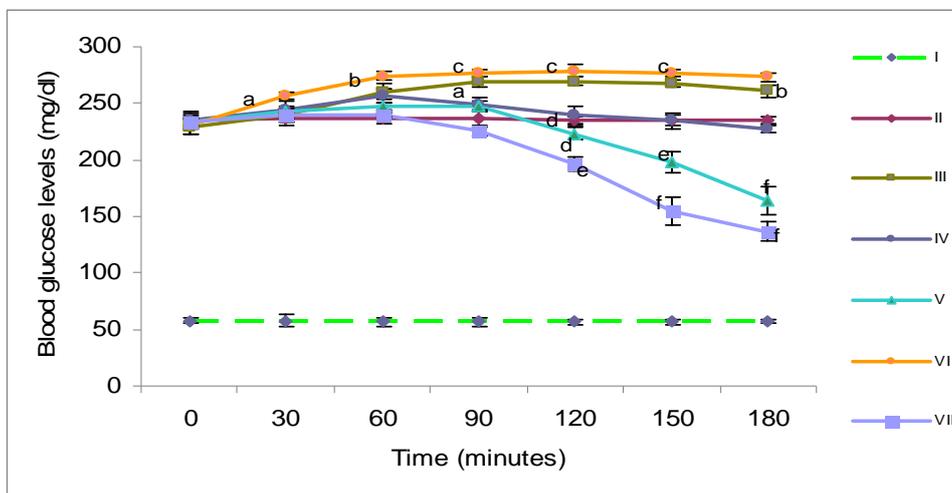


Figure 7: Effect of 50 mg/kg HU, HU_{Af}, HU_n and erinidine on the postprandial blood glucose concentrations in alloxan-induced hyperglycemic rats

^{a, b} and ^c represent significant increases in postprandial glucose concentrations at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, when compared to the basal values at 0 min while ^{d, e} and ^f represent significant decreases at $p < 0.05$, $p < 0.01$ and $p < 0.001$ in the postprandial glucose concentrations when compared to values at 60 min.

I = normoglycemic rats + 10 ml/kg of 10 ml/kg of 10%Tween 80-distilled water *p.o.*; II = alloxan-induced hyperglycemic rats + 10 ml/kg 10%Tween 80-distilled water *p.o.* pretreatment + 3 g/kg of D-glucose *p.o.*; III = alloxan-induced hyperglycemic rats + 50 mg/kg HU in 10%Tween 80-distilled water *p.o.* pretreatment + 3 g/kg of D-glucose *p.o.*; IV = alloxan-induced hyperglycemic rats + 50 mg/kg HU_{Af} in 10%Tween 80-distilled water *p.o.* pretreatment + 3 g/kg of D-glucose *p.o.* V = alloxan-induced

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hyperglycemic rats + 50 mg/kg HU_n in 10%Tween 80-distilled water *p.o.* pretreatment + 3 g/kg of D-glucose *p.o.*; VI = alloxan-induced hyperglycemic rats + 50 mg/kg erinidine in 10%Tween 80-distilled water *p.o.* + 3 g/kg of D-glucose *p.o.*

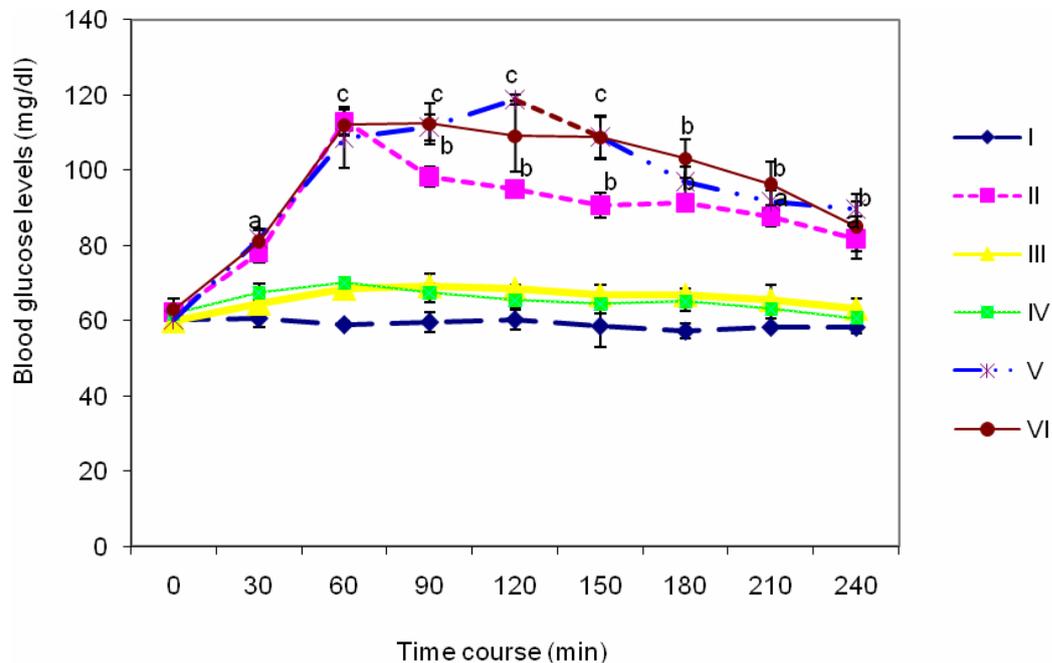


Figure 8: Acute time-course effect of 50 mg/kg HU, HU_{Af}, HU_n and erinidine pre-treatment on blood glucose levels in adrenaline-induced hyperglycemic rats

^{a, b} and ^c represent a significant rise at $p < 0.05$, $p < 0.01$ and $p < 0.001$ when compared to the basal value at 0 min.

I = 10 ml/kg 10%Tween 80-distilled water *p.o.* + 1 ml/kg of 10%Tween 80-distilled water *i.p.*; II = 10 ml/kg 10%Tween 80-distilled water *p.o.* + 50 µg/kg adrenaline *i.p.*; III = 50 mg/kg HU in 10%Tween 80-distilled water *p.o.* + 50 µg/kg adrenaline *i.p.*; IV = 50 mg/kg HU_{Af} in 10%Tween 80-distilled water *p.o.* + 50 µg/kg adrenaline *i.p.*; V = 50 mg/kg HU_n in 10%Tween 80-distilled water *p.o.* + 50 µg/kg adrenaline *i.p.*; VI = 50 mg/kg erinidine in 10%Tween 80-distilled water *p.o.* + 50 µg/kg adrenaline *i.p.*

Oral glucose tolerance test in alloxan-induced hyperglycemic rats

By the 5th day following single intraperitoneal injection with 120 mg/kg of alloxan, hyperglycemia became fully established in the treated rats. Oral treatment of alloxan-induced hyperglycemic rats with 3 g/kg of D-glucose was associated with significant ($p < 0.01$, $p < 0.001$) rise in the blood glucose levels at 30 min which peaked at 60 min and remained significantly high up to 240 min postprandial in Group II rats (Figure 7). However, pretreatment with 50 mg/kg of HU, HU_{Af} and erinidine was associated with varying and significant ($p < 0.05$, $p < 0.01$ and $p < 0.001$) attenuation of increases and eventual decreases in the postprandial blood glucose levels, with erinidine exhibiting the most significant hypoglycemic effect in the treated alloxan-induced hyperglycemic rats (Figure 7). HU_n caused no significant alterations in the postprandial blood glucose levels.

Adrenaline-induced hyperglycemia studies

Intraperitoneal injection of 50 µg/kg of adrenaline to rats pre-treated with 10 ml/kg of 10% Tween 80-distilled water resulted in significant ($p < 0.001$) elevation in the blood glucose levels from 78.00 mg/dl at 30 min and peaking at 113.00 mg/dl at 60 min post-injection (Figure 8). The elevated blood glucose levels then gradually decreased from 98.33 mg/dl at 90 min to 81.67 mg/dl at 240 min (Figure 8). Oral pre-treatment with 50 mg/kg of HU and HU_{Af} attenuated a noticeable elevation in the blood glucose levels following intraperitoneal administration of 50 µg/kg of adrenaline (Figure 8). However, oral pre-treatment with HU_n and erinidine did not attenuate a rise in the blood glucose levels induced by intraperitoneal injection of adrenaline (Figure 8).

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Discussion

The physico-chemical property of erininide isolated and other fractions of HU obtained in this study appear to be the same with those previously reported by Adeneye et al. (2012b). Bioengineered cell culture lines such as RIN β -cell, HIT-T15, β -TC-6, MIN6, INS-1 and 3T3-L1 cells have been used to establish and facilitate studies of mechanisms of both insulin secretion and β -cell dysfunction, being also the target to the study of natural products (Poitout et al., 1996; Maddux et al., 2001; Karalee et al., 2001). These cell lines release mainly insulin and small amounts of glucagon and somatostatin. Although the behavior of none of these cell lines perfectly mimics primary β -cell physiology, they are extremely valuable tools for the study of molecular events underlying β -cell function (Persuad et al., 1999; Fröde and Medeiros, 2008). Other *in vitro* antihyperglycemic screening techniques such as alpha-glucosidase inhibition, DPP-IV inhibition, glycogen phosphatase inhibition, and aldose reductase inhibition have been designed to simulate specific events occurring at the molecular, cellular and sub-cellular levels in the pancreatic β -cell and as such could serve as an alternative or complement to *in vivo* models (Fujita et al., 1996; Gerbhardt, 2000; Rotshteyn and Zito, 2004). These screening techniques usually offer fast, reliable and cost-effective screening alternative in addition to requiring only very small amounts of experimental compound, and minimal technicalities (Rotshteyn and Zito, 2004). In the present study, insulin-secretion property of 0.125 and 0.250 mg/ml of erinidine was studied using HIT-T15 cell culture, with erinidine failing to increase insulin secretion in the cultured cells suggesting that erinidine may unlikely possess sulfonyleurea-like activity. This observation is in complete agreement with earlier report that the crude water seed extract of *Hunteria umbellata* does not mediate antihyperglycemic action via hyperinsulinemia mechanism (Adeneye and Adeyemi, 2009a; Adeneye and Adeyemi, 2009b).

In the *in vivo* screening of erinidine and other fractions of HU for their possible antihyperglycemic potential, intraperitoneal adrenaline was used to induce acute hyperglycemia in the treated Spargue-Dawley rats. Adrenaline produces hyperglycemia by inhibiting insulin release, stimulating glycogenolysis in muscle and thus providing substrate in the form of lactate for hepatic gluconeogenesis, stimulating glucagon secretion and stimulating ACTH secretion which, in turn, stimulates glucocorticoid secretion from the adrenal cortex (Kraus-Friedmann, 1984; Krusteva, 1992; Alada et al., 2001). However, the test compounds did not significantly reduce the adrenaline induced hyperglycemia suggesting that they probably do not mediate their action via inhibition of adrenaline-induced stimulation of α_2 receptors in β -cells of pancreas and thus promoting hyperinsulinemia. Acute transient hyperglycemia is known to occur following absorption of glucose into the porta blood from the gastro-intestinal tract after oral ingestion of a meal. This post-absorptive hyperglycemia is often accompanied by hyperinsulinemia, particularly within the first few hours postprandial. This physiological response is aimed at lowering the postprandial hyperglycemia and maintains it within normal range, as insulin is known to promote intracellular glucose transport. However, this mechanism is very unlikely for this experiment as the injected alloxan (a potent cytotoxin) would have destroyed the pancreatic β -cells to a large extent for hyperglycemia to become established. It is, thus, plausible that erinidine and other fractions could be mediating their antihyperglycemic effects via inhibition of intestinal glucose uptake and improvement in the oral glucose tolerance in the treated rats, an action similar to those of standard *alpha glucosidase* inhibitors such as acarbose, miglitol and voglibose. Although, results of the *in vitro* studies showed erinidine to have a weak alpha glucosidase inhibition property but produced a significant intestinal glucose uptake inhibitory effect *in vivo*. This observation further suggests that erinidine may require biotransformation to its active metabolite(s) for its glucose uptake inhibitory effect to manifest. This observation may be explained by the fact that some extracts may not show good biological activities in *in vitro* assay but may become pharmacologically active after undergoing *in vivo* metabolism into their active metabolites (Farnsworth, 1993). Further studies are currently underway to provide insight into the pharmacokinetic and pharmacodynamic profile of this new compound.

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Dedication

This study is in loving memory of one of the greatest mentors and father of modern Pharmacognosy, Late Professor Emeritus Norman R. Farnsworth (1930-2011), formerly of Department of Pharmacognosy and Medicinal Chemistry, College of Pharmacy, University of Illinois at Chicago, U.S.A.

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