Biomedicine & Pharmacotherapy 149 (2022) 112833

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

# Hypoglycaemic activity of Oleanonic acid, a 3-oxotriterpenoid isolated from *Aidia Genipiflora* (DC.) Dandy, involves inhibition of carbohydrate metabolic enzymes and promotion of glucose uptake

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ARTICLE INFO

*Keywords:*
Streptozotocin-induced diabetes
Glucose uptake
α-Glucosidase
α-Amylase
Oleanonic acid
*Aidia genipiflora*

ABSTRACT

The present study evaluated the antidiabetic activities of the 70% ethanol stem bark extract of *Aidia genipiflora* (AGB) and one of its constituents, oleanonic acid in streptozotocin (40 mg/kg)-induced diabetic rats. *In vitro* assays of glucose uptake and inhibition of carbohydrate metabolizing enzymes were then used to investigate their mechanism(s) of hypoglycaemic action. *In silico* evaluation of the pharmacokinetic and toxicity properties of the compound was also carried out. Administration of AGB (100–400 mg/kg) and oleanonic acid (15 – 60 mg/kg) resulted in significant reductions ($p < 0.001$) in the blood glucose and considerable decrease ($p < 0.05$) in the elevated lipid parameters of the diabetic animals. AGB activity at 200 and 400 mg/kg; and oleanonic acid at 60 mg/kg were comparable to glibenclamide (5 mg/kg). The extract and its isolate strongly inhibited α-glucosidase and α-amylase activity with $IC_{50}$ values of (10.48 ± 1.39 μg/mL and 14.51 ± 1.26 μg/mL) and (36.52 ± 1.95 μM and 105.84 ± 1.08 μM) respectively. The glucose uptake assays showed that AGB and oleanonic acid exerted both insulin-dependent and independent promotional effect of glucose transport into the periphery by upregulating the expression of PI3K and PPARγ transcripts with a concomitant increase in GLUT-4 transcripts. Although oleanonic acid was predicted to be teratogenic, it was found to be generally non-lethal with favourable pharmacokinetics properties making it suitable for further studies. The study has shown that the stem bark of *A. genipiflora* is a source of new hypoglycaemic agents and that oleanonic acid possesses hypoglycaemic and anti-hyperlipidaemic activities.

## 1. Introduction

The menace of diabetes mellitus and its complications is a major global concern in healthcare. The International Diabetes Federation (IDF) estimates 463 million diabetic patients worldwide with this number expected to rise to 700 million by 2045 [1]. The increasing epidemic of diabetes has been attributed to factors such as unhealthy eating habits and sedentary lifestyles, population ageing, urbanization, and economic development [2]. Nearly half a million people are at risk of the disease with 4.2 million people dying from it in the year 2019 [3]. The management of diabetes mellitus is a global health issue with a cure yet to be discovered. Currently available medications for diabetes include insulin and various oral hypoglycaemic drugs like the biguanides (metformin), sulfonylureas (glibenclamide, glipizide and glimepiride), α-glucosidase inhibitors (miglitol and

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https://doi.org/10.1016/j.biopha.2022.112833
Received 31 January 2022; Received in revised form 11 March 2022; Accepted 16 March 2022
Available online 19 March 2022

acarbose), meglitinides and the thiazolidinediones (pioglitazone and rosiglitazone) [4]. Though effective, the use of these agents is associated with serious adverse effects and therefore the search for more effective and safer antidiabetic drugs is a pressing need. Almost 80% of the adult diabetic population and those that suffer the most debilitating complications of the disease reside in low- and middle-income countries because they do not have access to and/or cannot afford the oral hypoglycaemic medicines and therefore rely mostly on traditional medicines for their treatment [5].

The application of traditional practices in treating diseases has existed since the beginning of time across all ethnic groups. It is estimated that about 85% of the world's population especially those dwelling in the rural areas of developing nations rely exclusively on traditional medical practices for their primary healthcare [6]. Over the past decades, herbal preparations and medicinal herbal products have been shown to be effective in the management of diabetes and prevent the onset of secondary complications [7]. Whereas some medicinal plants have been proven to exert their activity through the stimulation of regenerated and/or surviving $\beta$-cells of the pancreas to increase insulin release, others have been shown to help by stimulating the uptake of glucose into the peripheral tissues and inhibiting carbohydrate metabolizing enzymes [8]. Furthermore, some medicinal plants have also been shown to modulate the key effector molecules of the PI3K/Akt pathway to exert their antidiabetic actions [9] whiles others have been shown to possess antioxidant and anti-hyperlipidaemic activities in addition to their hypoglycaemic effects.

*Aidia genipiflora* (DC) Dandy of the family Rubiaceae is a plant distributed across Central and West African tropical sub-regions. The leaves and stem bark of *A. genipiflora* are used in managing various communicable and non-communicable diseases in African Traditional Medicine including drowsiness, diabetes, oedema, wounds, and gout [10]. Anokwah et al. reported the antimicrobial activity of the stem bark of the plant and further isolated some of its constituents responsible for its use as an anti-infective agent in traditional medicine [11]. However, studies to validate the other traditional uses of the plant are yet to be conducted.

One of the bioactive constituents of *A. genipiflora* is oleanonic acid which has also been identified in several plant species [12–14]. The compound and its derivatives have been extensively investigated for its effects in the management of cancer and inflammation [15–18]. Similar studies have been conducted to establish its anti-infective activity which includes antiparasitic [19] and antibacterial [20] activities. Through computational studies, Petersen et al. identified oleanonic acid as a PPARγ agonist [21]. Elsewhere, the compound induced glucose uptake in L6 skeletal myotubes [22]. Interestingly, although both studies suggest that oleanonic may be useful in the management of diabetes, no studies have been conducted to identify its effect in an *in vivo* model. This study therefore seeks to investigate the antidiabetic potential of *A. genipiflora* and evaluate the *in vivo* hypoglycaemic activity of oleanonic acid in STZ-induced diabetic rats. The effect of oleanonic acid on glucose transport into the periphery as well as the effect of the compound on the activities of α-amylase and α-glucosidase are also reported.

## 2. Materials and methods

### 2.1. Reagents and chemicals

C2C12 myoblasts (CRL-1772) and 3T3-L1 pre-adipocytes (CL-173) were products of American Type Culture Collection (ATCC) (Manassas, Virginia, USA) whereas newborn calf serum (NBCS), foetal calf serum (FCS), L-glutamine, Dulbecco's modified Eagle's Medium (DMEM), penicillin/streptomycin and horse serum were bought from Gibco (Berlin, Germany). Dinitrosalicylic acid (DNS), streptozotocin (STZ), cytochalasin B, 3-Isobutyl-1-methylxanthine (IBMX), glibenclamide, acarbose, intestinal acetone powder, porcine pancreatic α-amylase, accutase, 2-deoxy-D-glucose and *p*-nitrophenyl-α-D-glucopyranoside were obtained from Sigma Aldrich (St. Louis, MO, USA). Ultima Gold XR and radioactive [$^3$H] 2-deoxyglucose were sourced from PerkinElmer (Waltham, MA, USA).

All organic solvents used were of analytical grade and obtained from BDH, Laboratory Supplies (Merck Ltd, Lutterworth, UK).

### 2.2. Plant collection, extraction and isolation of oleanonic acid

The stem bark of *A. genipiflora* was collected from Kwahu Asakraka in the Eastern Region, Ghana in April 2020 and authenticated at the Institute of Traditional and Alternative Medicine (ITAM), University of Health and Allied Sciences (UHAS) where voucher specimen (Voucher number: UHAS/ITAM/2020/SB010) has been kept. The collected plant material was thoroughly washed under running water, chopped into pieces, air-dried for 7 days and ground into powder. Afterwards, 1.3 kg of the powdered material was Soxhlet-extracted using 70% ethanol for 24 h. The extract obtained was evaporated under reduced pressure to obtain a brown residue (AGB, 138 g) of which 125 g was reconstituted in distilled water and sequentially extracted with petroleum ether (pet ether), chloroform ($CHCl_3$), ethyl acetate (EtOAc) and n-butanol (ButOH) and the solvents evaporated off to obtain five fractions: AGB-Pet-ether, AGB-$CHCl_3$, AGB-EtOAc, AGB-ButOH and aqueous portions [23].

The ethyl acetate portion (AGB-EtOAc, 20 g) was column chromatographed (CC) over Silica gel 60 (70–230 mesh) using $CHCl_3$ – methanol (MeOH) mixtures (100:1, 90:1, 80:1, 70:1–1:1) to obtain 108 eluates (100 mL) which were pooled into 5 fractions (F1-F5). Purification of fraction F3 (5.6 g) on CC by gradient elution using pet ether:/EtOAc mixtures followed by Sephadex LH-20 column eluting with $CHCl_3$: MeOH (1:1) yielded oleanonic acid (1.8 g) as a white amorphous powder.

The identity of the isolated compound as oleanonic acid was determined using NMR spectroscopic methods and by comparing to reported literature. The NMR spectroscopic data are provided in the supplementary material.

### 2.3. Intestinal α-glucosidase inhibition

Rat intestinal acetone powder (300 mg) was homogenized in 600 mL of phosphate buffer (0.1 M, pH 6.9) and centrifuged at 12 000 g for 30 min to obtain the enzyme solution used in the test. 100 μL of the enzyme solution obtained were incubated with 50 μL of the test samples (AGB [0.1 μg/mL – 100 μg/mL]; oleanonic acid and acarbose [1–1000 μM]) at 25 °C for 10 min in 96-well plates. Afterwards, 50 μL of 5 mM p-nitrophenyl-α-D-glucopyranoside solution was added to each well and the plates incubated at 37 °C for 30 min. The absorbances of the plates were then measured at 405 nm on a microplate reader (Thermomax, Molecular Device Corp., Sunnyvale, CA). Blank determination was carried out using 50 μL of buffer solution without any test sample [24].

### 2.4. Inhibition of pancreatic α-amylase

Porcine pancreatic α-amylase powder (350 mg) was homogenized in 700 mL of 0.02 M phosphate buffer (pH 6.9) to get the enzyme solution employed in the assay. 500 μL of the test samples (AGB [0.1 μg/mL – 100 μg/mL]; oleanonic acid and acarbose [1–1000 μM]) were mixed with 500 μL starch solution (1% *w*/*v*) and incubated at 25 °C for 10 min. Afterwards 500 μL enzyme solution was added to each mixture and incubated for 10 min at room temperature. A mL each of dinitrosalicylic acid colour reagent was then added to each mixture to end the reaction. The mixtures were then heated for 10 min at 100 °C, diluted and their absorbances measured at 540 nm [23].

### 2.5. Glucose transport assay

#### 2.5.1. Glucose transport in 3T3-L1 adipocytes

Fully differentiated and confluence (90%) 3T3-L1 adipocytes grown

in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 1% antibiotics and 10% NBCS in 5% $CO_2$ at 37 °C; and differentiated in DMEM augmented with 1 μmol/L dexamethasone, 1% antibiotics, 3-isobutyl-1-methylxanthine (IBMX, 0.5 mmol/L), 10 μmol/L human insulin and 10% FCS were serum starved in 24- well plates for 4 h before treating with the test samples (AGB [0.1 −100 μg/mL], oleanonic acid [0.1 −100 μM] and Insulin [0.01 – 10 nM]) for an hour. Afterwards, the cells were washed with KRP-buffer (2 ×) and incubated with 400 μL glucose uptake-buffer (KRP-buffer supplemented with 10 μM 2-deoxyglucose and 1 μCi/mL radioactive [$^3$H] 2-deoxyglucose) for 10 min. Glucose uptake was then halted by the addition of 10 μM Cytochalasin B in KRP buffer (CB-stop solution) and the cells iced for 10 min before rinsing thrice with cold phosphate buffered saline (PBS) solution, lysed 0.2 M NaOH and mixed with liquid scintillation cocktail (4 mL). The radioactivity present in the lysed cells were then measured in triplicate with a scintillation counter. The protein contents of the lysed cells were measured using the BCA-Protein Assay Kit after which the glucose uptake was calculated as radioactive count per min (cpm)/mg of protein. Final data were expressed as the percentage of control. Cytochalasin B (10 μM) was used to determine the non-specific glucose uptake which was subtracted from each value [23].

#### 2.5.2. Glucose transport in C2C12 myotubes

Our previously published method was employed [25]. Fully differentiated and confluence (90%) myotubes grown in DMEM containing 1% penicillin/streptomycin, 10% FCS, 2% horse serum and 2 mM glutamine in 5% $CO_2$ at 37 °C were incubated in serum-free DMEM for 4 h in 24- well plates and treated with the test samples (AGB [0.1 −100 μg/mL], oleanonic acid [0.1 −100 μM] and Insulin growth factor-1 (IGF-1) [0.1 – 100 nM]) for 60 min. The cells were then rinsed 3 × with HBS-buffer and treated with 400 μL glucose uptake-buffer (HBS-buffer containing 2-deoxyglucose (10 μM) and radioactive [$^3$H] 2-deoxyglucose (2 μCi/mL)) for 5 min. The glucose transport reaction was then stopped by the addition of CB-stop solution (Cytochalasin B (10 μM) in HBS buffer) and the cells kept on ice for 15 min before rinsing with ice-cold PBS solution (3 ×). The myotubes were then lysed with NaOH (0.2 M), added to 4 mL Liquid Scintillation Cocktail and the [3 H] 2-deoxyglucose content measured in triplicate. The protein content of the lysed cells was also determined using the BCA-Protein Assay Kit after which the glucose uptake was calculated as radioactive count per min (cpm)/mg of protein. Final data were expressed as the percentage of control. Cytochalasin B (10 μM) was used to determine the non-specific glucose uptake which was subtracted from each value.

#### 2.5.3. Reverse transcriptase-polymerase chain reaction (RT-PCR)

A previously described reverse transcriptase-polymerase chain reaction (RT-PCR) method with slight modifications was employed [26]. Briefly, differentiated C2C12 myotubes grown in 12-well plates were treated with the $EC_{50}$ of test samples (AGB, oleanonic acid, and IGF-1) obtained from the glucose transport assay in C2C12 myotubes and rosiglitazone (10 μM) for 24 h. After incubation, the total RNA from the cells were isolated using TRIzol (Invitrogen) in accordance with the manufacturer's instructions. Thereafter, cDNA was synthesized using SuperScript first-strand synthesis kit (Invitrogen) and analysed by PCR using an Applied Biosystems 7500 Fast PCR machine. The primers used were Glucose transporter-4 (GLUT-4): sense, 5′-CGG GAC GTG GAG CTG GCC GAG GAG-3′; anti-sense, 5′-CCC CCT CAG CAG CGA GTG A-3′; phosphoinositide-3-kinase (PI3K): sense, 5′-TGA CGC TTT CAA ACG CTA TC-3′; antisense, 5′-CAG AGA GTA CTC TTG CAT TC-3′; and peroxisomal proliferator-activated receptor-gamma (PPARγ): sense, 5′-GGA TTC ATG ACC AGG GAG TTC CTC- 3′; anti-sense, 5′-GCG GTC TCC ACT GAG AAT AAT GAC-3′. For PCR reaction, cDNA mixture (1 μL) was mixed with PCR reaction solution consisting of 10X PCR buffer, 10 pM of paired primers, 2 mM dNTP and 2 units of Taq polymerase.

### 2.6. Streptozotocin-induced diabetes assay

#### 2.6.1. Experimental animals

Male Sprague-Dawley rats weighing between 180 and 205 g bought and kept at the animal house of the Department of Pharmacology, Kwame Nkrumah University of Science and Technology (KNUST) were housed in stainless steel cages lined with saw dust under environmental conditions of temperature (25 ± 2 °C) and 12 h light: dark cycle. The animals were fed on standard pellet diet (R36, GHAFCO, Tema, Ghana) and freely allowed to drink water.

Male Sprague-Dawley rats were employed in the study because their pancreatic β-cells are susceptible to the necrotic actions of streptozotocin and produces stable diabetes model easily [27].

The National Institute of Health (NIH) Guidelines for the care and use of laboratory animals (NIH, Department of Health and Human Services publication No 5, revised 1985) were adhered to in the treatment of experimental rats.

Ethical clearance was obtained from the Department of Pharmacology Ethics Committee, KNUST (CHRPE/AP/212/19).

#### 2.6.2. Acute toxicity studies

The toxicity of *A. genipiflora* 70% ethanol stem bark extract (AGB) and oleanonic acid on male Sprague Dawley rats were evaluated as per the Organization of Economic Cooperation and Development (OECD) Guidelines number 425. Overnight fasted (18 h) experimental animals in groups of five were orally treated with a single dose of the test samples [AGB: 50, 500, 5 000 mg/kg; oleanonic acid: 10, 100 mg/kg or normal saline (0.9% 10 mL/kg)]. The rats were observed closely for toxicity signs, behavioural changes, or death at 0, 15, 30, 60, 120 and 180 min, 24 h and 14 days after administration of the test samples.

#### 2.6.3. Induction of diabetes and experimental design

Diabetes was induced in the experimental rats by injection of STZ (40 mg/kg [*i.p*]) freshly dissolved in 0.1 M cold sodium citrate buffer (pH 4.5). The diabetes induced rats were allowed to drink freely 5% glucose solution for the next 12 h to prevent initial drug-induced hypoglycaemic mortality. Hyperglycaemia was confirmed by elevated levels of blood glucose concentrations determined after 72 h using OneTouch Select glucometer (LifeScan, Inc. Milpitas, CA 95035 USA) [28]. Diabetic rats selected for the study had fasting blood glucose (FBG) above 10 mmol/L with demonstratable signs of hyperglycaemia such as polyuria, polydipsia and hyperphagia [29].

The diabetic animals were randomly selected and put into groups of six animals - negative diabetic control group (vehicle: 2% tragacanth solution), diabetic treatment groups (test samples: 100, 200 and 400 mg/kg for AGB [*p.o.*]; 15, 30 and 60 mg/kg for oleanonic acid [*p.o.*]) and diabetic positive control group (glibenclamide: 5 mg/kg [*p.o.*]). A negative normal control group (vehicle: 2% tragacanth solution [1 mL]) was also created with normoglycaemic rats. The treatment was started a day after diabetes was confirmed, and this was considered day one (1) of the study which continued for 3 weeks [30].

#### 2.6.4. Determination of glucose level and lipid parameters of experimental animals

The plasma glucose concentrations of the experimental rats were measured prior to administering the first dose and 6 h into the study from blood obtained from their tail veins after the application of lignocaine. The Fasting blood glucose (FBG) levels were determined weekly for 21 days.

The experiment was concluded on day 22 and the rats sacrificed by cervical decapitation in the morning under anaesthesia with ketamine (24 mg/kg [*i.p*]). Blood samples were collected by cardiac puncture for analysis of lipid parameters which include total cholesterol (TC), triglyceride (TG), and high-density lipoprotein cholesterol (HDL-C) using the BS-200 biochemistry analyser (www.mindray.com). Low density lipoprotein cholesterol (LDL-C) was then determined by the Friedewald equation [31].

### 2.7. In silico predictions of the pharmacokinetic and toxicity profile of oleanonic acid

The pharmacokinetic and toxicity properties of oleanonic acid was investigated using QikProp, admetSAR and ADMETlab [32,33]. Molecular descriptors for drug-likeness investigated include molecular weight, molecular polar surface area (PSA), number of hydrogen bond acceptors and donors and partition coefficient (cLogP), solubility and number of primary metabolites. The ADME properties estimated were Caco-2 permeability, blood brain barrier (BBB) penetration, cytochrome P450 metabolism and inhibition and human oral absorption. Toxicity prediction included reproductive toxicity, genotoxicity, carcinogenicity, hepatotoxicity, Ames mutagenicity, and toxicity dose levels.

### 2.8. Statistical analyses

Data from the *in vivo* assays are expressed as mean ± SEM whiles data from *in vitro* assays are displayed as mean ± SD. Analyses were carried out using one-way analysis of variance (ANOVA) with GraphPad for Windows version 8 (GraphPad Prism Software, San Diego, USA). Multiple comparisons between treatment groups for *in vivo* assays were done using Dunnet's post hoc test whereas Tukey's post hoc test was employed comparison between groups for *in vitro* experiments. $p < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Acute toxicity studies

Experimental animals treated with 70% ethanol stem bark extract of *A. genipiflora* (AGB) up to 5 000 mg/kg and oleanonic acid up to 100 mg/kg did not exhibit any difference in body weight and food intake compared to saline solution-treated animals. They also did not demonstrate any changes in behavioural patterns. Furthermore, no gross pathological differences were observed between the treated groups and the control. None of the treated animals (extract and compound) exhibited any sign of toxicity 14 days after administration (data not shown).

### 3.2. Antidiabetic activity of A. genipiflora stem bark extract (AGB) in STZ-induced diabetic rats

#### 3.2.1. Effect of AGB on the blood glucose of the diabetic rats

Treating the diabetic rats with the stem bark extract of *A. genipiflora* (AGB) and glibenclamide resulted in a significant ($p < 0.001$) hypoglycaemic effect which was dose and duration dependent compared to the diabetic control group. AGB reduced the blood glucose concentration of the experimental animals by 41.36%, 32.52% and 17.41% at 400, 200 and 100 mg/kg respectively 6 h into the study whereas glibenclamide caused a reduction of 49.26% by the same period (Table 1). The daily oral supplementation of the diabetic animals with AGB (100 – 400 mg/kg) and glibenclamide (5 mg/kg) reduced drastically the fasting blood glucose (FBG) of the diabetic rats bringing them below the 10 mmmol/L threshold at 200 and 400 mg/kg at the end of the study while the FBG of the untreated diabetic rats remained persistently high throughout the 21-day period (Fig. 2).

**Table 1**
Effect of *A. genipiflora* stem bark extract on the blood glucose of diabetic rats within 6 h of the study.

| Experimental Groups | 0 h | 6th hour | Variation (%) |
|---|---|---|---|
| Normal control | – | – | – |
| Diabetic control | 28.90 ± 0.53 | 30.98 ± 0.43 | -7.20 |
| AGB (100 mg/kg) | 29.30 ± 0.64 | 24.20 ± 0.75 *** | 17.41 |
| AGB (200 mg/kg) | 28.20 ± 0.97 | 19.03 ± 0.47 *** | 32.52 |
| AGB (400 mg/kg) | 29.28 ± 0.63 | 17.17 ± 0.55 *** | 41.34 |
| Glibenclamide (5 mg/kg) | 30.15 ± 0.54 | 15.40 ± 0.94 *** | 49.26 |

**Table 2**
Effect of *A. genipiflora* stem bark extract on the lipid parameters of diabetic rats.

| Experimental Groups | Lipid parameters (mg/dL) | | | |
|---|---|---|---|---|
| | **Total cholesterol** | **Triglycerides** | **LDL-C** | **HDL-C** |
| Normal control | 76.08 ± 2.42 | 70.94 ± 2.54 | 17.48 ± 1.49 | 39.91 ± 1.48 |
| Diabetic control | 125.50 ± 1.40 | 147.20 ± 0.92 | 77.84 ± 2.01 | 18.29 ± 0.25 |
| AGB (100 mg/kg) | 112.80 ± 0.81[a] | 136.30 ± 2.61 b | 69.20 ± 1.16 | 24.64 ± 0.92[a] |
| AGB (200 mg/kg) | 104.20 ± 2.67[c] | 128.10 ± 0.73[a] | 62.38 ± 3.29[a] | 27.58 ± 1.64[a] |
| AGB (400 mg/kg) | 86.80 ± 2.04[c] | 107.80 ± 5.03[c] | 54.32 ± 2.88[c] | 29.95 ± 1.43[c] |
| Glibenclamide (5 mg/kg) | 76.70 ± 2.57[c] | 95.75 ± 2.52[c] | 35.21 ± 4.62[c] | 31.62 ± 1.45[c] |

[a] $p < 0.01$
[b] $p < 0.05$
[c] $p < 0.001$ compared to diabetic control. AGB: 70% ethanol stem bark extract of *A. genipiflora*.

Values are expressed as mean ± SEM (n = 6), *** $p < 0.001$ compared to diabetic control. AGB: 70% ethanol stem bark extract of *A. genipiflora*.

#### 3.2.2. Effect of AGB on the lipid profile of experimental rats

The 70% ethanol stem bark extract of *A. genipiflora* (AGB) exhibited a significant ($p < 0.05$) hypolipidaemic activity on the lipid parameters of the STZ-induced diabetic rats. High plasma levels of triglycerides (TG), total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) experimental observed in the diabetic rats were considerably ($p < 0.05$) reduced whereas the low high-density lipoprotein cholesterol (HDL-C) appreciated significantly ($p < 0.01$).

### 3.3. In vitro hypoglycaemic activity of A. genipiflora stem bark extract (AGB) and oleanonic acid

#### 3.3.1. Effect of AGB and oleanonic acid on the activities of α-glucosidase α-amylase

The ethanol stem bark extract of *A. genipiflora* (AGB) and oleanonic acid inhibited considerably the activities of intestinal α-glucosidase and pancreatic α-amylase. The extract demonstrated strong inhibitory activities against the enzymes with $IC_{50}$ values of 10.48 ± 1.39 μg/mL and 14.51 ± 1.26 μg/mL against α-glucosidase and α-amylase respectively (Table 3) while oleanonic acid exerted a higher inhibitory activity against α-glucosidase (~5-fold higher than acarbose) than α-amylase. However, the α-amylase inhibitory activity of oleanonic acid was lower than acarbose respectively.

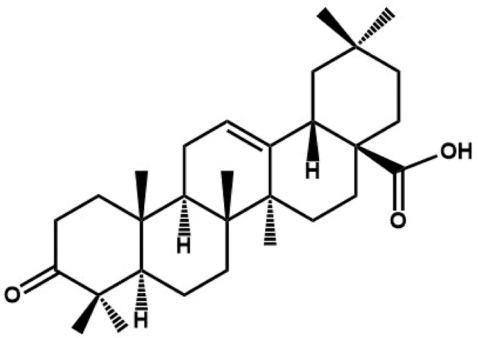


**Fig. 1.** Structure of oleanonic acid.

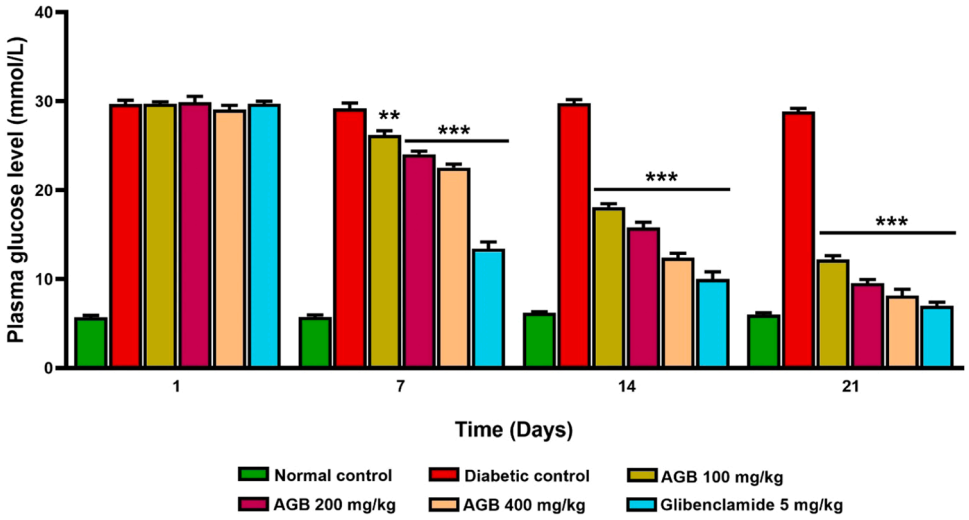


**Fig. 2.** Effect of *A. genipiflora* stem bark extract on fasting blood glucose of diabetic rats. Values are expressed as mean ± SEM (n = 6). ** $p < 0.01$, *** $p < 0.001$ compared to diabetic untreated group. AGB: 70% ethanol stem bark extract of *A. genipiflora*.

**Table 3**
Effect of *A. genipiflora* stem bark extract and oleanonic acid on carbohydrate metabolising enzymes.

| Test samples | $IC_{50}$ | |
|---|---|---|
| | **α-Glucosidase** | **α-Amylase** |
| AGB | 10.48 ± 1.39 μg/mL | 14.51 ± 1.26 μg/mL |
| Oleanonic acid | 36.52 ± 1.95 μM | 105.84 ± 1.08 μM |
| Acarbose | 183.68 ± 1.49 μM | 98.36 ± 1.08 μM |

Values displayed as mean ± SD (n = 3). AGB: 70% ethanol stem bark extract of *A. genipiflora*.

### 3.3.2. Effect of AGB and oleanonic acid on the glucose transport in C2C12 myotubes and 3T3-L1 adipocytes

The activity of the stem bark extract of *A. genipiflora* (AGB) and oleanonic acid on the enhancement of glucose transport were evaluated in C2C12 myotubes and 3T3-L1 adipocytes. AGB and oleanonic acid exerted considerable stimulation of glucose uptake above the control with $EC_{50}$ of 32.05 ± 0.58 μg/mL and 15.57 ± 0.93 μg/mL for AGB and 16.28 ± 0.30 μM and 7.26 ± 0.88 μg/mL for oleanonic acid in in C2C12 myotubes and 3T3-L1 adipocytes, respectively. Their activities were however lower than the positive controls (Table 4).

### 3.3.3. Effect of AGB and oleanonic acid on GLUT-4, PI3K and PPARγ transcripts expression

*A. genipiflora* stem bark extract (AGB) and oleanonic acid significantly ($p < 0.001$) increased the expression GLUT-4 and PI3K genes above the control which was statistically like IGF-1 and rosiglitazone. Their increase of GLUT-4 transcripts was 3.20- and 3.50-folds respectively above the control whereas they increased PI3K transcripts by 2.55- and 2.83-folds respectively when compared to the control. However, oleanonic acid stimulated a higher increase (2.5-fold above control) of the PPARγ gene transcripts than AGB (~1.7-fold above the control). Rosiglitazone on the other hand demonstrated the highest upregulation of PPARγ gene transcripts with an increase of 3.0-fold above the control (Fig. 3).

**Table 4**
Effect of *A. genipiflora* stem bark extract and oleanonic acid on glucose uptake in C2C12 myotubes and 3T3-L1 adipocytes.

| Test samples | $EC_{50}$ | |
|---|---|---|
| | **C2C12 myotubes** | **3T3-L1 adipocytes** |
| AGB | 32.05 ± 0.59 μg/mL | 15.57 ± 0.93 μg/mL |
| Oleanonic acid | 16.28 ± 0.30 μM | 7.26 ± 0.88 μM |
| IGF-1 | 2.85 ± 0.11 nM | – |
| Insulin | – | 0.76 ± 0.04 nM |

Values displayed as mean ± SD (n = 3). AGB: 70% ethanol stem bark extract of *A. genipiflora*.

## 3.4. In vivo antidiabetic activity of oleanonic acid

### 3.4.1. Effect on blood glucose of diabetic rats

The activity of oleanonic acid (15 – 60 mg/kg) on the blood glucose of the diabetic animals prior to and 6 h after administration of the initial doses are presented in Table 5 whereas its effect on the FBG of the diabetic animals recorded weekly over the 21 days study are shown in Fig. 4. The compound reduced the blood glucose level of the experimental animals with a considerable ($p < 0.05$) reduction at 60 mg/kg 6 h after treatment with the first dose and continued to significantly ($p < 0.05$) decrease the FBG over time till the end of the study compared to the diabetic control group.

### 3.4.2. Effect on the lipid profile of the diabetic animals

Oleanonic acid demonstrated considerable anti-hyperlipidaemic activity in the diabetic rats reducing significantly ($p < 0.001$) the elevated plasma levels of TC, TG and LDL-C and at the same time producing a significant ($p < 0.01$) increase in the plasma HDL-C levels mostly at 30 and 60 mg/kg (Table 6).

## 3.5. Predicted ADMET profile of oleanonic acid

Computer-aided analysis showed that oleanonic acid fell within the stipulated ranges of Lipinski's rule howbeit with one violation, lipophilicity (cLogP ≤5) (Table 7). The compound was predicted to be devoid of blood-brain permeability (BBB-). Although oleanonic acid was predicted to exhibit poor solubility, it was shown to have > 90% oral absorption and great permeability. It could also undergo passive absorption via P-glycoprotein however it was neither a P-glycoprotein substrate nor inhibitor. Apart from CYP450 3A4, oleanonic was found to be neither a substrate nor an inhibitor of Cytochrome P450 family of isozymes. In terms of its toxicity, the compound was predicted to generally non-toxic possessing no hERG inhibitory potential, non-carcinogenicity, non-hepatotoxicity, and non-genotoxicity. However, it was predicted to be teratogenic (Table 8).

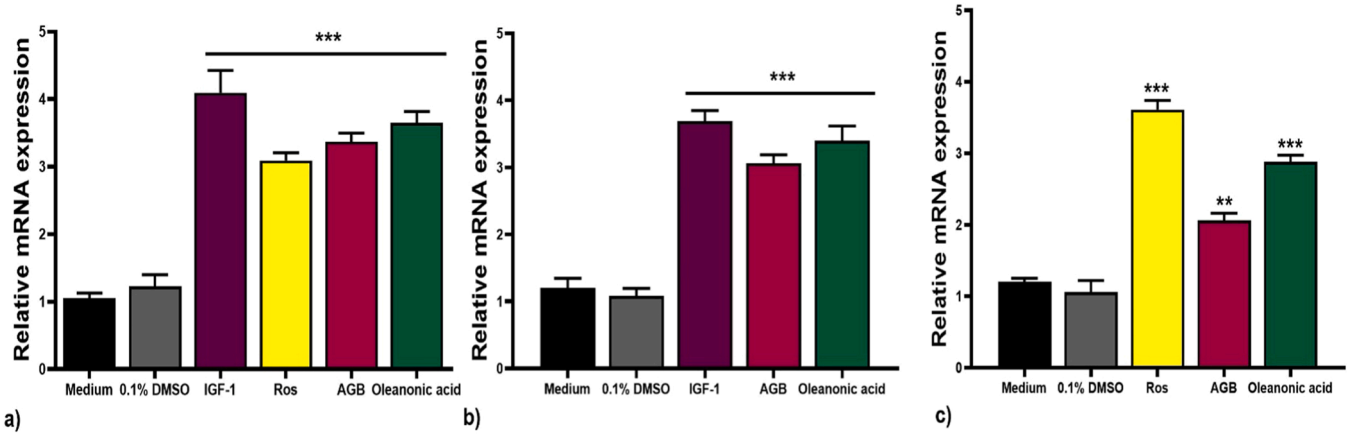


**Fig. 3.** Effect of *A. genipiflora* stem bark extract and oleanonic acid on a) GLUT-4 b) PI3K and c) PPARγ gene expression in C2C12 myotubes. Values are expressed as mean ± SD (n = 3). * * $p < 0.01$, *** $p < 0.001$ compared to control (medium). Ros: Rosiglitazone. AGB: 70% ethanol stem bark extract of *A. genipiflora*.

**Table 5**
Effect of oleanonic acid on the blood glucose of diabetic rats after 6 h of initial treatment.

| Experimental Groups | 0 h | 6th hour | Variation (%) |
|---|---|---|---|
| Normal control | 5.32 ± 0.46 | 5.70 ± 0.09 | – |
| Diabetic control | 30.40 ± 1.13 | 29.55 ± 1.56 | 2.80 |
| Oleanonic acid (15 mg/kg) | 30.05 ± 1.35 | 26.30 ± 1.82 | 12.48 |
| Oleanonic acid (30 mg/kg) | 31.20 ± 1.58 | 24.81 ± 1.60 | 20.50 |
| Oleanonic acid (60 mg/kg) | 30.70 ± 1.02 | 23.20 ± 1.51 * | 24.43 |
| Glibenclamide (5 mg/kg) | 29.92 ± 1.26 | 16.81 ± 1.34 *** | 43.80 |

Values are expressed as mean ± SEM (n = 6), * $p < 0.05$, *** $p < 0.001$ compared to diabetic control.

## 4. Discussion

In our continuous search for alternative treatments for diabetes, we evaluated the hypoglycaemic and hypolipidaemic activities of the hydroethanolic stem bark extract of *A. genipiflora* (AGB) in rat experimental model of diabetes using streptozotocin (STZ, 40 mg/kg) as the inducing agent. The extract exhibited a fast onset of action reducing drastically ($p < 0.001$) the elevated blood glucose levels of the diabetic rats by 17 – 42% at 100–400 mg/kg within 6 h indicating its tendency to manage sudden spikes in blood glucose concentration in diabetic patients. It continued to lower the fasting blood glucose (FBG) of the diabetic animals throughout the study and at 200 and 400 mg/kg brought the FBG to below 10 mmol/L by the 21st day which was comparable to glibenclamide.

Inhibiting the activity of carbohydrate metabolizing enzymes, α-glucosidase and α-amylase, and thereby delaying the digestion of dietary carbohydrate to glucose has been shown as an effective method to hinder the progression to diabetes in pre-diabetic individuals and high-risk persons as well as a way to prevent the development of diabetic complications [34]. The extract considerably inhibited the activities of α-glucosidase and α-amylase with $IC_{50}$ of 10.48 ± 1.39 μg/mL and 14.51 ± 1.26 μg/mL respectively. The inhibition of these enzymes by AGB could account for the observed hypoglycaemic activity of the extract in the diabetic rats and indicate the presence of plant constituents with such mechanisms of hypoglycaemic action within the extract.

The tendency of AGB to promote the transport of glucose into the peripheral tissues as another possible mechanism of hypoglycaemic activity was evaluated in mouse C2C12 skeletal muscles and 3T3-L1 adipocytes. The extract significantly promoted the uptake of radioactive [$^3$H] 2-deoxyglucose into the cell lines with $EC_{50}$ of 32.05 ± 0.58 μg/mL and 15.57 ± 0.93 μg/mL in C2C12 myotubes and 3T3-L1 adipocytes respectively. The considerably enhanced glucose uptake observed in the skeletal muscle cell line correlated with significant ($p < 0.001$) increase in the expression of glucose transporter-4 (GLUT-4)

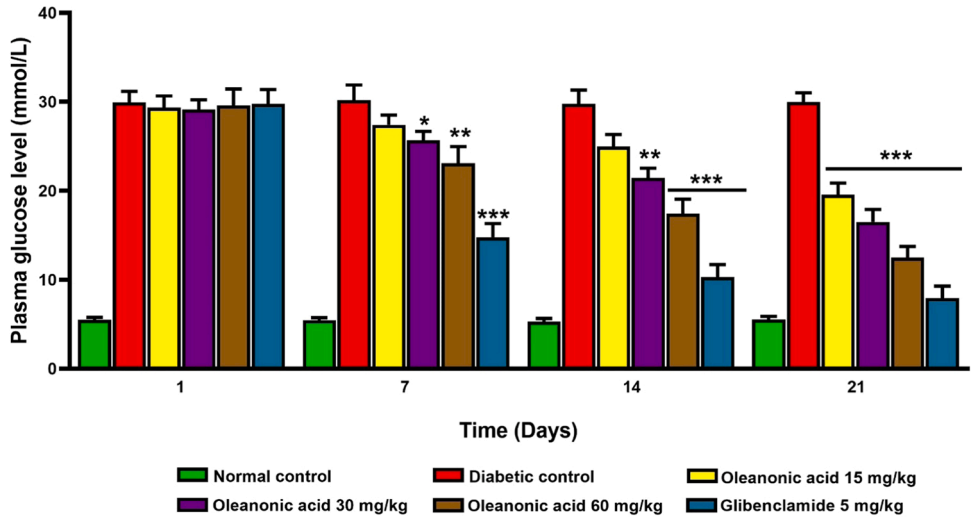


**Fig. 4.** Effect of oleanonic on fasting blood glucose of diabetic rats. Values are depicted as mean ± SEM (n = 6). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to diabetic untreated group.

**Table 6**
Effect of oleanonic acid on the lipid profile diabetic rats.

| Experimental Groups | Lipid parameters (mg/dL) | | | |
|---|---|---|---|---|
| | **Total cholesterol** | **Triglycerides** | **LDL-C** | **HDL-C** |
| Normal control | 76.72 ± 3.44 | 67.75 ± 2.00 | 17.51 ± 0.80 | 40.56 ± 2.13 |
| Diabetic control | 123.90 ± 1.39 | 150.90 ± 2.66 | 86.97 ± 1.70 | 17.16 ± 1.16 |
| Oleanonic acid (15 mg/kg) | 120.00 ± 2.48 | 145.30 ± 1.72 | 78.08 ± 4.25 | 21.19 ± 0.56 |
| Oleanonic acid (30 mg/kg) | 115.40 ± 3.14 | 134.20 ± 2.19 *** | 70.16 ± 2.84 *** | 23.94 ± 0.46 ** |
| Oleanonic acid (60 mg/kg) | 94.30 ± 2.23 *** | 121.39 ± 4.72 *** | 64.31 ± 3.39 *** | 25.44 ± 1.55 *** |
| Glibenclamide (5 mg/kg) | 80.14 ± 2.08 *** | 83.44 ± 1.77 *** | 36.54 ± 1.90 *** | 29.47 ± 1.31 *** |

* * $p < 0.01$, * ** $p < 0.001$ compared to diabetic control.

**Table 7**
Molecular properties of oleanonic acid.

| Descriptor | Calculated value | Standard value/range |
|---|---|---|
| Molecular weight | 454.69 | ≤ 500 |
| clogP | 6.18 | ≤ 5 |
| H-bond donor | 1 | ≤ 5 |
| H-bond acceptor | 3 | ≤ 10 |
| LogS | -7.325 | > −5.7 |
| Polar surface area | 54.37 | 140 Å$^2$ |
| No. of Primary metabolites | 3 | < 7 |

**Table 8**
Predicted pharmacokinetic and toxicity properties of oleanonic acid.

| Model | Result | Probability/ Value |
|---|---|---|
| Absorption | | |
| Blood-Brain Barrier | BBB - | 0.9970 |
| Human Intestinal Absorption | HIA + | 0.9919 |
| Caco-2 Permeability | Caco-2 + | 0.5282 |
| P-glycoprotein Substrate | Non-Substrate | 0.6903 |
| P-glycoprotein Inhibitor | Non-inhibitor | 0.8289 |
| Renal Organic Cation Transporter | Non-inhibitor | 0.9985 |
| Bioavailability ($F_{20\%}$) | $F_{20\%}$ - | 0.157 |
| Distribution | | |
| Plasma Protein Binding | High protein-bound | 93.35% |
| Volume of Distribution | Optimal VD | 0.846 |
| Fraction unbound in plasma | Low Fu | 3.899% |
| Subcellular localization | Lysosomes | 0.6599 |
| Metabolism | | |
| CYP450 3A4 Substrate | Substrate | 0.6063 |
| CYP450 2D6 Substrate | Non-substrate | 0.8683 |
| CYP450 2C9 Substrate | Non-substrate | 0.6106 |
| CYP450 1A2 Inhibitor | Non-inhibitor | 0.8772 |
| CYP450 3A4 Inhibitor | Non-inhibitor | 0.8315 |
| CYP450 2C9 Inhibitor | Non-inhibitor | 0.8584 |
| CYP450 2C19 Inhibitor | Non-inhibitor | 0.8273 |
| CYP450 2D6 Inhibitor | Non-inhibitor | 0.9514 |
| CYP Inhibitory Promiscuity | Non- CYP Inhibitory Promiscuity | 0.9345 |
| Excretion | | |
| Clearance (CL) | Low | 2.818 |
| Half-life | short half-life (<3 h) | 0.181 |
| Toxicity | | |
| AMES Toxicity | Non-AMES toxic | 0.8500 |
| Human Ether-a-go-go-Related Gene Inhibition | Non-inhibitor | 0.5098 |
| Carcinogens | Non-carcinogen | 0.9303 |
| Teratogenicity | Teratogenic | 0.9333 |
| Hepatotoxicity | Non-hepatotoxic | 0.7000 |
| Acute Oral Toxicity | III | 0.7802 |

and phosphoinositide-3-kinase (PI3K) transcripts which is characteristic of the classical pathway for insulin-dependent glucose uptake mechanism where there is the translocation of GLUT-4 from the cytosol to the membranes of peripheral cells via the activation of PI3-kinase [35]. AGB also significantly ($p < 0.01$) increased the peroxisomal proliferator-activated receptor-gamma (PPARγ) transcripts. The activation of this enzyme also results in the increased utilisation of glucose in the periphery by the expression and translocation of GLUT-4 through an insulin-independent glucose uptake process [36]. Thus, the observed glucose uptake effect of AGB is because of its tendency to stimulate both insulin-dependent and independent glucose uptake mechanisms. This makes the extract an effective alternative in the management of both type 1 and type 2 diabetes mellitus.

Hyperlipidaemia remains the major risk factor in the development of hypertension, atherosclerosis, and other cardiovascular diseases [37]. Thus, the reduction in total cholesterol (TC), triglycerides (TG) and low-density lipoprotein-cholesterol (LDL-C) is considered a suitable approach in preventing such diabetic cardiovascular complications. Elevated levels of serum TC, TG and LDL-C of the diabetic animals were significantly ($p < 0.01$) reduced with a concomitant increase ($p < 0.01$) in high-density lipoprotein-cholesterol (HDL-C) levels upon treatment with AGB which can be attributed to the presence of hypolipidaemic compounds in the extract and enhancement in glucose homeostasis through improved insulin secretion and action due to the continuous supplementation of *A. genipiflora* stem bark extract. The quick onset and long duration of hypoglycaemic activity coupled with the observed hypolipidaemic activity of AGB in the diabetic animals indicate that the stem bark extract of *A. genipiflora* is a good therapeutic adjunct in the management for the diabetes.

Phytochemical studies led to the purification of oleanonic acid from the ethanol stem bark extract of *A. genipiflora*. Its hypoglycaemic activity was then established through *in vitro* and *in vivo* experiments. Oleanonic acid inhibited α-glucosidase and α-amylase activities with $IC_{50}$ values of 36.52 ± 1.95 μM and 105.84 ± 1.08 μM, respectively. Molecular docking studies have shown that triterpenoids form stable binding interactions such as hydrogen bonds, hydrophobic interactions with amino acids and some H-Pi interactions in non-polar amino acids in the binding sites of α-glucosidase and α-amylase to exert their inhibitory activities [38,39]. Thus, the more and stronger interactions the triterpene has with the enzymes, the higher the inhibitory effect. This may therefore explain the higher inhibitory effect of oleanonic acid on α-glucosidase (~3 ×) than α-amylase. The inhibition of these enzymes also shows that oleanonic can reduce effectively postprandial hyperglycaemia and prevent diabetes-related complications.

Oleanonic acid exerted significant ability to promote glucose uptake into the periphery with $EC_{50}$ of 16.28 ± 0.30 μM and 7.26 ± 0.88 μM in C2C12 myotubes and 3T3-L1 adipocytes respectively corroborating its previously reported ability to enhance glucose uptake in L6 muscle cells [22]. It also produced a corresponding increase in GLUT-4, PI3K and PPARγ gene transcripts indicating that oleanonic acid promotes glucose uptake through GLUT-4 translocation via both insulin and non-insulin signalling pathways. As previously stated, glucose transport through the insulin signalling pathway involves the activation of PI3K and the subsequent translocation of GLUT-4 from the cytoplasm to the cell membrane in peripheral cells. On the other hand, activating PPARγ through its agonists, the thiazolidinediones, also enhance glucose transport through GLUT-4 translocation. Oleanonic acid stimulated a comparable increase in PPARγ transcripts to rosiglitazone confirming the earlier findings of Petersen et al. (2011) [21] that the compound is also a PPARγ agonist.

Following the *in vitro* assays, the hypoglycaemic and anti-hyperlipidaemic activity of oleanonic acid was evaluated in STZ-induced diabetic rats. The compound at the doses (15 – 60 mg/kg) administered demonstrated significant ($p < 0.001$) glucose-lowering activity and corrected to some extent the dyslipidaemia present in the animals at the end of the study by lowering the high levels of TC, TG and

LDL-C and at the same time increasing HDL-C levels. Generally, the overall antidiabetic activity of the *A. genipiflora* extract was higher than oleanonic acid indicating the contribution of unidentified active compounds such as flavonoids, glycosides, sterols and other triterpenoids to the bioactivity of the plant extract. These compounds may be more potent than and/or act synergistically or additively with oleanonic acid which was isolated in this study.

Interestingly oleanolic acid, the reduced derivative of oleanonic acid has also been shown to exert antidiabetic activity in rats [40]. Loza-Rodríguez et al. (2020) [41] revealed that oleanolic acid enhances GLUT-4 translocation through PPARγ/α expression to exert its anti-hyperglycaemic activity in C2C12 myoblasts. Several plant triterpenoids have also been shown to possess hypoglycaemic activities with some exhibiting similar mechanisms of action as oleanonic acid and its reduced derivative, oleanolic acid [42–44].

*In silico* pharmacokinetics prediction showed that oleanonic acid was within stipulated ranges except for one violation to Lipinski's rule, falling short of lipophilicity (cLogP = 6.18). The compound was found to lack the tendency to cross the blood brain barrier and therefore devoid of any central nervous system (CNS) activity. Although the poor solubility and non-polar nature could impair intestinal absorption, oleanonic acid was predicted to be highly orally absorbed and possess great permeability which could be because of its tendency undergo absorption via transporter carriers such as the P-glycoproteins [45].

Toxicity wise, oleanonic was predicted to be generally non-toxic and non-lethal possessing no carcinogenicity, no hepatotoxicity and no genotoxicity. However, it was found to be exhibit teratogenic potentials and should not be given to, including the plant extract from which it was isolated from, pregnant and lactating women [46]. The compound was found not to exhibit any hERG inhibitory potential and therefore would not interfere with cardiac conduction and function. The inhibition of hERG is a red flag in drug development which must be assessed at the early stages of the drug development process [47]. Oleanonic acid was identified as a category III drug for its acute oral toxicity with $LD_{50}$ between 500 and 5000 mg/kg, far above doses employed in this study (15 – 60 mg/kg). This was confirmed in the acute toxicity studies where treated animals even at 100 mg/kg did not exhibit any signs of toxicity.

The present study has demonstrated the hypoglycaemic activity of the 70% ethanol stem bark extract of *A. genipiflora* (AGB) and its isolate, oleanonic acid using *in vitro* and *in vivo* assays and given scientific credence to the use of the plant as an antidiabetic agent in traditional medicine as well as a potential source of novel compounds for the discovery of new hypoglycaemic drugs.

## Conclusion

In conclusion, the antidiabetic activity of the ethanol stem bark extract of *A. genipiflora* and oleanonic acid in STZ-induced diabetic rats has been demonstrated in this study. The extract and its constituent have also been shown to exert their hypoglycaemic effects through the promotion of glucose uptake in C2C12 myotubes and 3T3-L1 adipocytes through both insulin-dependent and independent pathways; as well as the inhibition of α-glucosidase and α-amylase. Thus, *A. genipiflora* and oleanonic acid may be useful in the management of diabetes. Further phytochemical studies could be carried out to unearth the other antidiabetic constituents of the plant. The antidiabetic activity coupled with the *in silico* studies also showed that oleanonic acid could serve as template for further hypoglycaemic drug development.

## Funding

The authors received no specific funding for this work.

## Data availability

No data was used for the research described in the article.

## Acknowledgments

The authors wish to thank the technical staff of the staff of the animal house of the Department of Pharmacology, KNUST and the technical staff of the School of Pharmacy, UHAS for their assistance.

### *CRediT authorship contribution statement*

**Benjamin Kingsley Harley:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Validation, Writing – original draft, Writing – review & editing. **Isaac Kingsley Amponsah:** Writing – original draft, Writing – review & editing. **Inemesit Okon Ben:** Data curation, Formal analysis, Investigation, Methodology. **Nana Ama Mireku-Gyimah:** Formal analysis, Writing – original draft, Writing – review & editing. **Daniel Anokwah:** Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **David Neglo:** Writing – original draft. **Cedric Dzidzor K. Amengor:** Formal analysis, Investigation, Writing – review & editing. **Theophilus Christian Fleischer:** Conceptualization, Formal analysis, Methodology, Supervision, Writing – review & editing.

### *Competing Interest*

The authors wish to declare no competing interest.

### *Supplementary Materials*

The NMR spectra of oleanonic acid are included in the supplementary data.

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2022.112833.

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