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Antidiabetic Andantioxidant Properties, and α-amylase and α-glucosidase Inhibition Effects of Triterpene Saponins from *Piper auritum*

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Abstract Bioactivity-guided fractionation of methanol extracts from leaves of *Piper auritum* produced four triterpenoid saponin compounds **1**-**4**. Structures were established based on interpretation of mass spectrometry (MS), nuclear magnetic resonance (NMR) data. 21-(*p*-methoxycinnamoyl)-olean-12-ene-28oic cid-3-*O*-α-L-arabinopyranosyl-(1→2)-β-D-glucopyranoside (**1**) and olean-12-ene-28 methyl ester-3-*O*-α-L-arabinofuranosyl-(1→2)-β-D-glucopyranoside (**2**) were orally administered to diabetic mice at dosage of 10 mg/kg of body weight per day for 30 days and resultant biochemical parameters were studied. Both compounds significantly (p<0.05) decreased serum glucose, total cholesterol, and triglyceride levels, compared with controls. Low density lipoprotein and high density lipoprotein cholesterol levels were ameliorated. The effects of lipid peroxidation and oxidative stress in the liver, pancreas, and kidney were reversed, with reductions insulin resistance and stimulation of insulin production. β-Glucosidase activities were studied *in vitro*. Compounds **1** and **2** can be used to improve glucose and lipid metabolism and to reduce the imbalance between generation of reactive oxygen species and scavenging enzyme activities for prevention of diabetic complications.

Keywords: *Piper auritum*, triterpenoids, antidiabetic, hyperlipidaemia, hypoglycaemic

Introduction

Diabetes is accompanied by impaired antioxidant defenses and consequently an increased production of free radicals, causing oxidative stress damaging pancreatic beta cells (1). The main target in the treatment of diabetes is to control blood glucose levels which involves the use of thiazolidinediones, biguanides, sulfonylureas, D-phenylalanin, thiazolidinediones, and α-amylase, α-glucosidase and lipase inhibitors (2). However, due to unwanted side effects, the efficacies of these compounds are debatable and there is a demand in obtaining new drugs to treat diabetes (3). Hypertriglyceridemia, hypertriglyceridemia, and hypercholesterolemia are risk factors that can accelerate the development of diabetes and atherosclerosis (4). Pancreatic α-amylase is a key enzyme in the digestive system and acts as a catalyst in the initial step of the hydrolysis of starch to maltose and finally to glucose. Degradation of the dietary starch proceeds rapidly and leads to elevated post prandial hyperglycemia (5). Hence, the retardation of starch digestion by inhibition of enzymes like α-amylase and α-glucosidase could play a key role in the control of diabetes.

Piper auritum known by the name of Hoja Santa, is used for their spicy aromatic scent and flavor. It has an important presence in Mexican cuisine. It has been reported to possess pharmacological properties like antioxidant (6), and hypoglycemic (7). The aim of this study was to test the effect of triterpenes from *Piper auritum* on diabetic mice.

Materials and Methods

General experimental procedures Infrared spectra (IR) were obtained using a PerkinElmer 1720-FTIR (PerkinElmer, Waltham, MA, USA). A Bruker DRX-300 NMR spectrometer, operate at 599.19 MHz for ^{1}H and 150.86 MHz for ^{13}C, was used for nuclear magnetic resonance (NMR) experiments with the UXNMR software package Chemical shifts were expressed as δ (ppm) using TMS as an internal standard. DEPT ^{13}C, ID TOCSY, ^{1}H-^{1}H DQF-COSY, and HMBC NMR experiments were carried out using previously described conventional pulse sequences (8). Mass spectrum values were measured using a JEOL HX 110 mass spectrometer (JEOL, Tokyo, Japan). Precoated TLC silica gel 60 F254 aluminium sheets from Sigma-Aldrich (St. Louis, MO, USA) were used. Column chromatography was carried out using

Silica gel 60 (230-400 mesh; Merck & Co., Kenilworth, NJ, USA). Solvents used as eluents were obtained from Fermont, CA, USA.

Plant material Fresh leaves of *Piper auritum* were collected in Amecameca de Juarez, Mexico, in May of 2013 were identified by staff members at the Herbarium of the Metropolitan Autonomous University-Xochimilco, Mexico DF, Mexico. A representative specimen was deposited in this herbarium (No. 7345) for further reference.

Experimental animals Healthy male CD1 mice (18 to 21 g), aged 6 weeks old, were provided by the bioterium of the National School of Biological Sciences Mexico DF, Mexico. Animals were housed in microlon boxes in a controlled environment (temperature 25±2°C) and fed a standard laboratory diet (Mouse Chow 5015; Purina, St. Louis, MO, USA) and water ad libitum. Before experiments, mice were acclimatized to laboratory conditions for 3 days. All animal experimental procedures were performed in accordance with the International Guidelines for Care and Use of Laboratory Animals (9) and were approved by the Animal Ethical Committee of the Escuela Nacional de Ciencias Biologicas (NOM-062-ZOO-1999) (10).

Chromatographic purification and isolation Air-dried (aircirculating oven, Maincer, Castellón, Spain) in leaves of *P. auritum* (9 kg) were ground in a hand mill-210 (Finca Casarejo, DF, Mexico) and subjected to extraction successively 3 times using methanol under reflux (3 h). Methanol extract was evaporated in vacuo in a BÜCHI rotavapor r-200 (Büchi, Flawil, Switzerland) to yield the crude residue (879 g). The methanol extract was initially separated using silica gel chromatography column and eluted using chloroform-ethyl ether (10:1). Each fraction (75 mL) was monitored based on TLC. Fractions with similar TLC patterns were combined to yield the 4 major fractions F1 to F4. Each fraction was monitored for a hypoglycaemic effect in diabetic mice. Fraction F1 was re-chromatographed on a silica gel column, (2 kg) and eluted using hexane-chloroform (2:3) to produce the 9 subfractions F1-1 to F1-9. Hypoglycemic subfraction F1-1 (40.6 g) was subjected to silica gel column chromatography and eluted using chloroform-hexane:acetone (2:3.5:0.5) to produce the 6 subfractions F11-1 to F11-6. The F11-6 subfraction was further purified using preparative chromatography eluted using chloroform-acetone (2:1) to produce the 4 subfractions F1161-F1164, with yields of 1 (200 mg), 2 (150 mg), 3 (89 mg), and 4 (125 mg).

Induction of severe diabetes (SD) Severe diabetes was induced in overnight fasted male mice using a single intraperitoneal injection of 60 mg/kg of streptozotocin (STZ) dissolved in a cold citrate buffer (pH 4.5) at a volume of 1 mL/kg body weight (10). Hyperglucemia was confirmed by measurement of the blood glucose level with FreeStyle Lite glucose meters (FreeStyle, MO, USA) both 72 h and 7 days after injection of streptozotocin. Mice with a permanent high fasting blood glucose level >300 mg/dL were used for experiments.

Induction of mild diabetes (MD) Mild diabetes was induced in overnight fasted mice using a single intraperitoneal injection of STZ at 45 mg/kg of body weight in a 0.1 mol/L cold citrate buffer (pH 4.5) 15 min after intraperitoneal administration of 120 mg/kg of nicotinamide. STZ treated animals were provided a 5% glucose solution overnight to overcome drug induced hypoglycemia. After a 10 day period for development of diabetes, mice with moderate diabetes exhibit persistent glycosuria and hyperglycaemia (blood glucose >250 mg/dL) were used for experiments (11).

Experimental design in diabetic mice

***Effects of a single oral administration of isolated compounds 1 and 2 on glucose levels in severe and mild diabetic mice*:** After mice had been denied access to food/water overnight, they were randomly divided into 33 groups (6 mice per group). Normal (control) mice were administered distilled water. Diabetic group mice were orally administered 10 mg/kg of body weight of the triterpenoid saponins **1** and **2** suspended in Tween 80, (1%) via gavage. Diabetic mice received glibenclamide (GB) at a dosage of 5 mg/kg of body weight as a standard drug for comparison purposes. Blood samples were collected from the tail vein at 0, 2, 4, 6, 8, and 12 h after administration of all treatments. The plasma glucose-concentration was determined using an enzymatic colorimetric method (12) with a commercial kit (Sigma-Aldrich).

Antidiabetic testing in severe and mild streptozotocine-induced diabetic mice In a parallel study, 11 groups (*n*=10) of diabetic mice were used to determine thechronic effect of compounds **1** and **2**. Normal (control), severe (SD), and milddiabetic (MD) mice groups were fed a normal diet and provided water ad libitum. Saline was administered via gastric gavage. Severe and mild diabetic mice that received the triterpenoid saponins **1** and **2** by gastric gavage (10 mg per kg of body weight) every day were designated as groups SD+**1** (severe diabetes more compound 1) SD+2 (severe diabetes more compound 2) , and MD+**1** (mild diabetes more compound **1**) and MD+**2** (mild diabetes more compound **2**). Two groups of severe (SD+GB) and mild diabetic mice (MD+GB) were administered glibenclamide (GB) at 5 mg/kg as a positive control.

Determination of body weight and food intake The body weight of each rat was measured once each week and the total amounts of food and water consumed were recorded 3 times per week.

Oral glucose tolerance and insulin tolerance testing Mice in each group were orally administered compounds **1** and **2** at dosages of 10 mg/kg body weight on a daily basis for 30 days. At the end of the experimental period, an oral glucose tolerance test (OGTT) was performed to assess animal sensitivity to a high glucose load. Overnight-fasted mice were fed orally 2 g of glucose/kg of body weight Blood samples were collected at 0 min (immediately after

glucose loading) from the caudal vein through a small incision at the end of the tail and at 30, 60, 90, and 120 min after glucose administration. On day 18, insulin tolerance testing (ITT) was performed using overnight fasted mice from all groups. Thirty min after administration of extracts, mice were intraperitoneally injected with 0.15 U/kg of insulin as recombinant Human Insulin Injection (Lilly, Indianpolis, IN, USA). Blood samples were collected in the same manner as indicated for OGTT testing and blood glucose levels were determined at 0, 30, 60, and 120 min after insulin injection.

Antioxidant parameter levels in the serum, liver, pancreas, and kidney The activity of serum superoxide dismutase (SOD) was measured following the xanthine oxidase method (13) using commercial kits. Absorbance values were measured using a spectrophotometer at 550 nm. Serum catalase (CAT) and glutathione peroxidase (CSHPx) activities were measured following the colorimetric method (14) based on measurement of absorbance value (Shimadzu, Kyoto, Japan) at 405 and 412 nm, respectively. Glutathione reductase (GSH) levels were determined based on measurement of the rate of NADPH oxidation at 340 nm. All assay kits were purchased from Cayman Chemical (Ann Arbor, Michigan, MI, USA), and all procedures followed kit instructions. The pancreatic protein concentration was determined using the Bradford method (15) following the Bio-Rad protein assay kit instructions. The lipid peroxidation (LPO) level was determined using thiobarbituric acid reactive substances (TBARS) assay (Shimadzu) following the method of Fraga *et al*. (16) and expressed as mMol/mg of protein.

Serum lipid profiles and glucose levels At the end of the experimental period the effect of each treatment was determined based on serum levels of total cholesterol (TC), triglyceride (TG), and HDL-cholesterol using a commercial diagnostic kit (Genzyme Diagnostics, Cambridge, MA, USA). The LDL-cholesterol level was calculated as the difference between total cholesterol and HDL levels. Blood glucose levels were determined following the glucose oxidase-peroxidase (GOD-POD) method (17).

Determination of serum and pancreatic insulin levels Serum and pancreatic insulin levels were measured using an enzyme linked inmunosor-bent assay (ELISA) with a kit (Boehringer Mannheim Diagnostic, Mannheim, Germany). The level of insulin was expressed as μIU/mL.

Determination of the α-glucosidase inhibitory activity The α-glucosidase inhibitory activity of extracts was evaluated using the method described by Tsujii *et al*. (18). Briefly, α-glucosidase was dissolved in a phosphate buffer (50 mM, pH 6.7). Specific concentrations (2-2002 μg/mL) of the compounds were then added. The substrate1 mM *p*-nitrophenyl glycoside was added to initiate the enzyme reaction carried out at 37°C for 30 min. Then, a 3x volume of 1 M Na_2CO_3 (1 M) was added to stop the reaction. The enzymatica ctivity was quantified based on measurement of the absorption value at 405 nM (Shimadzu).

Inhibition of α-amylase A starch solution (0.5% w/v) was prepared by stirring 0.125 g of potato starch in 25 mL of a 20 mM sodium phosphate buffer with 6.7 mM sodium chloride at pH 6.9 in a boiling water bath for 15 min. The enzyme solution was prepared by mixing of 1 unit/mL of α-amylase in the same buffer. A colorimetric reagent was prepared by mixing an equal volume of a sodium potassium tartrate tetrahydrate solution and a 96 mM 3,5-dinitro salicylic acid solution. The previously mixed starch solution (1 mL) was mixed with increasing concentrations (50, 100, 200, 400, and 800 μg/mL) of the inhibitor acetic acid (0.1 mL), then 1 mL of the enzyme solution was added and left to react with the starch solution at 25°C for 3 min. After this, 1 mL of a colorimetric reagent was added and the contents were heated for 10 to 15 min in a boiling water bath. The final volume was made up using distilled water and the absorbance was measured at 540 nm spectrophotometrically (Shimadzu) (19).

After addition of 450 μL of ferrocyanide (20 mM), the resulting mixture was incubated (Thermo Fisher Scientific, Waltham, MA, USA) for 5 min at room temperature, and the absorbance was measured at 420 nm using a spectrophotometer.

$ONOO^-$ scavenging assay The $ONOO^-$ (peroxynitrite) scavenging activity was measured following a modified method (21). To synthesize peroxynitrite ($ONOO^-$) 5 mL 0.6 M KNO_2 was mixed with an acidic solution (0.6 M HCl) of 5 mL H_2O_2 (0.7 M) on ice bath for 1 min and 5 mi of ice-cold 1.2 M NaOH was added to the solution. The solution was subjected to treatment with granular MnO_2 prewashed with 1.2 M NaOH to remove the excess H_2O_2. The reaction mixture was left overnight at −20°C. Peroxynitrite solution was collected from the top of the frozen mixture and the concentration was measured spectrophotometrically at 302 nm (€=1670 M^{-1} cm^{-1}). To measure peroxynitrite scavenging activity an Evans Blue bleaching assay was used. The reaction mixture contained 50 mM phosphate buffer (pH 7.4), 0.1 mM DTPA, 90 mM NaCl, 5 mM KCl, 12.5 μM Evans Blue, various doses of compounds (0-200 μg/mL) and 1 mM peroxynitrite in final volume of 1 mL. The absorbance was measured at 611 nm (Shimadzu) after incubation at 25°C for 30 min. The percentage scavenging of ONOO- was calculated by comparing the results of the test and blank samples. All tests were performed six times.

O_2 scavenging assay The O_2 scavenging activity was assessed based on spectrophotometric determination of the reduction product of NBT in an X-XOD system (22). In brief, following 10 min of incubation (Thermo Fisher Scientific) of the triterpenes 1 and 2 at room temperature in a reaction mixture of 50 μmol/L of NBT, 50 μmol/L of xanthine, and 0.05 U/mL of XOD (final concentrations), the absorbance was measured at 560 nm (Shimadzu).

Trolox equivalent antioxidant capacity (TEAC) assay $ABTS^{+\cdot}$ radical

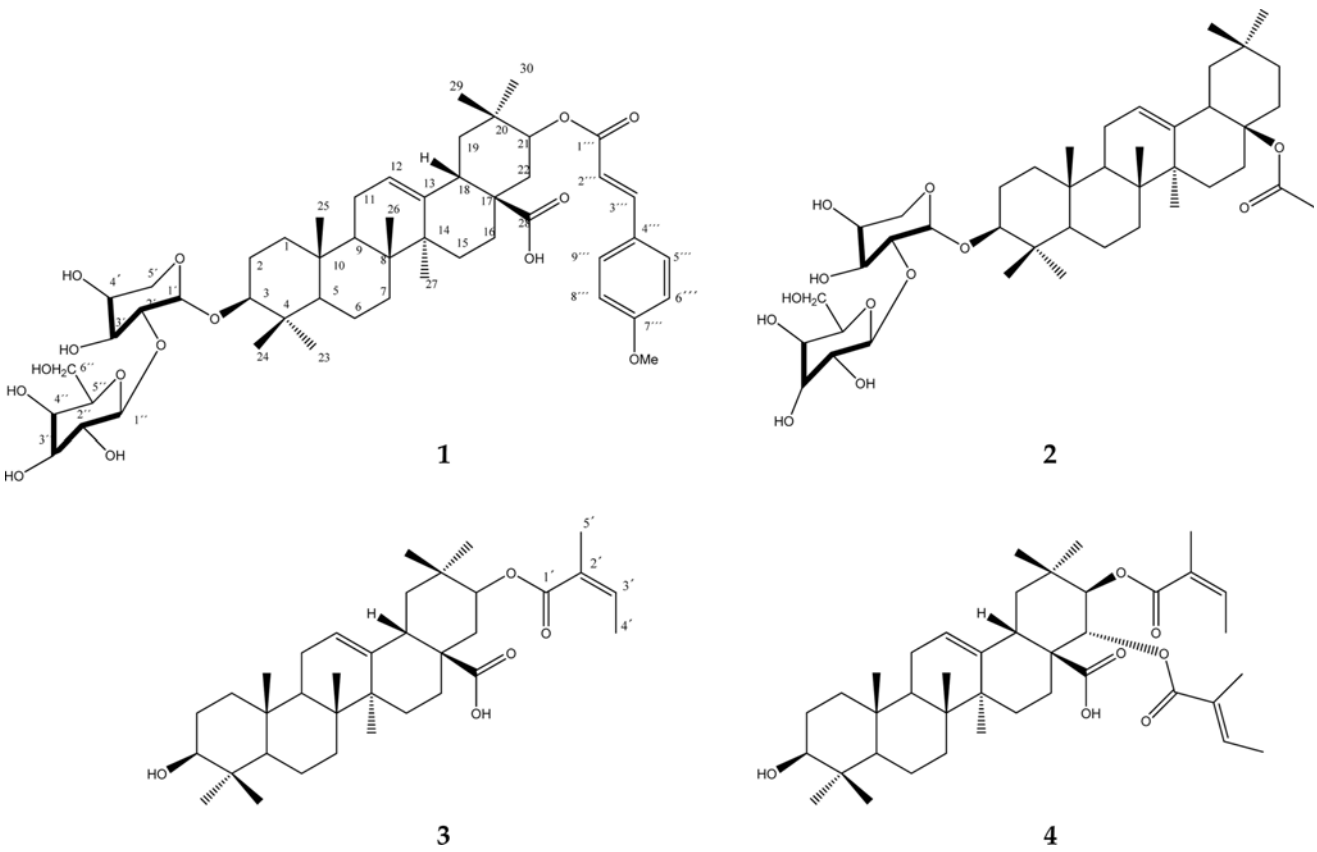

Fig. 1. Oleanane triterpenoids islated from *Piper auritum*.

cations were prepared based on mixing of the ABTS stock solution (7 mM in water) with 2.45 mM potassium persulfate ($K_2S_2O_8$). This mixture was left for 12-24 h in the dark until the reaction was complete and the absorbance was measured at 734 nm. 5 μM of the compounds **1** and **2** (1 mL) were reacted with 1 mL of the ABTS solution and the absorbance was measure at 734 nm after 7 min using a spectrophotometer (23).

Oxygen radical absorbance capacity assay An oxygen radical absorbance capacity assay was performed as per the method described by Cao *et al*. (24). Compounds **1** and **2** were incubated (Thermo Fisher Scientific) with fluoresce in as a free radical probe and AAPH as a free radical generator. The kinetics of fluorescein degradation were determined using a Spectra XMS Gemini microplate reader (Molecular Devices, Sunnyvale, CA, USA). Trolox was used to generate a standard curve. The antioxidant capacity was expressedas ORAC units, where 1 ORAC unit equaled the net protection produced by 1 μM Trolox.

Ferrous ion chelating ability The method of Decker and Welch (25) was used to investigate the ferrous ion chelating activity. A reaction mixture containing 1.0 mL of different concentrations (ranging between 50-8,000 μg/mL) of the compounds **1** and **2** was mixed with 3.7 mL of methanol, 0.1 mL of 2 mM ferrous chloride, and 0.2 mL of 5 mM ferrozine to initiate a reaction, and the mixture was vortexed vigorously and left to stand at room temperature for 10 min. The absorbance of the solution was measured at 562 nm (Shimadzu). The percent chelating effect against the ferrozine-Fe^{2+} complex was then calculated. Ferrous metal ions chelating activity was calculated as follows:

$$\text{Metal ions chelating activity (\%)}=(1-\text{As/Ac})\text{x}100$$

Where the Ac is the absorbance of the control reaction and As is the absorbance of the test sample.

Nitric oxide radical scavenging assay A nitric oxide radical scavenging assay was performed according to the method described by Sreejayan and Rao (26). A reaction mixture (3 mL) containing 10 mM nitroprusside in phosphate buffered saline, and the compounds **1** and **2** or the extracts at concentrations of 50-800 μg/mL were incubated (Thermo Fisher Scientific) at 25°C for 150 min. 0.5 mL of the incubated reaction mixture was removed at 30 min intervals, followed by addition of 0.5 mL of Griess reagent. The absorbance was measured at 546 nm (Shimadzu). Assay of the inhibition effect against lipid peroxidation: A solution of 2 mg of β-carotene in 10 mL of chloroform was prepared and 1.0 mL of this solution was pipetted into 20 mg of linoleic acid and 200 mg of a Tween 40 emulsifier. The chloroform was completely evaporated

(Büchi rotavapor r-200; Büchi). Aliquots of 5.0 mL of this emulsion were transferred into a series of tubes containing different concentrations (10-50 μg/mL) of the compounds **1** and **2** The absorbance was measured (Shimadzu) immediately (t=0) and after 90 min at 470 nm. The tubes were incubated (Thermo Fisher Scientific) at 50°C in a water bath during the testing procedure (22).

Superoxide radical scavenging assay Superoxide anions were generated using a nonenzymatic PMS/NADH system (22). A reaction mixture contained 1 mL of test solution, 1.9 mL of 0.1 M phosphate buffer (pH, 7.4), 1 mL of 20 μM PMS, 156 μM NADH, and 25 μM NBT in the phosphate buffer (pH, 7.4). After 2 min of incubation (Thermo Fisher Scientific) at 25°C, the color was read using a spectrophotometer at 560 nm against blank samples that contained no PMS.

Hydroxyl radical scavenging assay Hydroxyl radical scavenging activity was measured by the ability of the triterpenes 1 and 2 scavenge the hydroxyl radicals generated by the Fe^{3+}-ascorbate EDTA-H_2O_2 system (Fenton reaction) (27). The reaction mixture in a final volume of 1.0 mL contained 100 μL of 2-deoxy2-ribose (28 mM in 20 mM KH_2PO_4 buffer, pH 7.4), 500 μL of the the triterpenes 1 and 2 at various concentrations (50-800 μg/mL) in buffer, 200 μL of 1.04 mM EDTA and 200 μM $FeCl_3$ (1:1 v/v), 100 μL of 1.0 mM hydrogen peroxide (H_2O_2) and 100 μL of 1.0 mM ascorbic acid. Test samples were kept at 37°C for 1 h. The free radical damage imposed on the substrate, deoxyribose was measured using the thiobarbituric acid test. One mL of 1% thiobarbituric acid (TBA) and 1.0 mL 2.8% trichloroacetic acid (TCA) were added to the test tubes and were incubated at 100°C for 20 mino After cooling, the absorbance was measured at 532 nm against a blank containing deoxyribose and buffer.

Hydrogen peroxide scavenging assay A hydrogen peroxide solution (2 mM/L) was prepared using a standard phosphate buffer (pH 7.4). Concentrations of the compounds **1** and **2** 25-400 μg/mL in distilled water were added to 0.6 mL of a hydrogen peroxide solution. The absorbance was measured at 230 nm (Shimadzu) after 10 min against a blank solution containing the phosphate buffer without hydrogen peroxide. The percent scavenging activity at different concentrations 25-400 μg/mL of the compounds was determined and was compared with the α-tocopherol standard (23).

Statistical analysis The effect of extracts 1 and 2 on each assay was determined using a one-way analysis of variance (ANOVA). Individual differences among groups were analyzed using Dunnett's test with SPSS software. Statistical significance was defined as $p<0.05$. Data were expressed as mean±standard (SEM) for 10 mice in each group.

Results and Discussion

Characterization of triterpenes Compound **1** is a powder that is positive in the Molish and Liebermann-Buchard reaction test, suggesting that it has the basic triterpenoid skeleton. The molecular formula of compound **1**, $C_{51}H_{74}O_{15}$, was determined by HRMS (*m/z* 926.5028). Analysis of IR spectrum of **1** showed the presence of hydroxyl at 3394, cm^{-1}, carbonyl (CO) (1707 cm^{-1}) and double bond (1644 cm^{-1}) groups. DEPT experiment differentiation of 52 resonances into eight methyls, 11 methylenes, 21 methines, 11 quaternary carbons. HMBC experiment showed correlations between δ_H 0.97, 0.95, 1.01, 0.92, 0.99, 0.86 and 0.96 (3H each, s, H3-23, 24, 25, 26, 27, 29, 30), with the carbons at δ_C 27.74, 18.14, 17.65, 17.97, 14.27, 29.23 and 22.89 respectively. A distorted triplet at δ_H 5.21 (t, *J*=1.5 Hz), characteristic of a vinylic proton to H-12, and two methines bearing and oxygen function [4.01 (dd, *J*=11.7, 4.3 Hz, H-3), 3.52 m (m, H-21)] indicated that the aglycone possessed an olean-12-ene skeleton (28). The presence of an *E-p*-methoxycinnamoyl moiety was evident from the resonances at δ_H 7.62 (d, *J*=8.5 Hz), δ_H 7.52 (2H, J 8.5 Hz), 7.50 (d, *J*=15.90 Hz) and 6.26 (d, *J*=15.90 Hz), and 3.79 (s, 3H) and δ_C 126.45, 138.28, 114.32, 157.41 and 55.74 (29). The HMBC spectrum confirmed the substitution pattern from the correlations between H-2''' and C-1''' and C-4'''; H-3''' and C-1''',C-2''',and C-5'''; H-5''' and C-21;and H-6'''and C-4'''. The position of the *p*-methoxycinnamoyl group attached to C-21 is indicated from the correlation between H-21 and C-1'''. In the HMBC spectrum correlated two anomeric carbons at δ_C 111.20 (C-1') and δ_C 112.11 (C-1'') with two glucopyranosyl moieties, α-arabinopyranosyl-1' [δ_H 4.95 (d, $J_{1,2}$=4.6 Hz, H-1')] and β-D-glucopyranosyl-1'' [δ_H 5.16 (d, $J_{1,2}$=4.6 Hz, H-1'')]. Also the long-range correlations between the anomeric proton signals at δ_H 5.02 (H-1') and δ_C 89.41 (C-3) suggested that the arabinose was linked to the aglycone at C-3 and correlations between the anomeric proton signals at δ_H 4.95 (H-1'') and the carbon at δ_C 81.0 (C-2') of arabinopyranoside, indicated that the glucose was attached to position 2 of the arabinose. Glucose was assigned as β configuration based on the larger coupling constant H-3 (dd, *J*=11.7, 4.3 Hz) of 3 α-H at δ_H 4.01 (30). NMR spectrum were consistent with the mass spectral fragmentation pattern with peaks at *m/z* 777 $[M+H\text{-}ara\text{-}H_2O]^+$, 759 $[M+H\text{-}ara\text{-}2H_2O]^+$, 741 $[M+H\text{-}ara\text{-}3H_2O]^+$, 605 $[M\text{-}ara\text{-}glu\text{-}COOH\text{-}H_2O]^+$, 747 $[M+H\text{-}glu\text{-}H_2O]^+$, and 632 $[M\text{-}ara\text{-}glu]^+$ indicating that the arabinose unit was directly attached to the hydroxyl group of C-3 of the aglycone. The simultaneous loss of fragment *m/z* 133 (arabinose) and 162 (glucose) was indicative of two terminal sugars. Other important ions were observed at *m/z* 381 and 191 to the retro-Diels-Alder. The structure of compound **1** was therefore elucidated as 21-(*p*-methoxycinnamoyl)-olean-12-ene-28oic acid-3-*O*-α-L-arabino-pyranosyl-(1→2)-β-D-glucopyranoside

Table 1. Effect of a single oral administration of compounds **1** and **2** on blood glucose levels in normal, severe, and mild diabetic mice

Groups	At the time of grouping	Blood glucose levels (mg/dL) at different time intervals (h)				
		2	4	6	8	12
Non-diabetic control	96.64±5.30	97.37±4.20	95.31±2.80	98.11±3.10	96.18±1.90	97.04±3.70
Non-diabetic+**1**	94.32±1.48	74.28±7.43[a1)]	66.82±5.74[a]	61.31±2.58[a]	57.17±6.44[a]	67.89±3.34[a]
Non-diabetic+**2**	97.36±4.60	81.24±3.29[a]	76.32±3.87[a,]	68.44±5.28[a,]	62.12±6.21[a]	74.19±4.89 [a]
Non-diabetic+GB[2)]	103.56±3.27	82.48±4.75	64.30±2.58[a]	60.27±1.48[a]	63.18±2.39[a]	70.42±2.31
Diabetic control SD	279.51±9.71	283.20±8.65	284.29±1.39	278.42±10.28	280.01±14.39	281.25±7.42
SD+**1**	310.41±15.78	275.21±12.86[b]	219.27±10.38[b]	188.16±14.06[b]	175.23±13.48[b]	249.42±16.32[b]
SD+**2**	329.65±18.62	310.14±9.74	264.29±13.65[b]	214.41±17.58[b]	184.33±15.22[b]	264.43±12.48[b]
SD+GB	335.90±4.29	263.46±2.87[b]	220.70±3.69[b]	201.19±4.04[b]	208.52±2.29[b]	252.67±1.83[b]
Diabetic control MD	247.74±14.68	245.81±12.70	248.19±11.80	249.11±13.59	250.01±10.90	248.21±12.33
MD+1	277.32±19.21	233.78±11.45	211.56±3.42[b]	188.51±19.82[b]	169.51±12.24[b]	179.58±15.71[b]
MD+2	230.29±10.28	219.27±10.88	194.90±18.40[b]	184.55±14.20[b]	173.29±14.52[b]	199.11±12.25[b]
MD+GB	252.11±5.61	197.42±5.96[b]	176.74±4.83[b]	137.26±2.77[b]	148.52±2.63[b]	155.31±2.83[b]

[1)]Each value represents a mean±SD (n=6). [a]p<0.05 compared to normal group mice (ANOVA) followed by Dunnett's test. [b]p<0.01 compared to diabetic group mice (ANOVA) followed by Dunnett´s test.
[2)]GB, Gibenclamide; SD, severe diabetes; MD, mildly diabetes

Table 2. Effects of **1** and **2** on blood glucose level of repeated administration in severe and mild diabetic rats. Effects on food intake body weight, water intake

Group	At the time of grouping	Blood glucose levels (mg/dL)		Physico-metabolic symptoms		
		15 day	30 day	Food intake (g/day)	Weight (gain/loss, 30 days)	Water intake (mL/day)
Control no diabetic	96.4±2.9	97.2±4.2	99.1±5.8	21.6±6.8	22.4±6.1	41.7±5.7
Control Diabetic SD	294.7±7.6	307.8±10.6	345.1±9.2	36.2±5.7	-42.1±4.0	141.6±17.8
Control Diabetic MD	210.4±6.3	266.4±11.0	287.9±8.4	33.8±5.2	-37.5±3.8	134.2±16.6
SD+**1**	337.2±4.7	302.5±7.7[a1)]	175.6±2.6[a]	30.5±3.2[a]	39.3±7.2[b]	123.1±10.4[a]
SD+**2**	350.4±2.3	263.1±7.4[a]	210.6±1.9[a]	31.4±7.0[a]	37.8±5.4[b]	130.7±11.1[a]
MD+**1**	240.0±2.5	180.4±6.9[b]	120.2±3.6[b]	27.3±3.7[a]	41.6±4.7[b]	110.3±12.8[b]
MD+**2**	223.5±5.9	196.7±7.2[b]	130.4±4.3[b]	28.6±6.2[a]	42.5±8.0[b]	105.7±13.5[b]
Diabetic SD+GB	280.2±9.7	239.6±7.2[b]	219.6±7.2[b]	28.1±3.4[a]	45.4±5.3[b]	117.6±4.2[b]
Diabetic MD+GB	215.4±5.5	155.0±2.9[b]	145.0±2.9[b]	27.5±4.1[a]	43.9±2.1[b]	100.4±7.8[b]

[1)]Values are expressed as a mean±SD (n=6), [a]Significantly (p<0.01) different from normal mice. [b]Significantly (p<0.05) different from diabetic mice, where the significance was performed by oneway ANOVA followed by post hoc Dunnett´s test.

(Fig. 1).

Based on similar analysis, The NMR data for compound **2** were almost superimposable with **1** except for a lack of *p*-methoxy-cinnamoyl functionality. The structure for compound **2** is identified as olean-12-ene- 28 methyl ester-3-*O*-α-L-arabinofuranosyl-(1→2)-β-D-glucopyranoside (Fig. 1).

Compound **3** was obtained as an amorphous solid that showed absorption bands of hydroxyl and carboxyl groups in the IR spectrum. Again, the molecular formula $C_{35}H_{54}O_5$ of **3** was deduced from the HRESIMS (observed *m/z* 554.3961). DEPT spectral data indicated 35 carbons attributable to nine methyls, nine methylene, seven methine, and ten quaternary carbon. A hydroxymethine signal (H-3) at δ_H 3.95 (dd, *J*=11.8, 4.5 Hz), which was characteristic of a triterpene with 3β-hydroxy-olean-12-en-28-oic acid (31). It also contained an angeloyl group as indicated by its characteristic NMR signals at δ_H 5.98 (1H, dq, *J*=7.3, 1.6 Hz, H-3'), 2.16 (3H, dd, *J*=7,2, 1.5 Hz, H-4') and 2.04 (3H, s, H-5') (32). Finally, the position of the angeloyl unit in compound **3** was characterized by HMBC experiments, in which long-range correlations were observed between H-21 and C-1'. Consequently, the structure of **3** was elucidated as 3β-hydroxy-21-O-angeloyl-olean-12-en-28-oic acid.

Compound **4** had the molecular formula $C_{40}H_{60}O_7$ as determined by HRESIMS (*m/z* 652.4345), which was supported by the DEPT and ^{13}C NMR spectra. The ^{13}C NMR spectrum contained 40 signals, of which 30 were assigned to a triterpenoid moiety and 10 to the acyl groups. The ^{13}C NMR spectrum showed carbonyl groups at δ_c 178.25, 168.72 and 167.43. Detailed NMR analysis suggested that both compounds **3** and **4** had the same acyl group (angeloyl) at C-21. The two characteristic proton signals at δ_H 6.37, 1.63 and 1.96 demonstrated that the other acyl group in compound **4** was tigloyl. HMBC correlation of H-21 (δ_H 6.25, 1H, d, *J* = 9.2) with C-1' (δ_c 168.72) of the angeloyl group and H-22 (δ_H 6.08, 1H, d, *J*=9.2) with C-I'' (δ_c 167.43) of the tigloyl group provided definitive evidence that the angeloyl and tigloyl groups were linked to C-21 and C-22 respectively. Consequently, the structure of **4** was elucidated as 3β-hydroxy-21-angeloyl-22- tigloyl-olean-12-en-28-oic acid.

Table 3. Effects of compounds **1** and **2** on glucose loading (OGTT) in SD and MD diabetic mice

Groups	Blood glucose levels (mg/dL)				
	0 min	30 min	60 min	90 min	120 min
Control no diabetic	91.46±1.82	179.65±3.17	160.43±2.83	127.49±2.49	95.90±1.53
Control Diabetic SD	341.25±3.47	422.19±4.76[a1)]	441.49±6.12[a]	449.32±5.10[a]	379.87±2.83[a]
Control Diabetic MD	268.78±5.18	344.16±1.62[a]	369.23±5.21[a]	373.41±5.16[a]	293.53±3.70[a]
SD+**1**	342.21±3.96	390.31±6.90[a]	321.29±7.12[c]	264.18±4.38[c]	180.08±5.73[c]
SD+**2**	313.56±6.39	373.23±5.72[a]	324.62±5.94[c]	270.42±4.86[c]	198.12±6.43[c]
MD+**1**	256.16±3.13[a]	316.86±7.28[a]	232.28±6.27[c]	208.36±7.23[c]	169.10±4.68[c]
MD+**2**	243.62±8.32[a]	325.29±9.41[a]	242.82±5.39[c]	219.90±8.11[c]	177.31±5.37[c]
Diabetic SD+GB	315.28±6.48	364.21±4.37[a]	309.37±4.69[c]	274.20±6.51[c]	169.48±3.12[c]
Diabetic MD+GB	265.30±8.00	315.25±2.25[a]	295.05±4.40[c]	263.11±4.78[c]	167.36±1.83[c]

[1)]Values are expressed as a mean±SD (n=6), Significantly (p<0.05) different from normal mice. [b]Significantly (p<0.05) different from diabetic mice. (p<0.05) different from diabetic mice. [c]Significantly (p<0.05) different from normal and diabetic mice, based on a-way ANOVA followed by a post hoc Dunnett's test.

Effect of compounds 1 and 2 on fasting blood glucose levels in normoglycemic, STZ induced severe-diabetic, and STZ-nicotinamide induced mildy-diabetic mice for 2-12 h Oral administration of compounds **1** and **2** at dosage of 10 mg/kg produced a significant hypoglycaemic effect in normal fasted mice after 4 h. The most pronounced effect of compound 1 was observed after 6 h (Table 1). A dosage of 10 mg/kg reduced the blood glucose level of normal fasted mice from an initial mean value of 94.32±1.48 at the initial time (0 h) to a mean value of 61.31±2.58 with a 35% reduction after 6 h. However, in normal mice administered compound 2, a reduction in the blood glucose level of 30% was observed after 6 h at dosages of 10 mg/kg. Oral treatment with glibenclamide (5 mg/kg) caused a slight reduction in blood glucose levels. Reduction in the blood glucose level caused by compounds **1** and **2** at all doses were larger than for standard drug glibenclamide (Table 1). The plasma glucose level in STZ-induced diabetic mice (MD and SD) treated with 10 mg/kg of compounds **1** and **2** for 2-12 h is shown in Table 1. Serum glucose levels of diabetic control mice were markedly higher than levels of normal control mice, and the blood glucose concentrations in compounds **1** and **2** treated diabetic mice were significantly (p<0.05) decreased after the treatment period, compared with diabetic control mice. The effect on blood glucose levels of the compounds **3** and **4** was not significantly therefore not presented in this study.

Effect of triterpenes 1 and 2 on fasting blood glucose levels in STZ induced severe-diabetic mice and STZ-nicotinamide induced midly diabetic mice after 4 weeks The antihyperglycemic effects of compounds **1** and **2** on fasting blood glucose levels in STZ induced type 1 and STZ-nicotinamide-induced type 2 diabetic mice are shown in Table 2. Compounds **1** and **2** administered at the same concentrations (10 mg/kg) produced significant (p<0.05) antihyperglycaemic effects in streptozotocin-induced severe and mildly diabetic mice after 2 weeks continuing for up to 4 weeks, compared with controls (Table 2). Treatment of diabetic mice with glibenclamide (5 mg/kg) produced a significant (p<0.05) fall in blood glucose levels after 4 weeks (68%), compared with controls. Maximum decreases were observed a dosage of 10 mg/kg after administration of compounds **1** and **2** (48 and 40% respectively) to SD mice. Values for MD mice were 50 and 42% respectively. Compounds **1** and **2** after administration at a dosage of 20 mg/kg gradually decreased blood glucose levels for 4 weeks in both groups MD and SD (61 and 53% respectively). In MD mice treated with 1 and 2 after 4 weeks, normal blood glucose levels were restored.

Effect of triterpenes on body weight, and food and water intake Changes in body weights of normal and experimental diabetic mice are shown in Table 2. Body weight was significantly (p<0.05) lower in diabetic mice, compared with controls (33). Oral administration of compounds **1** and **2** significantly (p<0.05) increased body weight and reduced food and water intake of diabetic mice, compared with untreated diabetic mice. Differences in body weights between compounds **1** and **2** treated mice (10 mg/kg of body weight) and STZ treated control mice, were significantly (p<0.05)different, indicating that compounds **1** and **2** maintained the normal body weight, compared with STZ control mice (Table 2). Compounds **1** and **2** prevented increases in food and drink intake induced as a consequence of diabetes. The blood glucose level was reduced within 1 week, indicating that compounds **1** and **2** possessed hypoglycaemic activities. This may bedue to an increase muscle wasting. Also weight loss in diabetic mice indicate a break down of tissue proteins produced to unavailability of carbohydrates as an energy source (34). Diabetic mice treated with triterpenes **1** and **2** or glibenclamide showed and increase in body weight which may be due to better glycemic control produced by the improved of metabolism in muscle cells.

Effects of compounds 1 and 2 on oral glucose tolerance in mice The effects of compounds **1** and **2** on glucose tolerance was determined after 3 days of treatment. Postprandial blood glucose levels in mice showed a significant (p<0.05) change after glucose loading, compared with controls, increasing rapidly in all diabetic mice within the first 30 min and remaining high over the next 120

Table 4. Effect of compounds **1** and **2** on serum levels of HDL-cholesterol, LDL-cholesterol, VLDL- cholesterol, total cholesterol, triglycerides, and the TBARS level in the liver and kidney of STZ- induced diabetic mice

Groups	Mean Concentration (mg/g)±SEM							
	HDL-c (mg/dL)	LDL-c (mg/dL)	VLDL-c (mg/dL)	T cholesterol (mg/dL)	Triglycerides (mg/dL)	TBARS (mM/mg protein)		
						Pancreas	Liver	Kidney
Normal control	39.2±1.73[b1)]	46.8±15.19[b]	18.1±5.19[b]	95.6±1.56[b]	94.2±2.93[b]	2.28±0.10[b]	0.98±0.007	1.9±0.03
SD control	19.6±2.65[a]	156.5±13.65[a]	35.3±4.78[a]	249.3±3.54[a]	181.0±6.31[a]	3.66±0.47[a]	1.60±0.08[a]	2.8±0.01[a]
MD control	20.2±3.56[a]	148.4±16.83[a]	35.3±4.78[a]	191.56±5.76[a]	138.2±5.12[a]	3.66±0.38[a]	1.59±0.07[a]	2.6±0.09[a]
SD+**1**	23.5±3.32[a,b]	139.8±10.54[c]	21.3±2.98[a,c]	144.6±6.77[a,b]	125.3±1.78[a,d]	2.32±0.25[a,b]	1.09±0.08[b]	2.0±0.03[b]
SD+**2**	22.7±4. 56[a,b]	131.1±12.94[c]	19.3±5.72[a,c]	149.2±5.79[a,b]	131.4±2.19[a,d]	2.29±0.63[b]	1.10±0.06[b]	2.1±0.07[b]
MD+**1**	25.5±3.32[a,b]	130.6±16.37[a,b]	23.7±4.87[a,c]	140.1±6.90[a,b]	98.2±4.18[a,b]	2.39± 0.21[a,b]	0.99±0.05[b]	1.8±0.01[b]
MD+**2**	24.7±4.42[a,b]	133.5±1 3.39[a,b]	22.1±3.80[a,c]	142.6±3.84[a,b]	108.6±6.79[a,b]	2.35±0.63[b]	1.00±0.04[b]	1.9±0.04[b]
SD+GB	25.7±2.17[a,b]	98.4±17.58[a,b]	22.7±2.42[a,c]	141.2±4.31[a,b]	91.8±5.97[a,d]	2.34±0.55[a,b]	1.04±0.02[b]	1.9±0.03[b]
MD+GB	26.3±2.48[a,b]	99.3±7.10[a,b]	24.8±3.92[a,c]	148.1±2.75[a,b]	96.0±2.54[a,b]	2.42± 0.76[a,b]	0.94±0.01[b]	1.8±0.09[b]

[1)]Values are expressed as a mean±SEM, n=10. [a]p<0.05 compared with normal control group mice, [b]p<0.01 compared with diabetic control group mice, based on a one-way ANOVA followed by a post hoc Dunnett´s test. Plasma insulin values at 0 h before drug administration were significantly different compared to the value at days 30 after drug treatment. Significant difference between diabetic control mice and normal control mice [a]p<0.001. Significant difference between treated group mice and diabetic control mice [b]p<0.01, [c]p<0.05, [d]p<0.01 compared with glibenclamide (5 mg/kg) treated group mice.

Table 5. Antioxidative status of mice and biochemical parameters at the end of the experimental period

Organ	Groups	SOD (U/1/min)	CAT (U/1/s)	CSH-Px (U/1/mL)	GSH (U/1/mL)	MDA (nmol/1/mL)
Kidney	N	3.2±0.32[a1)]	0.76±0.004[a]	68.56±1.39[a]	25.16±3.62[a]	29.3±3.48[a]
	SD	2.0±0.44[b]	0.60±0.003[a]	278.52±4.64[b]	5.28±0.99[b]	22.7±4.12[c]
	MD	2.1± 0.22[b]	0.63±0.009[a]	259.70±2.10[b]	7.22±1.74[b]	25.1±5.20[b]
	SD+**1**	3.3±0.67[c]	0.65±0.005[a]	76.32±5.80[a]	18.23±2.43[c]	26.9±6.20[a]
	SD+**2**	3.0±0.28[c]	0.67±0.002[a]	80.02±5.13[a]	17.55±4.32[c]	27.1±4.39[a]
	MD+**1**	3.5±0.12[b]	0.77±0.006[a]	66.49±3.81[a]	20.38±5.32[c]	28.4±3.47[a]
	MD+**2**	3.3±0.20[b]	0.82±0.004[a]	67.59±6.39[a]	19.21±3.19[c]	27.6±5.88[b]
Liver	N	6.4±0.23[a]	0.82±0.011[a]	99.42±7.35[a]	48.76±5.91[a]	27.1±4.28[a]
	SD	5.1±0.17[a]	0.58±0.005[b]	39.18±5.22[c]	25.28±4.47[b]	23.2±3.19[b]
	ND	5.3±0.61[a]	0.61±0.003[b]	45.88±3.89[c]	28.01±7.25[c]	25.0±2.74[a]
	SD+**1**	5.8±0.72[a]	0.69±0.001[a]	83.21±5.58[b]	38.47±4.26[d]	24.1±4.61[a]
	SD+**2**	5.7±0.42[a]	0.65±0.006[a]	79.86±4.96[b]	36.38±2.80[d]	24.9±5.65[a]
	MD+**1**	6.0±0.18[a]	0.75±0.004[a]	87.79±6.36[b]	41.18±6.29[d]	26.7±1.48[a]
	MD+**2**	5.9±0.63[a]	0.72±0.003[a]	84.18±4.13[d]	40.32±7.29[e]	26.1±4.32[a]
Pancreas	N	3.9±0.45[a]	0.71±0.004[a]	359.71±4.06[a]	51.25±4.37[a]	28.4±5.61[a]
	SD	2.7±0.26[b]	0.64±0.002[b]	319.27±8.44[b]	15.48±1.70[b]	23.5±3.87[b]
	ND	2.9±0.51[b]	0.67±0.007[b]	325.55±7.15[b]	19.24±3.17[c]	24.2±2.67[a,c]
	SD+**1**	3.1±0.34[a]	0.68±0.005[a]	341.32±6.90[c]	43.29±7.11[d]	26.4±4.47[a]
	SD+**2**	3.0±0.19[a]	0.66±0.009[a]	339.87±5.38[c]	40.52±6.21[d]	25.2±4.52[a]
	MD+**1**	3.3±0.22[a]	0.70±0.008[a]	344.18±8.29[c]	46.23 ±6.37[d]	27.1±5.39[a]
	MD+**2**	3.2±0.41[a]	0.69±0.002[a]	342.39±5.31[d]	45.12±5.81[d]	25.5±6.90[a]

[1)]Each value represents a mean±SEM, n=6 mice. Values within columns bearing the same lower-case letters (a, b, c) were not significantly different at p<0.05, and are not in any particular order. Normal control (N), diabetic control (SD), diabetic control (MD)

min in diabetic control mice. Changes in the levels of blood glucose in normal, diabetic control, and experimental group mice after oral administration of glucose (2 g/kg) are shown in Table 3. Oral treatment (10 mg/kg) in diabetic mice with the 2 compounds produced a significant (p<0.05) reduction of blood glucose levels after 120 min, compared with STZ-diabetic mice, indicating a decrease of blood glucose levels. OGTT was performed to assess animal sensitivity to a high glucose load (2 g/kg). These compounds improved glucose tolerance, indicating a decrease in insulin resistance and maintenance of steady blood glucose levels, probably indicating induction of peripheral glucose use.

Effect of triterpene compounds 1 and 2 on serum lipid profiles

The effects of compounds **1** and **2** on serum lipid profiles is shown in Table 4. There was a significant (p<0.05) increase in serum triglyceride levels, along with an increase in total cholesterol and TBARS levels in control diabetic mice that received only distilled water. Daily administration of compounds **1** and **2** at a dosage of 10 mg/kg to

Table 6. Effect of compounds **1** and **2** on pancreatic and serum insulin levels. Inhibitory activities of compounds **1** and **2** against α-glucosidase and α-amylase

Groups (mg/kg) (μg/mL)	Serum insulin (mIU/mL)	Pancreatic insulin (mIU/g protein)	Compound	Concentration	% Inhibition	
					α-amylase	α-lucosidase
Normal control	15.65±1.95[a1)]	27.31±2.45[a]	**1**	50	23.29±4.62	13.67±3.62
SD control	11.32±1.81[b]	16.14±3.19[b]		100	31.14±6.76	22.29±6.28
MD control	12.62±4.29[b]	18.10±5.23[b]		150	49.53±7.24	41.42±4.27
SD+**1**	14.45±1.76[c]	24.19±2.78[c]		200	62.25±3.78	58.17±5.23
SD+**2**	13.41±1.48[c]	21.30±2.35[c]		250	76.61±5.64	65.08±6.13
MD+**1**	14.33±2.65[c]	25.51±6.80[c]	**2**	50	19.41±1.33	10.43±2.38
MD+**2**	13.67±4.70[c]	24.13±4.86[c]		100	25.37±4.33	18.43±5.19
SD+GB	13.90±2.54[c]	26.26±2.73[b]		150	37.69±6.72	31.60±4.20
MD+GB	14.52±5.14[c]	26.87±4.13[b]		200	55.21±7.08	47.17±2.59
				250	68.36±5.18	53.69±3.28

[1)]Data are expressed as a mean±SEM: *n*=10. *S*uperscripts (a, b, c, d) (p<0.05)

diabetic mice for 30 days significantly (p<0.05) reduced the serum total cholesterol level, compared with controls. In contrast, the lipoprotein HDL level was significantly (p<0.05) lower in diabetic group mice, compared to control mice (Table 4). After 30 days of continuous administration of compounds **1** and **2**, there was a considerable (p<0.05) increase in the HDL levels similar to normal control group mice, indicated that compounds **1** and **2** controlled development of diabetes via improvements in the lipid metabolism. Lipid peroxidation (LPO) was measured based on the thiobarbituric acid (TBA) reaction with malondialdehyde (MDA), a product formed due to peroxidation of membrane lipids. TBARS levels in the pancreas, liver, and kidneys of diabetic mice also significantly (p<0.05) decreased after treatment with compounds **1** and **2**, compared with controls (Table 4).

Effect of triterpenes on SOD, CAT, GSH, and GPx enzyme levels in the pancreas One of the main consequences of chronic hyperglycaemia is enhanced oxidative stress resulting from an imbalance between production and neutralization of reactive oxygen species (ROS) (35). Antioxidant effects of triterpenes 1 and 2 on tissue oxidative markers were studied (Table 5). Diabetic mice showed significant (p<0.05) reductions in superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GSH) levels in the pancreas, hepatic, and renal tissues, compared with controls. Levels of these enzymes were restored to near normal values after treatment with compounds **1** and **2**, indicating that compounds **1** and **2** prevented oxidative stress, acted as suppressors of liver cell damage, and inhibited progression of the liver dysfunction induced by chronic hyperglycemia. Chronic hyperglycemia and dyslipidemia are associated with avariety of metabolic disorders in diabetic humans and animals causing oxidative stress and depletion of the activity of the antioxidative defense system, resulting in elevated levels of ROS (36).

Drugs with antioxidant properties can support the endogenous defense systems and reduce both ROS initiation and propagation. Results of this study clearly showed lowering of antioxidant enzymes and increase of LPO in diabetic groups. There are sign of oxidative stress due deleterious imbalance between production and removal of free radicals. The LPO level was decreased in compounds **1** and **2** treated diabetic mice possibly by suppression of free radical formation and increasing antioxidant enzyme levels (CAT, SOD, GSH, and GPx) in the liver, kidney, and pancreas. In addition, administration of compounds **1** and **2** to diabetic STZ-treated mice caused a significant (p<0.05) increase in the MDA concentration a secondary product of LPO, compared with controls. The LPO induced by STZ was associated with decreased enzymatic activities. Oxidative stress affects cellular integrity only when antioxidant mechanisms are no longer able to cope with detoxification of generated free radicals. Thus supplementation withan antioxidant can support cellular processes.

Effect of compounds 1 and 2 on serum insulin and pancreatic insulin levels The serum insulin level was significantly (p<0.05) decreased in streptozotozin induced diabetic mice to levels as low as for STZ untreated mice. After 3 weeks of administration of compounds **1** and **2**, there was a significant (p<0.05) elevation of the serum insulin level, compared with controls. The effect of compounds **1** and **2** on insulin levels is shown in Table 6. Repeated administration of compounds **1** and **2** resulted in hypoglycemia with reduced plasma insulin levels. Thus, compounds **1** and **2** improved hyperinsulinemia in type 2 diabetic (MD) mice as insulin resistance in peripheral tissues is known to be one of the major pathogenic factors in type 2 diabetes.

Effect of compounds 1 and 2 against α-glucosidase and α-amylase The inhibitory activities of triterpenoid saponins were established against α-amylase and α-glucosidase in this study. The percentage inhibition displayed by each compound is shown in Table 6. Compounds **1** and **2** showed α-amylase inhibitory activities of 76.61 and 68.36%, respectively, at a concentration 250 g/mL. At a concentration 1000 μg/mL, compounds **1** and **2** showed percentage inhibition values of 65.08 and 53.69% for α-glucosidase, respectively. Saponins play a role in the carbohydrate metabolism due to reduction in the

Table 7. Antioxidant activities of triterpene compounds **1** and **2**

	1	2	Trolox	BHA	Several standard
•OH scavenging	89.67±6.39[e1)]	99.45±9.19[a]	36.21±1.73[b]	28.26±2.65[f]	-
$•O_2^-$ scavenging	815±72.33[a]	844.31±30.22[d]	2231±58.87[b]	2276±62.34[b]	-
Fe^{2+} chelating	534.19±10.2[d]	629.04±15.62[a]	28.63±4.72[b]	ND	-
DPPH• scavenging	42.50±8.05[e]	71.23±13.64[c]	4.65±0.69[b]	12.49±1.72[a]	-
$ABTS^{+•}$ scavenging	38.21±9.72[d]	42.81±6.58[c]	9.73±2.19[a]	7.85±1.29[b]	-
ORAC (1 ORAC unit/1 μmol trolox)	15.34±5.29[b]	13.21±2.43[a]	-	-	Quercetin 8.56±2.43[d]
NO• (%)	62.62±8.34[a]	70.04±7.23[c]	-	-	Curcumin 59.25±2.90[a]
ONOO• (%)	71.12±5.29[a]	75.08±9.83[c]	-	-	Quercetin 72.46±5.19[c]
H_2O_2 (%)	65.56±8.18[a]	59.82±7.41[c]	74.41±4.72[b]	77.69±8.41[c]	α-Tocopherol 67.82±5.34[a]
HOCl (%)	60.21±9.36[c]	66.17±5.92[b]	-	-	Quercetin 27.63±2.57[a]
SOD scavenging (%)	43.41±10.22[c]	40.26±8.90[b]	44.73±4.65[b]	49.43±3.18[c]	α-Tocopherol 44.63±4.60[b]
Lipid Peroxidation (%)	59.87±11.53[a]	55.64±7.45[c]	86.50±9.78[b]	80.74±6.52[b]	α-Tocopherol 69.36±5.14[c]

[1)] IC_{50} value (μg/mL) and expressed as a mean±SD (n=4). Means values with different superscripts in the same row are significantly different (p<0.05), the same superscripts indicate no significant different (p<0.05). ND, not detected

extent of carbohydrate absorption by inhibition of the enzymatic actions of α-amylase and α-glucosidase. α-Amylase is the enzyme responsible for hydrolysis of complex starch to oligosaccharides, whereas α-glucosidase hydrolyses oligosaccharides, trisaccharides, and disaccharides into glucose, and other monosaccharides. Inhibition of these enzymes produces a postprandial antihyperglycemic effect by a reduction in the rate and extent of glucose absorption in the small intestine with a modest reduction of hemoglobin glycosylation in diabetes (37). The potency of postprandial antihyperglycemic and glucose tolerant effects of compounds **1** and **2** may be derived from synergism due to concurrent inhibition of α-amylase and α-glucosidase. Inhibition of postprandial carbohydrate metabolism has a short term blood glucose level lowering effect and a long-term effect indicated by small reductions in glycated hemoglobin levels.

Effect of compounds 1 and 2 on ROS scavenging The total phenolic compounds of ethanol:water extracts of Mexican *Piper auritum* were previously determined to be in the range of 6.79-68.03 mg of galic acid (GA)/g dry solids. In addition, antioxidant activities also were evaluated using the phenol Folin Ciocalteu reagent and the ABTS methods (23). The ROS scavenging activities of compounds **1** and **2**, Trolox, BHA, quercetin, and curcumin as a reference antioxidant are shown in Table 7. Compound 1 showed the highest radical scavenging activities against HOCl, $ONOO^-$, O_2^-, and DPPH. In contrast to activity differences between compounds **1** and **2** shown in other assays, the O_2^- scavenging activities were similar, indicating that antioxidants were more reactive toward O_2^- than toward the other ROS varieties. HOCl can inactivate the antioxidant enzyme catalase via break down of the heme-prosthetic group and, thus, is a more efficient scavenger than standard quercetin. Addition of compounds **1** and **2** removes hydroxyl radicals and prevents damage. The observed IC_{50} values (is the drug concentration causing 50% inhibition of the desired activity) indicated that compounds **1** and **2** were weaker hydroxyl radical scavengers than the standards Trolox and BHA. The hydroxyl radical is an ROS variety formed in biological systems that causes DNA strand breakage, leading to carcinogenesis, mutagenesis, and cytotoxicity (38). Compounds **1** and **2** were fast and effective scavengers of the ABTS radical and this activity was lower than for Trolox and BHA. The ORAC assay is commonly used for evaluation of the total antioxidant activities of plant foods. Oxygen radical absorbing capacity (ORAC) was reduced significantly after **1** and **2** addition (Table 7). Antioxidants may respond in a different manner to different radical involving multiple mechanisms. No single assay will accurately reflect the antioxidant effectof an extract or a compound, so that in this study compounds **1** and **2** was tested in different ROS including singlet oxygen, superoxide, hydroxyl, nitric oxide, peroxynitrite, hydrogen peroxide, and hypochlorous acid among others. The ABTS method is based on radical-scavenging capacity to react with $ABTS^+$ generated in the system. The assay is widely used to determine antioxidant activity in biological systems and foods and a high level of antioxidant activity is indicated to high TEAC value. The TEAC method is the Trolox equivalent antioxidant capacity. The ORAC assay determine the inhibition of the peroxyl-radical generated in the system. Data suggested that the consumption of leaves of *P. auritum* assists the reduction of diabetic-complications.

Nitric oxide (NO) is a free radical that plays an important role in the pathogenesis of pain and inflammation. The NO scavenging activities of compounds **1** and **2** were higher than the activity of curcumin, perhaps due to antioxidants that compete with oxygen to react with nitric oxide, thereby inhibiting generation of peroxynitrite (ONOO). The reaction of NO with the superoxide radical generates the highly reactive peroxynitrite anion (ONOO-) that highly toxic for living cells (39). The production of ROS, such as superoxide an ion radicals, is catalyzed by free iron through Haber-Weiss reactions (40). A reduced form of $O_2^·$ (superoxide radical) has been implicated in initiating oxidation reactions associated with aging. A decrease in absorbance values at 560 nm due to antioxidants thus indicates consumption of the superoxide anion in a reaction mixture peroxide radical scavenging activities at 100 μg/mL for compounds **1** and **2** in comparison with the same dosage of α-tocopherol are shown in Table 7. Compounds

1 and **2** exerted a significantly (p<0.05) weaker superoxide radical scavenging activity than α-tocopherol at 100 μg/mL. The superoxide anion is also implicated as a harmful ROS with a detrimental effect on cellular components in a biological system. The superoxide radical indirectly initiates lipid oxidation by generation of singlet oxygen. The 2 oxidation states of iron, Fe^{2+} and Fe^{3+}, donate or accept electrons through redox reactions that are important for biological reactions, but which may also be harmful to cells. Excess iron is involved in $O_2^{\cdot}$ and hydrogen peroxide conversion into an extremely reactive hydroxyl radical (Haber-Weiss reaction) that causes severe injury to membranes, proteins, and DNA and decomposes lipid hydroperoxides into peroxyl and alkoxyl radicals that are responsible for the chain reaction of lipid peroxidation Iron chelating properties for compounds **1** and **2** are shown in Table 7. In conclusion, the triterpene saponin compounds **1** and **2** from methanol extracts of the leaves of *P. auritum* exhibited antioxidant and antihyperglycemic activities, enhanced glucose uptake in adipose and muscle tissues, and exhibited a beneficial lipid regulation ability. The mechanism of the antidiabetic activity may involve an antioxidant effect, improvement of insulin resistance, and a protective effect for pancreatic β-cells to secret insulin. The precise mechanism is worthy of further study.

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References

1. Fufeng C, Xiong H, Wang J, Ding X, Shu G, Mei Z. Antidiabetic effect of total flavonoids from Sanguis draxonis in type 2 diabetic mice. J. Ethnopharmacol. 149: 729-736 (2013)
2. Genet S, Kale RK. Baquer NZ. Alterations in antioxidant enzymes and oxidative damage in experimental diabetic rat tissues: Effect of vanadate and fenugreek (*Trigonellafoenum graecum*). Mol. Cell Biochem. 236: 7-12 (2002)
3. McKenney JM. Pharmacotherapy of dyslipidemia. Cardiovasc. Drug. Ther. 15: 413-422 (2001)
4. Malviya N, Jain S. Malvia S. Antidiabetic potential of medicinal plants. Acta Pol. Pharm. 6: 7113-7118 (2010)
5. Garcia A, Leyva M, Martinez J, Stashenko E. Determination of the composition chemical and antioxidant activity *in vitro* of essential oil of *Piperauritum Kunth* (*Piperaceae*). Sci. Tech. 33: 439-442 (2007)
6. Perez GRM, Cotera LFC, Neira GAN. Evaluation of the antioxidant and antiglycation effects of the hexane extract from *Piper auritum* leaves *in vitro* and beneficial activity on oxidative stress and advanced glycation end-productmediated renal injury in streptozotocin-treated diabetic mice. Molecules 17: 11897-11919 (2012)
7. Perez GRM. Effect of the hexane extract of *Piper auritum* on insulin release from β-cell and oxidative stress in streptozotocin-induced diabetic rat. Pharmacogn. Mag. 8: 308-313 (2012)
8. Nolis P, Parella T. Spin-edited 2D HSQC-TOCSY experiments for the carbohydrates and peptides. J. Magn. Reson. 176: 15-26 (2005)
9. Guide Laboratory Animals for the Care and Use of Laboratory Animals. 8th ed. National Academy of Sciences, Washington DC, USA (2011)
10. Manual Organization of Ethics Committee in Research of the National School of Biological Sciences (CEI-ENCB) del IPN. (1999)
11. Zhou J, Zhou S, Zeng S, Zhou J, Jiang M, He Y. Hypoglycemic and hypolipidemic effects of ethanolic extract of Mirabilis jalapa L. root on normal and diabetic mice. Evid-Based Compl. Alt. Article ID 257374 (2012)
12. Trinder P. Determination of blood glucose using an oxidase-peroxidase system with a non- carcinogenic chromogen. J. Clin. Pathol. 22: 158-161 (1969)
13. Hashimoto S. A new spectrophotometric assay method of xanthine oxidase in crude tissue homogenate. Anal. Biochem. 62: 426-435 (1974)
14. Erel O. A new automated colorimetric method for measuring total oxidant status. Clin. Biochem. 38: 1103-1111 (2005)
15. Bradford MA. A rapid and sensitive method for the quantitation of microgram quantities of rotein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254 (1976)
16. Fraga CG, Leibovitz BE, Tappel AL. Lipid peroxidation measured as thiobarbituric acid-reactive substances in tissue slices: Characterization and comparison with homogenates and microsomes. Free Radical Bio. Med. 4: 155-161 (1998)
17. Park SE, Cho MH, Lim JK, Kim JS, Kim JH, Kwon DY, Park CS. New colorimetric method for determining the isomerization activity of sucrose isomerase. Biosci. Biotech. Bioch. 71: 583-586 (2007)
18. Tsujii E, Muroi M, Shiragami N. Nectrisine is a potent inhibitor of α-glucosidases, demonstrating activities similarly at enzyme and cellular levels. Biochem. Bioph. Res. Co. 220: 459-466 (1996)
19. Conforti F, Statti G, Loizzo MR, Saccherti G, Poli F. *In vitro* antioxidant effect and inhibition α-amylase of two varieties of Amaranthus caudatus seeds. Biol. Pharm. Bull. 28: 1098-1102 (2005)
20. Yu L, Haley S, Perret M, Harris J, Wilson J, Qian M. Free radical scavenging properties of wheat extracts. J. Agr. Food Chem. 50: 1619-1624 (2002)
21. Balavoine GG, Geletii YV. Peroxynitrite scavenging by different antioxidants. Part I: Convenientassay. Nitric Oxide-Biol Ch. 3: 40-54 (1999)
22. Karadag A, Ozcelik B, Saner S. Review of methods antioxidant capacities. Food Anal. Method. 2: 41-60 (2009)
23. Re R., Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improvement ABTS radical cation decolourization assay. Free Radical Bio. Med. 26: 1232-1237 (1999)
24. Cao G, Alessio HM, Cutler RG. Oxygen-radical absorbance capacity assay for antioxidant. Free Radical Bio. Med. 14: 303-311 (1993)
25. Decker EA, Welch B. Role of ferritin as lipid oxidation catalyst in muscle food. J. Agr. Food Chem. 38: 674-677 (1990)
26. Sreejayan N, Rao MNA. Free radical scavenging activity of curcuminoids. Drug Res. 46: 169-173 (1996)
27. Fenton R, Chemizmu DK. Fenton reaction-controversy concerning the chemistry. Ecol. Chem. Eng. S 16: 347-358 (2009)
28. Ikuta A, Itokawa H. Triterpenoids *Akebia quinata* callus tisuue. Phytochemistry 25: 1625-1628 (1986)
29. Yoshikawa M, Murakami T, Yoshizumi S, Murakami N, Yamahara J Matsida H. Bioactive saponins and glycosides. V. acylated polyhydroxyolean-12-ene triterpene oligoglycosides, camellia saponins A1, A2, B1, B2, C1, and C2, from the seeds of *Camellia japonica* L.: Structures and inhibitory activity on alcohol absorption. Chem. Pharm. Bull. 44:1899-1890 (1996)
30. Takedaa Y, Okada Y, Masuda TC. O-bisglycosylapigenins from the leaves of *Rhumnella maequilotera.* Phytochemistry 65: 463-468 (2004)
31. Qiao A, Wang Y, Xiang L, Zhang Z, HeX. Triterpenoids of sour jujube show pronounced inhibitory effect on human tumor cells and antioxidant activity. Fitoterapia 98: 137-142(2014)
32. Zhang Z, Koike Z, Zhonghua J, Nikaido T, Guo D, Zheng J. New saponins from the seeds of *Aesculus chinensis*. Chem. Pharm. Bull. 47: 1515-1520 (1999)
33. Sharma M, Siddique MW, Shamirn AM, Gyanesh S, Pillai KK. Evaluation of antidiabetic and antioxidant effects of seabuckthom (*Hippophae rhamnoides* L.) in streptozotocin-nicotinamide induced diabetic rats. Open Conf. Proc. J. 2: 53-58 (2011)
34. Pepato MT, Migliorini RH, Goldberg A, Kettelhut IC. Role of different proteolytic pathways in degradation of muscle protein from streptozotocin-diabetic rats. Am. J. Physiol. 1271: E340-E347 (1996)
35. Ravi K, Ramachandran B, Subrarnanian S. Effect of *Eugenia jambolana* seed kernel on antioxidant defense system in streptozotocin-induced diabetes mice. Life Sci. 75: 2717-2731 (2004)
36. Maghrani, M, Zeggwagh NA, Lemhadri A. Study of the hyperglycemic activity of *Fraxinus excelsior* and *Silybum marianum* in an animal model of type 2 diabetes mellitus. J. Ethnopharmacol. 91: 309-316 (2004)
37. Kim Y, Jeong Y, Wang M, Lee W, Rhee H. Inhibitory effect of pine extract on α-glucosidase activity and postprandial hyperglycemia. Nutrition 21: 756-761 (2005)
38. Bolzan AD, Bianchi MS. Genotoxicity of streptozotocin. Mutat. Res. 512: 121-134 (2003)
39. Yamaji Y, Nakazato Y, Oshirna N, Hayashi M, Saruta T. Oxidative stress induced by iron released from transferrin in low pH peritoneal dialysis solution. Nephrol. Dial. Transpl. 19: 2592-2597 (2004)
40. Kehrer JP. The Haber-Weiss reaction and mechanisms of toxicity. Toxicology 149: 43-50 (2000)