Hypoglycemic Effects of Sesquiterpene Lactones from *Byrsonima crassifolia*

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Abstract The novel dimeric guaianolides sesquiterpene lactone Byrsoninas A and B from seed hexane extract of *Byrsonima crassifolia* were identified. Streptozotocin-induced mildly diabetic and severely diabetic mice were treated with these oral administrations at a dosage of 20 mg/kg of body weight per day for 30 days. Also the protective effect *in vitro* in RIN-5F cells against oxidative stress was investigated and TNF- α and IL-6 levels were measured. Both Byrsonina types reduced blood glucose, cholesterol, triglyceride, lipoprotein, and transaminase levels and increased HDL-cholesterol, antioxidant enzymes, and TBARS-reactive substance levels. Byrsoninas A and B both improved this glucose metabolism by reducing insulin resistance and by stimulating insulin production due to protection effect for pancreatic β -cells against oxidative stress, lipid abnormalities were reduced and, chronic inflammation responses were alleviated producing a hepatoprotective role.

Keywords: guaianolides, antidiabetic, hyperlipidemia, *Byrsonima crassifolia*

Introduction

Diabetes mellitus (DM) is characterized by chronic hyperglycemia resulting from defects in insulin secretion or action, or both, with impaired carbohydrate, lipid, and protein metabolism. DM is divided into Type 1, or severe diabetes (also called insulin-dependent), characterized by a fasting glucose level greater than or equal to 235 mg/dL, and Type 2, or mild diabetes (also called non-insulin dependent) diabetes, characterized by a fasting glucose level greater than 126 mg/dL (1). The pathogenesis of type 2 diabetes is complex, involving progressive development of insulin resistance in the liver and peripheral tissues accompanied by defective insulin secretion from pancreatic β -cells leading to overt hyperglycemia (2).

Chronic exposure of pancreatic β -cells to elevated glucose levels results in β -cell dysfunction and, ultimately, β -cell death, a phenomenon termed β -cell glucose toxicity. Glucose toxicity is likely an important contributor to progressive β -cell deterioration and development of overt diabetes. Recently, accumulating evidence has suggested that oxidative stress is increased in pancreatic β -cells in diabetic animal models and diabetic patients (3). Since the islet cells have at lowest intrinsic antioxidant capacity, compared with other tissues, oxidative stress can contribute to β -cell glucose toxicity (4).

Byrsonima crassifolia is a tropical tree, commonly known as “nanche”, that is distributed widely in México, Central, and South

America (5). Bright yellow ripe nanche fruit is edible with sweet taste and a slightly bitter after-taste. In México, nanche is consumed as a juice, liquor, jelly, and candy. Since prehispanic times, nanche has been used as a medicine and ethnobotanical applications for dysentery, infections, wound healing, anti-inflammatory, and diabetes (6).

Phytochemical studies and pharmacological studies of this plant have been conducted since the 1970s and esters (7), epicatechins (8), glycolipids (9), flavonoids (10), triterpenes, and napthoquinones (11) have been reported. *B. crassifolia* leaf and bark extracts displayed spasmogenic effects (12). Chloroformic extracts from bark showed an anti-inflammatory activity (13). Hexane extracts from seeds showed an anti-inflammatory activity (14). Antioxidant activities of extracts from leaves, fruits, and bark have been reported (15). Ethyl acetate extracts of roots exhibited an antibacterial activity (16). Also, the aqueous extracts of leaves inhibited some dermatophytes (17). Ethanol extracts of leaves showed trypanocidal activities against *Leishmania mexicana* promastigotes (18), and hexane extracts from seeds produced hypoglycemic effects in normal and streptozotocin (STZ) induced diabetic mice (19). This study was carried out to assess the antidiabetic properties of 2 new dimeric guaianolides isolated from hexane extracts of *Byrsonima crassifolia* in STZ-induced mildly diabetics and severely diabetics mice.

Materials and Methods

General experimental model IR spectra were recorded on a PerkinElmer FTIR 1720X spectrophotometer (PerkinElmer, Waltham, MA, USA). Optical rotations were measured using a PerkinElmer 192 polarimeter (PerkinElmer) equipped with a sodium lamp (589 nm). ^1H NMR and ^{13}C NMR spectra were obtained using a Bruker DRX-300 NMR spectrometer (Bruker, Karlsruhe, Germany), and the UxNMR software package was used for NMR experiments; Chemical shifts were reported as δ (ppm), down field relative to tetramethylsilane (TMS) as an internal standard. High resolution electron impact mass spectrometry (HREIMS) was conducted using a JEOL HX 110 mass spectrometer (JEOL, Tokyo, Japan). TLC was conducted with a pre-coated TLC silica gel 60 FF254 aluminum sheets (Sigma-Aldrich, St. Louis, MO, USA). Column chromatographies were conducted with Sepadex LH-20 (Sigma-Aldrich) and silica gel (silica gel 60, 230-400 mesh, Merck, Kenilworth, NJ, USA).

Plant material *Byrsonima crassifolia* L. (belonging to the Malpighiaceae family) fruit was collected in Morelos state, Mexico in August of 2013 and was taxonomically authenticated at the Herbarium of Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional. A voucher specimen of the plant was stored as reference (No. 8976).

Isolation of bioactive compounds from *B. crassifolia* Seeds from the fruit of *B. crassifolia* were air dried, for 15 days, in shade and indoor, then 10 kg of seeds was ground in electric mill (Casarejo, DF, Mexico) to produce a powder with grain size of 1-2 mm that was extracted twice using hexane for 3 h. Seeds extracts were combined and evaporated *in vacuo* to generate 572 g of residue that was loaded onto a silica gel column and eluted using petroleum ether-acetone-hexane (2:1:0.5). A total of 6 fractions (F1-F6) were obtained. Each fractions were pooled together according to their similarities provided by thin layer chromatography analysis. These fractions were then tested for hypoglycemic activity. Subfraction F1 showed hypoglycemic properties and was subjected to silica gel column chromatography with elution using ethyl ether-chloroform (1:5) to produce the 7 subsequent subfractions (F1-1 to F1-7). The active fraction F1-1 was subjected to chromatography using a silica gel column with chloroform-ethylacetate (5:1) to yield the 6 subfractions (F11-1 to F11-6). The F11-1 fraction was further purified using a preparative plate with chloroform-ethylacetate (11:2) to produce the 5 fractions (F111-1 to F111-5) and visualized using UV radiation at 254 nm. Fraction F111-1 was separated using Sephadex LH-20 with a gradient of CHCl_3 -methanol (from 10:1 to 5:1) to yield 124 mg of Byrsonina A and 112 mg of Byrsonina B.

Byrsonina A: IR (KBr) ν_{max} 2940, 1774, 1743, 1674, 1460, 1123, 1071, 742 $1/\text{cm}$; HREIMS: m/z 820.5461 (calcd. 820.5472 for $\text{C}_{48}\text{H}_{52}\text{O}_{12}$). ^1H NMR and ^{13}C NMR data are presented in Table 1.

Byrsonina B: IR (KBr) ν_{max} 1776, 1734, 1670, 1459, 1377, 1268, 1162, 1098, 721 $1/\text{cm}$; HREIMS: m/z 704.3928, (calcd. 704.4001 for $\text{C}_{44}\text{H}_{48}\text{O}_8$).

Experimental animals The male mice weighing 20-25 g before and during the experiments were fed a standard laboratory diet (Mouse Chow 5015; Purina, St. Louis, MO, USA) with free access to water and housed in a controlled environment at $25 \pm 2^\circ\text{C}$. The experimental protocol and animal handling throughout the study were performed following the guidelines stated in the Principles of Laboratory Animal Care (National Institute of Health publication (NIH) 85-23, in Mexican Official Normativity (NOM-062-Z00-1999) (20).

Induction of severe diabetes (SD) Severe diabetes was induced in overnight fasted male mice using a single intraperitoneal injection of streptozotocin (Sigma-Aldrich) at a dosage of 50 mg/kg of body weight dissolved in cold citrate buffer (pH 4.5) (21). Hyperglycemia was confirmed by measuring glucose levels 72 h after streptozotocin administration and 7 days after injection, confirming a high glucose level. Mice with a permanent high fasting blood glucose level >300 mg/dL were used for experiments.

Induction of mildly diabetes (MD) Mild diabetes was induced in overnight fasted mice by administration of a single intraperitoneal injection of a freshly prepared STZ solution of 60 mg/kg of body weight (b.w.) in 0.1 mol/L of cold citrate buffer (pH 4.5) 15 min after the intraperitoneal administration of 120 mg/kg of nicotinamide (Sigma-Aldrich). STZ treated animals were allowed to drink a 5% glucose solution overnight to overcome drug induced hypoglycemia. After 10 days of diabetes development, mice with moderate diabetes having persistent glycosuria and hyperglycemia (blood glucose >250 mg/dL) were used for further experimentation (22).

Experimental design A total of 132 mice were divided into 22 groups, with 6 animals in each group. Grouping of animals was based on mice blood glucose values and body weights relative equality of mean blood glucose values between different groups. Normal control mice, diabetic control mice (SD) and mild diabetic control mice (MD) control mice groups received normal saline (1 mL/kg); diabetic mice (SD) group received 20 mg/kg b.w. of Byrsonina A suspended in Tween 80, 1% via gavage; diabetic control mice (MD) for administration of Byrsonina A (20 mg/kg b.w.). The other diabetic groups (MD) were orally administered 20 mg/kg b.w. of Byrsonina A and Byrsonina B respectively. Diabetic treated mice received glibenclamide (GB) a dose of 5 mg/kg b.w. as standard drug. Blood samples were collected from the tail vein and the plasma glucose concentration was determined by an enzymatic colorimetric method using a commercial GOD-POD kit (Sigma-Aldrich). Drug treatment was given for duration of 30 days.

The optimum dosage of Byrsoninas A and B were determined based on oral glucose tolerance testing. All drugs solutions and vehicle were administered orally using gastric intubations once daily at 9:00 am for 30 days. At the end of the experimental period, mice were fasted overnight, the euthanized using anesthesia. A laparotomy was performed on each mouse and the pancreas and liver were

excised, trimmed free off at, then rinsed in phosphate buffered saline. Portions of the pancreas and liver were also stored at 40°C for later analysis of oxidative stress markers. Each organ was isolated immediately after sacrificing the animal and washed with ice cold saline. These was then finely minced and homogenized (Silverson, DF, Mexico) in a 50 mM phosphate buffer at pH 7.4 and centrifuged (CK-24; DESEGO, Morelia, Mexico) at 2,000× *g* for 10 min at 4°C.

Body weight Food intake, body weights of mice, and water were measured prior to induction of hyperglycemia at day 0 of treatment, and on a daily basis thereafter, for 4 weeks.

Oral glucose tolerance testing (OGTT) in diabetic rats At the end of the experimental period, OGTT was performed to assess animals sensitivity to a high glucose load in the blood. Glucose was administered at 2 g/kg of body weight 30 min after administration of Byrsoninas A and B at a dosage of 20 mg/kg of body weight and glibenclamide (5 mg/kg). Blood was drawn from the tail vein at 0, 30, 60, 90, and 120 min after glucose administration and the blood glucose level was measured using GOD-POD kit (Sigma-Aldrich).

Estimation of serum biochemical parameters Collected blood was used for estimation of serum biochemical parameters of serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), serum alkaline phosphatase (SALP), and total protein levels, using commercial diagnostic kits (Cayman Chemical, Ann Arbor, MI, USA).

Serum lipid profiles and glucose levels At the end of the experimental period the effect of each treatment was investigated based on determination of the serum total cholesterol (TC), triglyceride (TG), HDL-cholesterol levels, and LDL-cholesterol levels using a commercial Diagnostic Kit (Genzyme Diagnostics, Lexington, MA, USA).

Antioxidant levels in the serum, liver, pancreas, and kidney The serum malonyldialdehyde (MDA) concentration, and the superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GSH), and glutathione peroxidase (GPx) activities were measured using commercial kits purchased from Cayman Chemical, according to kits instructions. For the pancreas and liver, the protein concentrations were determined using the Bradford method (23) ascribed in the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA).

Estimational glucose metabolic enzymes activities in liver tissues The activities of glucokinase and glucose-6-phosphatase were assayed using commercial EUSA kits purchased from R&O System (Boca Raton, FL, USA). Liver tissue glycogen levels were estimated using commercial kits purchased from Cayman (Ann Arbor, MI, USA). All testing was carried out according to kit instructions.

Determination of the serum insulin level and the pancreatic insulin content Serum levels and pancreatic insulin contents were measured using an enzyme linked immunosorbent assay (ELISA) (24) with a kit (Boehringer Mannheim Diagnostic, Mannheim, Germany)

Protective effect in pancreatic β -cells against oxidative stress Using culture collection of RIN-5F cells which were obtained from a rat pancreas (American Type Culture Collection; number: CRL-2058). Byrsoninas A and B were investigated for protective effect against oxidative stress in these cells. RIN-5F cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS), streptomycin (100 μ g/mL), and penicillin G (100 U/mL) (10% FBS/RPMI 1640) under an atmosphere of 5% CO₂/95% humidified air at 37°C. Cells (5×10⁵ cells/well) were cultured in Nunc 12-place multiwell plates. Thereafter, RIN-5F cells received 1 mL of fresh medium (1% FBS/RPMI 1640) without or with Byrsoninas A and B and AGEs for another 3 h. The effect of Byrsoninas A and B and AGEs on oxidative stress was examined using measurement of intracellular ROS levels based on ROS-mediated conversion of non-fluorescent 2,7-dichloro-dihydrofluorescein diacetate (DCFH-DA) (25). The intensity of fluorescence reflects enhanced oxidative stress. After 3 h of incubation, RIN-5F cells were incubated with DCFH-DA (25 μ M) in 1% FBS/RPMI 1640 at 37°C for 20 min. At the end of incubation, DCFH fluorescence of the cells from each well was measured at an emission wavelength of 530 nm and an excitation wavelength of 488 nm using a flow cytometer (Becton Dickinson, San Jose, CA, USA).

Determination of interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α) levels Serum levels of IL-6 and TNF- α were measured using an ELISA kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

Data analysis Data were expressed as a standard deviation (SD) of multiple experiments. Paired Student's *t*-tests were used to compare 2 groups and an analysis of variance (ANOVA) with the Tukey range test was used for multiple comparisons with PRISM software (Graph Pad, San Diego, CA, USA). A *p* value of less 0.05 was considered to be statistically significant.

Results and Discussion

Characterization of Birsonina A Byrsonina A, obtained as a colorless gel-like substance, provided an ion at *m/z* 820.5461 from the positive HREIMS indicating a molecular formula of C₄₈H₅₂O₁₂ in agreement with the ¹³C NMR spectrum and DEPT experimental results. The IR spectrum revealed absorption bands for γ -lactone (1774 1/cm), ester (1743 1/cm), and 1674 1/cm (olefinic group). The ¹³C NMR, HMQC, HMBC, and DEPT spectra revealed 48 carbons consisting of 6 methyls, 5 methylenes, 24 methines, and 13

quaternary carbons. The NMR spectroscopic data of compound **1** were consistent with a skeleton of a guaia-1 (10) skeleton, oxygenated at 2β , and 8α , based on the presence of 2 lactone proton signals at δ_H 4.25 and δ_H 3.19. The NMR spectrum of Byrsonia A showed 2 doublets methyl signals at δ_H 1.24 (3H, d, $J=6.3$ Hz), δ_H 1.20 (3H, d, $J=6.3$ Hz), δ_C 13.91, and δ_C 14.21, which are characteristic of methyl protons of the α -methyl- γ -lactone commonly found in guaianolide sesquiterpene lactones (26). The 2 lactone carbonyl carbons (δ_C 172.98 and δ_C 173.44) and the 2 carbons bearing oxygen (δ_C 75.21 and δ_C 75.39) were assigned to 2 γ -lactone, respectively (Table 1). In addition, the methyl protons of acetyl groups (δ_C 172.48 and δ_C 22.88) produced 2 singlet peaks at δ_H 1.97 and δ_H 1.99. Byrsonia A was probably a dimeric sesquiterpene with 2 guaia-12,6-olide units.

Correlations observed between H-13 and H-6 indicated that they were oriented toward the β -face. In addition, a large coupling

constant ($J_{6,7}=9.3$ Hz) confirmed the trans-arrangement of H-6 and 7. In addition, Byrsonia A showed the presence of a benzoyl. In HMBC spectra, correlations of δ_H 5.23 (H8) with δ_C 168.78 (C-1') confirmed the presence of a benzoyl linkage at C-8. The ^1H NMR spectrum of a singlet at δ_H 2.40 could be assigned to a methyl tertiary group attached to a quaternary carbon (C-10). Assignments were supported by HMBC H_{3-14} to C-10, C-9, and C-1. ^{13}C NMR spectra revealed the occurrence of a tetra-substituted double bond (δ_C 132.1 and 146.62). The NOE correlation between the methyl of the acetyl group was assigned to the C-2 position. The small coupling constant value of $J_{2,3}=3.3$ Hz required an α -orientation of H-2, which was further substantiated by NOE difference experimental results, that showed effects between H-2 and H-3. The position of the acyl group was confirmed at C-2 (δ_C 65.10) by the cross peak between H-2 (δ_H 5.54) and C=O (δ_C 170.3) in the HMBC spectrum (Fig. 1). Moreover, key

Table 1. ^1H NMR and ^{13}C NMR data for compound Byrsonina A¹⁾

Position	^1H	^{13}C	HMBC
1	-	132.10	-
2 α	5.54 (1H, dd, $J=4.1, 3.7$)	65.10	C=O
3 α	2.40 (1H, dt, $J=14, 3.7$)	36.17	
3 β	1.83 (1H, dt, $J=14, 6.0$)		
4 β	2.40 (1H, m)	50.43	C-15, C-15'
5	3.33 (1H, br, d $J=10.1$)	38.56	C-10, C-7, C-6, C-1, C-4, C-2
6	4.25 (1H, dd, $J=6.8, 9.3$)	75.39	
7	3.19 (1H, m)	57.51	C13, C-11, C-9, C-8, C-6
8	4.30 (1H, td, $J=2.1, 10.3$)	68.22	C-14, C-10, C-7, C-8, C-1
9a	2.79 (1H, dd, $J=2.0, 13.4$)	37.41	C-14, C-10, C-8, C-7, C-1
9b	2.70 (1H, m)	-	
10	-	146.62	
11	2.67 (1H, m)	40.43	C-13, C-7, C-12
12	-	178.56	
13	1.34 (3H, d, $J=7.0$ Hz)	13.91	C-7, C-11, C-12
14	2.40 (3H, br, s)	20.52	C-10, C-9, C-8, C-1, C-2
15	2.18 (1H, m)	26.76	C-4
1'	-	132.65	
2'		66.48	
3'		36.38	
4'		143.76	
5'		56.68	C-10', C-7', C-6', C-4', C-1'
6'	4.28 (1H, dd, $J=7.1, 9.5$)	75.21	
7'		54.61	C13', C-11', C-6', C-5', C-9'
8'		67.49	
9'		37.98	C-14', C-10', C-7', C-8', C-5', C-1'
10'	-	147.65	
11'		41.23	
12'		178.73	
13'		14.21	
14'		21.45	
15'	5.59 (1H, brs)	133.56	C-4', C-5, C-3'
1''	-	168.78	
2''-6''	7.70 (1H, d, $J=7.6$)	130.98	
3''-5''	7.49 (d, $J=7.6$)	128.87	
CH ₃ COO	1.97	22.88	
CH ₃ COO	1.99	172.48	

¹⁾ δ in ppm and J (in Hz) are in parentheses. Recorder in CDCl₃ at 300 MHz and 125 MHz for ^1H and ^{13}C NMR, respectively.

Fig. 1. Key COSY (bold), HMBC (HH) correlations for 1

correlations of another guaianolide unit with an acetyl, a benzoyl groups and 1 exo-olefin signal (δ_c 142.78 and δ_c 126.3) were evident. The linkage between the two guaianolide units was characterized using HMBC spectra (Table 1) which indicated long-range correlations between H-4 (δ_H 2.40) and C-4' (δ_c 143.76). The coupling constants for H-6 ($J_{5,6}=6.8$ Hz, $J_{6,7}=9.3$ Hz) and H-6' ($J_{5',6'}=7.1$ Hz, $J_{6',7'}=9.5$ Hz) clearly indicated the presence of 2 *trans*-fused α -methyl- γ -lactone rings in Byrsonina A. The orientations of H-6 and H-11 were determined to be β and the orientation of H-5, H-7, CH₂-15, and Me-13 were determined to be α based on NOESY correlations (H-6/H-4, H-11; H-5/H-1; H-13/H-7, H-5) and by the coupling constant of H-6 to H-7. Therefore, compound **1** was a novel dimeric guaianolide assigned the name of Byrsonina A (Fig. 1).

Characterization of Byrsonina B was obtained as a colorless gel-like substance with an ion at m/z 704.3928 provided by a positive HREIMS, indicating a molecular formula of C₄₄H₄₈O₈ in agreement

with the ¹³C NMR spectrum and DEPT experimental results. The IR spectrum showed characteristic bands at 1776 (γ -lactone), 1743 (ester carbonyl), and 1674 (double bond). The structure of Byrsonina B deduced from NMR data, was closely related to Byrsonina A with similarities in chemical shifts, coupling constants, and 2D-NMR correlations. Byrsonina B had the same skeleton as Byrsonina A based on ¹³C NMR data. Byrsonina B did not have an acyl moiety (δ_H 2.56, m, H-2; δ_c 45.21, C-2) but did have the same stereochemistry at all centers. Thus, the structure of compound **2** was elucidated and the compound was named Byrsonina B (Fig. 1).

Effect of byrsonimas A and B on oral glucose tolerance in mice

Changes in the levels of postprandial blood glucose levels in normal, diabetic control, and experimental group mice after oral administration of glucose (2 g/kg) are shown in Table 2. Oral treatment (20 mg/kg of body weight) in diabetic mice with Byrsoninas A and B produced a

Table 2. Effect of Byrsoninas A and B on oral glucose tolerance test

S. No.	Groups	Time (min)				
		0	30	60	90	120
1	Non-diabetic control	94.61±4.74 ¹⁾	99.43±5.18	94.61±4.74	104.13±3.98	105.40±3.51
2	Diabetic control type SD	348.65±9.56	423.10±10.76	498.54±21.67	474.32±19.47	441.38±17.52
3	Diabetic control type MD	234.56±10.23	367.80±14.29	336.42±12.07	298.31±11.10	250.39±10.78
4	SD+Byrsonina A (15 mg/kg)	321.19±8.84	390.46±13.60	420.31±11.44 ^a	382.57±12.61 ^a	350.49±13.45 ^a
5	MD+Byrsonina B (15 mg/kg)	281.39±13.06	317.62±16.72	330.52±11.63	292.32±10.76	275.11±12.41
6	SD+Byrsonina A (20 mg/kg)	354.32±9.76	389.58±18.82	410.78±10.87	374.80±13.48 ^a	360.38±14.29 ^a
7	MD+Byrsonina B (20 mg/kg)	285.28±13.95	313.90±10.81	325.17±15.83	283.58±13.63 ^b	262.35±11.70 ^b
8	SD+Byrsonina A (30 mg/kg)	312.26±17.31	345.38±12.05	389.68±15.29 ^a	359.73±14.61 ^a	320.14±12.54 ^a
9	MD+Byrsonina B (30 mg/kg)	248.82±13.30	279.16±10.62	306.61±15.42 ^b	260.41±15.74 ^b	215.49±16.53 ^b
10	GB (10 mg/kg)	374.19±13.84	401.83±14.38	421.07±17.29 ^{ab}	361.20±12.64 ^{ab}	243.51±10.72 ^{ab}

¹⁾Results were represented as a mean±SD, of blood glucose levels obtained from 6 animals. Significant difference at ^a $p<0.05$, when compared to diabetic control type SD. Significant difference at ^b $p<0.05$ when compared to diabetic control type MD.

Table 3. Effect of on fasting blood glucose level in non-diabetic and diabetic mice in interval of 2-12 h

Group	Dose (mg/kg)	At the time of grouping	Blood glucose levels (mg/dL) at different time intervals (h)				
			2	4	6	8	10
Non-diabetic+Byrsonina A	-	101.28±1.34 ¹⁾	100.5±0.95	100.34±1.38	103.02±0.98	99.19±1.75	100.21±0.85
Non-diabetic+Byrsonina B	20	104.29±1.29	80.54±4.95 ^a	48.32±2.38 ^a	42.32±1.76 ^a	50.27±2.85 ^a	66.12±1.83 ^a
Diabetic control type SD	20	106.29±3.45	89.41±5.38 ^a	60.12±2.48 ^a	55.29±1.54 ^a	59.63±2.37 ^a	71.70±1.39 ^a
Diabetic control type MD	-	375.13±3.67	364.13±4.23	369.44±4.10	371.23±5.16	378.01±4.47	373.39±5.33
SD+Byrsonina A	-	287.38±3.96	291.04±6.11	297.30±4.27	288.54±5.03	295.61±4.23	290.21±6.25
SD+Byrsonina B	20	360.42±2.87	341.39±3.43 ^b	266.68±2.68 ^b	190.27±5.89 ^b	232.39±3.49 ^b	287.21±4.24 ^b
MD+Byrsonina A	20	338.69±4.21	301.29±5.17 ^b	258.48±3.36 ^b	192.01±4.19 ^b	204.38±1.87 ^b	236.36±3.98 ^b
MD+Byrsonina B	20	224.56±4.23	184.13±4.28	151.92±2.90 ^c	128.37±4.23 ^c	135.24±4.23 ^c	140.13±4.23 ^c
Diabetic+GB	20	252.11±5.61	197.42±5.96 ^c	176.74±4.83 ^c	137.26±2.77 ^c	149.52±2.63 ^c	153.31±2.83 ^c
Non-diabetic+Byrsonina A	5	348.89±5.79	272.68±6.87 ^b	201.35±2.59 ^b	209.43±2.94 ^b	219.60±1.98 ^b	266.29±2.83 ^b

¹⁾Each values represent mean±SD ($n=6$). ^a $p<0.05$ compared to Non-diabetic control (ANOVA) followed by Dunnett's test. Signification differences ^b $p<0.05$ when compare to diabetic control type SD. Signification differences ^c $p<0.05$ when compare to diabetic control type MD.

significant ($p<0.05$) reduction of the glucose level in blood at 120 min, showing a decrease of glucose in blood, compared with STZ-diabetic rats. This activity improved the glucose tolerance, suggesting a decrease in insulin resistance and helping to maintain blood glucose levels steady, which may indicate induction of peripheral glucose use.

Effect of fasting on blood glucose levels in normoglycemic, STZ induced severely diabetics, and STZ-nicotinamide induced mildly diabetics mice during the interval of 2 to 12 h Oral administration of Byrsoninas A and B at a dosage of 20 mg/kg of body weight produced a significant ($p<0.05$) hypoglycaemic effect in normal fasted mice after 4 h, compared with controls. The most pronounced effect of Byrsonina A was observed after 6 h (Table 3). Reductions in the blood glucose levels caused by Byrsoninas A and B at all dosage were higher than for the standard drug glibenclamide. The plasma glucose levels in STZ-induced diabetic mice (MD and SD) treated with Byrsoninas A and B at 20 mg/kg of body weight during the interval of 2 to 12 h are shown in Table 2. The serum glucose levels of diabetic control mice were markedly higher than of normal control mice, and the glucose concentrations the Byrsoninas A and B treated diabetic mice were significantly ($p<0.05$) decreased after the treatment

period, compared with diabetic control mice.

Effect of Byrsonina A and Byrsonina B on fasting blood glucose levels in STZ induced severely diabetics mice and STZ-nicotinamide induced mildly diabetics mice for 4 weeks The antihyperglycemic effects of Byrsoninas A and B on the fasting blood glucose levels in STZ induced type 1 and STZ-nicotinamide-induced type 2 diabetic mice are shown in Table 4. Byrsoninas A and B administered at the same concentrations produced a significant ($p<0.05$) antihyperglycaemic effects in STZ-induced severely and mildly diabetic mice after 2 weeks for up to 4 weeks, compared with controls. Maximum decreases of 58% and 46% for Byrsoninas A and B respectively were observed at a dosage of 20 mg/kg of body weight after administration severely diabetic mice. In MD, Byrsoninas A and B gradually decreased blood glucose levels for 4 weeks after administration at a dosage of 20 mg/kg (61 and 53%, respectively). These values showed restoration of normal blood glucose levels in treated mice.

Glibenclamide is reported to be as in effective when β -cells are destroyed (27). In this study, glibenclamide induced a significant ($p<0.05$) reduction in blood glucose levels in STZ-induced diabetic rats, compared with controls, suggesting partial destruction of

Table 4. Effect of Byrsoninas A and B after 30 days of treatment on the blood glucose level and plasma insulin levels and the pancreatic insulin content in STZ-induced severe and mildly diabetic mice

Group	Fasting blood glucose level (mg/dL)					Before administration (0)	Plasma insulin (μ U/mL)	Pancreatic insulin (μ U/mL)
	0	1	2	3	4			
Non-diabetic control	100.1 $\pm$ 4.8 ¹⁾	102.6 $\pm$ 3.6 ^a	101.4 $\pm$ 2.1	102.2 $\pm$ 1.9	101.3 $\pm$ 5.0	3.59 $\pm$ 0.49	3.60 $\pm$ 0.15	25.80 $\pm$ 3.80
Diabetic control type SD	358.7 $\pm$ 6.7	367.2 $\pm$ 8.4	381.5 $\pm$ 4.9	411.2 $\pm$ 9.6	432.8 $\pm$ 9.3	0.77 $\pm$ 0.082	1.50 $\pm$ 0.29	15.21 $\pm$ 4.58
Diabetic control type MD	251.27 $\pm$ 8.2	264.53 $\pm$ 5.9	269.25 $\pm$ 4.9	278.44 $\pm$ 3.7	279.40 $\pm$ 6.2	0.82 $\pm$ 0.065	1.67 $\pm$ 0.043	17.82 $\pm$ 7.31
SD+Byrsonina A	375.7 $\pm$ 4.7	333.1 $\pm$ 10.4 (11)	180.6 $\pm$ 8.1 ^a (48)	172.4 $\pm$ 5.9 ^a (54)	155.7 $\pm$ 4.0 ^a (58)	0.74 $\pm$ 0.024	3.24 $\pm$ 0.51 ^c	21.67 $\pm$ 6.43 ^c
SD+Byrsonina B	368.5 $\pm$ 5.8	331.5 $\pm$ 7.6 (10)	257.6 $\pm$ 7.9 ^a (30)	215.2 $\pm$ 8.3 ^a (41)	198.8 $\pm$ 9.0 ^a (46)	0.76 $\pm$ 0.030	2.96 $\pm$ 0.78 ^c	21.03 $\pm$ 6.37 ^c
MD+Byrsonina A	235.53 $\pm$ 3.2	206.76 $\pm$ 6.5 (12)	119.80 $\pm$ 3.3 ^b (49)	100.02 $\pm$ 6.3 ^b (57)	91 $\pm$ 5.8 ^b (61)	0.77 $\pm$ 0.016	3.27 $\pm$ 0.54 ^d	22.17 $\pm$ 5.48 ^d
MD+Byrsonina B	245.60 $\pm$ 3.9	215.1 $\pm$ 5.8 (12)	165.7 $\pm$ 3.6 ^b (32)	130.7 $\pm$ 7.4 ^b (47)	114.2 $\pm$ 5.6 ^b (53)	0.78 $\pm$ 0.039	3.07 $\pm$ 0.72 ^d	20.87 $\pm$ 5.31 ^d
Diabetic+GB	352.60 $\pm$ 6.6	270.5 $\pm$ 6.9 (14)	152.7 $\pm$ 9.2 ^a (51)	109.4 $\pm$ 5.3 ^a (65)	100.2 $\pm$ 2.1 ^a (68)	0.74 $\pm$ 0.061	3.49 $\pm$ 0.34 ^c	19.35 $\pm$ 3.23 ^c

¹⁾Each value represents a mean $\pm$ SD, ($n=6$), ANOVA followed by multiple two-tail "t" test. Blood glucose level showed a significant difference between treated group and diabetic control type SD mice ^a $p<0.001$. Significant difference between treated group and diabetic control mice (MD) ^b $p<0.01$. Insulin values at 0 h before drug administration are significantly different compared to respective days 28 after drug treatment ^c $p<0.05$ compared with diabetic control type SD group and ^d $p<0.01$ compared with corresponding value for diabetic control type MD. Glibenclamide (GB) at dosages of 5 mg/kg. () % inhibition.

pancreas β -cells. In normoglycaemic mice, sulfonylurea agents have been found to induce hypoglycaemia based on an ability to stimulate β -pancreatic cells to liberate insulin (28). These results suggest that the action mechanism of Byrsoninas A and B may be similar to sulfonylurea effects. Animals treated for 30 days showed a significant ($p<0.05$) reduction of blood glucose levels, compared with control simplying that Byrsoninas A and B may act in the long-term.

Effect of Byrsoninas A and B on serum insulin levels and the pancreatic insulin content Serum insulin levels and the pancreatic insulin content were significantly ($p<0.05$) decreased in STZ induced diabetic mice to values, as low as 1.5 μ U/mL (in STZ untreated mice), compared with non-diabetic control group mice (3.6 μ U/mL). The effects of 30 day treatment with Byrsoninas A, Byrsoninas B and glibenclamide produces an elevation of serum insulin and pancreatic insulin levels compared to STZ-diabetic groups (SD and MD) (Table 4).

Effect of Byrsoninas A and B on body weight food and water intake During the study period of 4 weeks, the body weight and food intake of all mice were recorded daily. Results are presented only for day 0 and for the last day of each subsequent week (Table 4). Byrsoninas A and B treatments at dosages of 20 mg/kg of body weight resulted in no significant ($p<0.05$) differences in the body, liver and kidney weight of treated mice compared with STZ control mice (Table 5).

Byrsoninas A and B prevented the increases in food and drink intake that are induced as a consequence of diabetes. Insulin resistance in a diabetic state usually causes an increase in body weight and increases in food and water intake. For chronic treatment, the results of this study indicated that; daily administration of Byrsoninas A and B for 4 weeks reduced the increase in food and drink intake that are

associated with diabetes (Table 5).

Effect of Byrsoninas A and B on the G6Pase, GK, and glycogen contents and the hexokinase activity in the liver In this study, effect of Byrsoninas A and B on some glycolytic enzymes (GK and G6Pase) are shown in Table 5. Hexokinase and glycogen content was evaluated and the results are given in Table 6. Byrsoninas A and B increased the hepatic glycogen content in liver and skeletal muscle activity of HK and GK, while the G6Pase activity was decreased in the Byrsoninas A and B diabetic groups when compared to the diabetic control groups. While the HK and GK activities in the Byrsoninas A and B group were comparable to that in the normal control group, a marked inhibition of G6Pase activity was observed. This is supported by the relatively higher glycogen content observed in the Byrsoninas A and B groups than in the normal control group. The observed activation of HK and GK along with inhibition of G6Pase, an enzyme that catalyzes the terminal step in both glycogenolysis and gluconeogenesis, further support the insulinogenic activity of the Byrsoninas A and B.

Effects of Byrsonina A and B on serum lipid profile The serum lipid profile in control and experimental animals are shown in Table 6. The rise in blood glucose was accompanied with increase in serum total cholesterol, triglyceride, low density lipoprotein, very low density lipoprotein and decreased in high density lipoprotein cholesterol in diabetic mice than those in normal control mice. Treatment with Byrsoninas A and B or glibenclamide to diabetic mice resulted in significant ($p<0.05$) decrease in these parameters compared to those in untreated diabetic mice. However, serum HDL levels were significantly increased in diabetic treated mice. Treatment of Byrsoninas A and B led

Table 5. Change in body weights of mice at four weeks of treatment of Byrsoninas A and B and physico-metabolic symptoms. Byrsoninas A and B effects on hepatic glucose regulation enzyme activities (G6Pase and GK) in diabetic mice

Group	Body weight (g)		Intake (g/d)			Final (g)		G6Pase	GK
	Initial	Final	Gain	Food	Water	Liver weight	Kidney weight	Activity (mU)	Activity (mU)
No-diabetic	24.6±5.3 ¹⁾	31.1±4.8	6.5±1.9 (26)	28.7±5.0	32.9±3.5	6.45±3.6	1.30±0.6	0.39±0.008	3.39±0.04
Diabetic control type SD	27.0±7.1	28.5±7.5	1.5±0.05 (5)	37.6±4.3	186.9±2.6	3.54±1.1	0.80±0.003	0.70±0.003	1.28±0.09
Diabetic control type MD	26.3±7.3	28.3±2.8	2.0±0.08 (7)	36.59±4.3	154.71±6.9	3.95±6.1	0.85±0.01	0.60±0.002	1.05±0.007
SD+Byrsonina A	22.6±5.8	26.4±3.9	4.8±1.1 ^a (18)	30.4±2.8 ^a	129.5±5.2 ^a	5.10±2.8 ^a	1.1±0.04 ^a	0.37±0.005 ^a	3.0±0.02 ^a
SD+Byrsonina B	26.4±5.0	30.2±5.3 ^a	3.8±1.6 ^a (14)	35.5±6.4 ^a	122.8±6.8 ^a	4.8±1.9 ^a	0.96±0.005 ^a	0.34±0.002 ^a	2.81±0.04 ^a
MD+Byrsonina A	25.5±5.6	28.8±5.1	3.3±1.04 ^b (15)	29.6±6.1 ^c	130.0±5.8 ^c	6.09±3.4 ^b	1.20±0.07 ^b	0.40±0.006 ^b	3.20±0.08 ^c
MD+Byrsonina B	23.8±6.0	26.7±4.8 ^b	2.9±0.9 ^b (12)	31.1±5.7 ^b	119.5±6.1 ^c	5.89±4.2 ^b	1.03±0.01 ^c	0.42±0.003 ^b	2.98±0.05 ^c
SD+GB	24.5±6.2	28.2±5.8 ^a	48.1±4.7 ^c (20)	28.2±2.5 ^b	113.3±5.0 ^c	6.10±3.7 ^c	1.29±0.7 ^c	0.36±0.009 ^b	3.11±0.04 ^c

¹⁾Each values represents a mean±SD, (n=6); ANOVA followed by multiple two tail "t" test. Significant differences between diabetic control mice SD and treated mice ^ap<0.001. Significant differences between treated group mice and diabetic control mice MD ^bp<0.01, ^cp<0.05. Glibenclamide 4 mg/kg treated group mice. () indicates %.

Table 6. Influence of compounds Byrsoninas A and B on the serum HDL-cholesterol, LDL-cholesterol VLDL-cholesterol, total cholesterol, triglycerides level and the glycogen content in the liver and the skeletal muscle, and the HK activity in STZ- induced diabetic mice (SD) and STZ-nicotinamide induced diabetic mice (MD)

Groups	Mean Concentration (mg/g)±SEM				Glycogen (mg/kg)			
	HDL-C (mg/dL)	LDL-C (mg/dL)	VLDL-C (mg/dL)	Total-C (mg/dL)	Triglycerides (mg/dL)	Liver	Skeletal muscle	HK activity
No diabetic control	39.8±2.19 ¹⁾	26.5±4.21	18.4±2.45	122.9±8.10	94.8±3.91	19.58±1.68	11.61±3.24	1.67±0.02
Diabetic control type SD	19.0±1.87	87.3±6.12	35.8±3.84	250.5±9.71	180.7±8.23	9.31±2.67	4.45±1.56	1.29±0.07
Diabetic control type MD	21.4±7.84	68.5±4.29	30.3±2.50	236.3±7.58	168.4±7.82	10.63±0.008	4.04±0.005	1.30±0.002
SD+Byrsonina A	34.5±6.12 ^a	39.3±2.67 ^b	23.6±3.90 ^a	140.3±7.17 ^a	110.4±12.41 ^a	17.03±2.39 ^b	10.38±1.29 ^a	1.54±0.08 ^a
SD+Byrsonina B	32.3±7.26 ^b	36.5±7.39 ^b	21.25±4.73 ^a	144.28±5.19 ^b	118.6±9.53 ^a	15.81±6.12 ^b	8.57±1.38 ^b	1.51±0.03 ^b
MD+Byrsonina A	36.3±8.24 ^d	37.1±10.42 ^c	26.6±7.19 ^c	135.1±7.37 ^c	102.9±4.19 ^d	18.21±6.43 ^c	10.97±2.67 ^c	1.58±0.09 ^b
MD+Byrsonina B	35.1±4.19 ^c	34.98±9.21 ^c	24.45±8.58 ^d	138.2±5.28 ^c	107.6±6.75 ^d	17.01±5.36 ^c	9.81±3.01 ^d	1.56±0.07 ^b
SD+GB	36.2±3.76 ^a	38.3±2.43 ^a	22.7±2.42 ^a	121.8±5.48 ^b	96.8±5.97 ^b	17.78±2.30 ^b	11.01±1.96 ^b	1.60±0.03 ^a

¹⁾Values are expressed as mean±SEM (n=6) ^ap<0.05, ^bp<0.01, vs diabetic control type SD. ^cp<0.05, ^dp<0.01, vs diabetic control type MD.

to significant reductions of glucose, triglyceride, and total cholesterol contents in diabetic mice (Table 6) which suggested that Byrsoninas A and B prevent the excessive glucose supply and abnormal lipid accumulation.

Serum biochemical parameters The hepatoprotective properties of Byrsoninas A and B were studied in STZ induced MD and SD mice. Treatments with Byrsoninas A and B at dosage of 20 mg/kg of body weight significantly ($p<0.001$) reduced the SGOT, SGPT, SALP, and serum protein levels, near normal values, compared with controls (Table 7). There was a significant ($p<0.05$) elevation in serum triglycerides, and total cholesterol levels, in diabetic controls. Daily administration of Byrsoninas A and B at a dosage of 20 mg/kg of body weight to diabetic mice for 30 days significantly ($p<0.05$) reduced serum total cholesterol and triglycerides levels compared

with controls. The serum level of HDLc, a useful lipoprotein, was decreased in diabetic group mice. After 30 days of continuous administration of Byrsoninas A and B, there was a considerable elevation in the serum HDLc level to levels-similar to control group mice, suggesting that Byrsoninas A and B control the development of diabetes through improvements in the lipid metabolism.

In STZ-induced diabetic mice, the liver was necrotized and an increase in the activities of SGOT, SGPT, and SALP in the plasma were probably due to leakage of these enzymes from the liver cytosol into the blood stream, which gives an indication of the hepatotoxic effect of STZ (29). Treatment of diabetic mice with Byrsoninas A and B reduced the activity of these enzymes in the plasma compared with diabetic untreated group mice, and consequently alleviated the liver damage caused by STZ-induced diabetes. Significant ($p<0.05$) indicated the hepatoprotective roles of Byrsoninas A and B in

Table 7. Influence of Byrsoninas A and B on serum biochemical parameters and TBARS levels in hyperglycemic type 1 and 2 mice

Group	IU/L		g/dL		TBARS (μM/g)		
	SGOT	SGPT	ALP	Total protein	Liver	Kidney	Pancreas
No diabetic control	20.9±3.2 ¹⁾	24.1±2.8	170.3±10.4	7.9±2.1	0.99±0.004	1.8±0.01	2.47±0.54
Diabetic control type SD	39.2±8.9 ^a	42.0±4.6 ^a	245.1±11.6 ^a	4.3±1.5 ^a	1.69±0.08 ^d	2.6±0.07 ^d	3.55±0.47 ^d
Diabetic control type MD	30.6±6.5 ^a	37.6±9.1 ^a	234.0±7.4 ^a	4.9±1.2 ^a	1.44±0.02 ^d	2.1±0.06 ^d	3.32±0.02 ^d
SD+Byrsonina A	26.1±7.4 ^b	29.6±7.2 ^b	198.9±9.7 ^b	6.5±1.9 ^b	0.91±0.004 ^{de}	1.7±0.08 ^{de}	2.40±0.12 ^{de}
SD+Byrsonina B	27.3±6.3 ^b	31.4±6.3 ^b	205.7±12.5 ^b	5.3±2.1 ^b	0.98±0.008 ^{de}	1.9±0.004 ^{de}	2.36±0.52 ^{de}
MD+Byrsonina A	25.8±5.9 ^c	28.2±9.0 ^b	190.8±8.9 ^c	6.7±2.0 ^c	0.90±0.03 ^{df}	1.6±0.04 ^{df}	2.34±0.19 ^{df}
MD+Byrsonina B	26.5±4.4 ^c	30.6±7.2 ^b	200.0±7.6 ^c	5.6±1.4 ^c	0.92±0.02 ^{df}	1.8±0.04 ^{df}	2.38±0.38 ^{df}
SD+GB	23.1±8.0 ^b	25.1±5.7 ^b	192.8±11.8 ^b	7.2±3.8 ^b	0.93±0.08 ^{de}	1.5±0.04 ^{de}	2.37±0.55 ^{de}

¹⁾Values are expressed as a mean±SD, (n=6). ^ap<0.001 compared to no diabetic control and ^bp<0.001 compared with STZ control group mice (SD). ^cp<0.05 compared to STZ control group mice (MD). All TBARS values are expressed as a mean±SEM (n=6). Values. ^dp<0.05 compared to no diabetic control group mice, ^ep<0.001 compared to diabetic control group mice (SD). ^fp<0.05 compared to diabetic control group mice (SD), where the significance was determined using a one-way ANOVA followed by a post hoc Dunnett's test. ALP, alkaline phosphatase; SGOT, serum glutamate oxaloacetate transaminase; SGPT, serum glutamate

Table 8. Effect Byrsoninas A (1) and B (2) on antioxidant enzyme activities in liver, kidney and pancreas in diabetic mice

Parameters	No diabetic Control	Diabetic control SD	Diabetic control MD	Diabetic SD+1	Diabetic SD+2	Diabetic MD+1	Diabetic MD+2	Diabetic SD+GB
SOD-Liver	7.54±2.19 ¹⁾	3.79±0.82 ^a	3.98±0.74 ^a	6.01±1.05 ^b	5.87±0.89 ^b	6.69±2.23 ^c	6.30±1.28 ^c	6.80±0.41 ^b
SOD-Kidney	13.82±3.51	7.35±1.37 ^a	8.12±2.62 ^a	11.47±3.41 ^b	10.24±2.63 ^b	12.59±3.70 ^c	11.79±3.38 ^c	13.025±1.57 ^b
SOD-pancreas	54.52±5.21	36.47±3.28 ^a	38.11±4.53 ^a	46.90±5.48 ^b	43.88±5.39 ^b	49.48±6.18 ^c	47.63±4.75 ^c	50.71±4.36 ^b
CAT-Liver	82.36±6.17	44.53±3.13 ^a	50.27±5.02 ^a	67.12±6.74 ^b	64.56±6.38 ^b	69.46±7.23 ^c	68.03±4.80 ^c	70.26±2.16 ^b
CAT-Kidney	35.61±2.33	20.76±1.65 ^a	22.44±2.78 ^a	32.07±2.53 ^b	29.56±2.81 ^b	33.58±7.48 ^c	31.43±6.90 ^c	34.17±1.79 ^b
CAT-pancreas	59.32±5.38	25.56±4.19 ^a	28.38±3.87 ^a	43.79±5.18 ^b	40.24±3.67 ^b	46.81±8.20 ^c	44.29±6.41 ^c	47.83±4.36 ^b
GSH-Liver	47.68±7.12	21.80±1.97 ^a	25.71±1.86 ^a	39.48±4.36 ^b	36.52±4.86 ^b	40.17±5.30 ^c	38.79±5.90 ^c	42.38±2.28 ^b
GSH-Kidney	24.45±3.71	5.79±2.06 ^a	9.13±2.03 ^a	17.20±3.53 ^b	15.29±3.83 ^b	18.81±5.80 ^c	16.52±3.53 ^c	19.27±3.58 ^b
GSH-pancreas	12.48±3.39	5.19±0.94 ^a	6.90±1.50 ^a	8.47±1.56 ^b	6.91±1.80 ^b	9.74±2.69 ^c	8.53±2.71 ^c	10.79±1.92 ^b
GPx-Liver	7.26±1.59	4.31±1.30 ^a	5.18±0.98 ^a	5.64±1.87 ^b	5.35±1.83 ^b	5.73±2.01 ^c	5.48±1.63 ^c	5.92±1.26 ^b
GPx-Kidney	5.92±1.42	3.53±0.92 ^a	3.90±0.76 ^a	4.60±1.73 ^b	4.53±1.34 ^b	4.64±1.20 ^c	4.04±1.46 ^c	4.78±0.94 ^b
GPx-pancreas	4.64±1.19	2.32±0.36 ^a	2.84±0.27 ^a	3.70±1.26 ^b	3.31±0.61 ^b	3.79±0.75 ^c	3.52±0.41 ^c	3.89±0.27 ^b

¹⁾All values are expressed as mean±SEM (n=6). Values. ^ap<0.01 when compared to no diabetic control group; ^bp<0.01, ^cp<0.05 compared to diabetic control group SD and MD respectively; where the significance was performed by one-way ANOVA followed by post hoc Dunnett's test. The values are given in U/mg of protein.

prevention of diabetic complications.

TBARS levels TBARS levels also decreased after treatment with Byrsonina A in severely diabetic mice by 46% in the liver, 35% in the kidneys, and 32% in the pancreas. Treatment with Byrsonina B produced a decrease in liver, kidneys, and pancreas TBARS levels of 42, 27, and 33%, respectively. However, treatment with Byrsonina A in mildly diabetic mice produced a decreases in liver, kidneys, and pancreas TBARS levels of 47, 38, and 34%, and treatment with Byrsonina B caused 45, 31, and 33% reductions, respectively. Dietary supplementation with these compounds resulted in a significant (p<0.05) diminution in TBARS levels compared with control, and levels moved toward values in control group mice (Table 7).

TBARS levels are an index of endogenous lipid peroxidation and oxidative stress with intensified free radical production. Therefore, measurement of TBA-reactive substance levels is frequently used to determine the level of oxidative stress in diabetic patients. In addition, increased lipid peroxidation in the pancreas implies a level

of susceptibility to diabetic oxidative stress. These elevated TBARS levels in diabetic mice might be due to stimulation of hepatic triglyceride synthesis as a result of a free fatty acid influx (28). Repeated separate administration of Byrsoninas A and B had a beneficial effect for reducing the degree of hyperlipidemia that is associated with hyperglycemia.

Effect of Byrsoninas A and B on SOD, CAT, GSH, and GPx activities in hepatic, renal, and pancreas tissues

The antioxidant effects of Byrsoninas A and B on tissue oxidative markers was studied (Table 8). Diabetic mice showed a significant (p<0.05) reduction in SOD, CAT, GSH, and GPx activities in hepatic and renal tissues in diabetic control groups indicating the presence of persistent oxidative stress. In addition, it is known that *B. crassifolia* possesses antioxidant compounds (15) that have abilities to prevent the deleterious effects of STZ. Decomposition of streptozotocin releases reactive oxygen species (ROS) that act on the cellular membrane and the DNA chain and cause cell death. Byrsoninas A and B apparently acted by increasing

Table 9. Effect of Byrsoninas A and B on AGEs-induced oxidative stress in RIN-5F cells based on serum IL-6 and TNF- α levels in mice

Groups	Intensity of DCFH-DA fluorescence	Groups	IL-6 (pg/mL)	TNF- α (pg/mL)
CNT (Res -)	9.16 $\pm$ 1.85 ¹⁾	No diabetic control	44.54 $\pm$ 6.29	34.51 $\pm$ 4.26
AGE1 (Res -)	19.12 $\pm$ 3.78	Diabetic control type SD	49.65 $\pm$ 6.13	44.72 $\pm$ 6.35 ^a
AGE2 (Res -)	28.46 $\pm$ 7.54	Diabetic control type MD	48.22 $\pm$ 4.94	42.19 $\pm$ 3.65 ^a
CNT+Byrsonina A	3.45 $\pm$ 0.67*	SD+Byrsonina A	46.23 $\pm$ 5.82	35.51 $\pm$ 5.65 ^b
CNT+Byrsonina B	4.04 $\pm$ 1.23*	SD+Byrsonina B	47.32 $\pm$ 5.82	36.21 $\pm$ 4.48 ^b
AGE1+Byrsonina A	10.67 $\pm$ 3.20*	MD+Byrsonina A	45.39 $\pm$ 4.50	35.76 $\pm$ 3.71 ^c
AGE1+Byrsonina B	8.97 $\pm$ 2.56*	MD+Byrsonina B	46.61 $\pm$ 5.64	34.73 $\pm$ 4.83 ^c
AGE2+Byrsonina A	13.74 $\pm$ 3.08*	GB 4 mg/kg	-	-
AGE2+Byrsonina B	11.88 $\pm$ 3.19*			

¹⁾Each value represents a mean $\pm$ SD, for 3 assays, * p <0.05. BSA alone (CNT) was used as a control. Effects of AGE1 and AGE2 on intracellular ROS levels in RIN-5F cells after 3 h of treatment with AGEs. ^a p <0.05 compared to normal control mice. ^b p <0.05 compared with diabetic control mice (SD); ^c p <0.05 compared with diabetic control mice (MD).

SOD, GPx, and CAT activities after 30 days of STZ administration in mice indicated that Byrsoninas A and B reduced levels of reactive oxygen free radicals by improving the activities of antioxidant enzymes. Associated with changes in lipid peroxidation, diabetic animals showed decreased activities of the key antioxidant enzymes SOD, CAT, and GPx, which all play important roles in scavenging the toxic intermediates of incomplete oxidation. A decrease in the activity of these enzymes can lead to excess availability of the superoxide anion (O_2^-) and hydrogen peroxide in the biological systems. This excess availability generates hydroxyl radicals, resulting in initiation and propagation of lipid peroxidation. Treatment with Byrsoninas A and B increased enzyme activities and probably helped to control free radicals.

The protective effect of Byrsoninas A and B in RIN-5F cells against AGEs-induced oxidative stress The effect of Byrsoninas A and B on the AGEs-induced oxidative stress was investigated in RIN-5F cells (Table 9). RIN-5F cells were treated with Byrsoninas A and B and the intracellular peroxide level was measured with DCFH-DA fluorescent probe (25). The fluorescence intensity of the Byrsoninas A and B treated group was significantly (p <0.05) lower than that of the control groups. The fluorescence intensity of AGE1 and AGE2-treated cells was significantly (p <0.05) higher than the intensity of CNT-treated cells. Addition of Byrsoninas A and B resulted in a reductions in oxidative stress. These results clearly indicated that Byrsoninas A and B protected pancreatic β -cells from AGEs-induced oxidative stress.

In the diabetic state, the glycation reaction is observed in different tissues and organs and for different kinds of glycated proteins (30). Therefore, the results of this study demonstrated that Byrsoninas A and B protected RIN-5F cells from AGEs-induced oxidative stress followed by decreasing insulin gene expression and secretion (Table 9) suggesting that Byrsoninas A and B partially rescued exhausted pancreatic β -cells from further AGEs-induced oxidative stress (30). AGEs might cause deterioration in the function of pancreatic β -cells in patients with long-term hyperglycemia. Therefore, Byrsoninas A and B can act as suppressors of pancreas cell damage and can inhibit

progression of the pancreatics dysfunction induced by chronic hyperglycemia.

TNF- α and IL-6 levels in the serum In diabetic mice, the level TNF- α in the serum were significantly (p <0.05) increased, compared with controls (Table 9). After treatment of diabetic mice with Byrsoninas A and B for 30 days, there were no significant (p <0.05) differences in serum IL-6 levels between any of the experimental groups. TNF- α levels were decreased significantly (p <0.05) after treatment of Byrsoninas A and B in SD and MD groups.

IL-6 and TNF- α are two of the most important proinflammatory cytokines in the serum (31). The increased plasma concentrations of IL-6 and TNF- α are associated with development of insulin resistance and type 2 diabetes since these cytokines have the potential to suppress the action of insulin through interference with insulin receptor-mediated signal transduction (32). To explore the possible molecular mechanism underlying the antidiabetic activity of Byrsoninas A and B, concentrations of IL-6 and TNF- α in the serum of MD mice were determined after treatment with Byrsoninas A and B and compared with vehicle treatment. The TNF- α levels were significantly (p <0.05) reduced after treatment with Byrsoninas A and B compared with with diabetic control groups. The results of this study indicated that repression of chronic inflammation appears to be involved in the antidiabetic activity of Byrsoninas A and B.

Byrsoninas A and B exerted effective anti-diabetic activities since they ameliorated glucose tolerance, suppressed hyperglycaemia, and restored normal liver function in diabetic mice. The antidiabetic effects of Byrsoninas A and B were apparently due to an insulinogenic action in helping regeneration of damaged pancreatic tissues and protecting pancreatic β -cells. Results also suggested reduction in glucose levels via both decreased insulin resistance and increased insulin production. Improvement in insulin resistance can account for the observed increase in the HDL cholesterol levels and the decrease in LDL cholesterol and triglyceride levels. Byrsoninas A and B may alter expressions of the pro-inflammatory cytokine biomarkers associated with diabetes. Byrsoninas A and B prevented the oxidative

damage that is caused by hyperglycemia and raised the endogenous antioxidant levels, thus protecting insulin producing islet cells against damage triggered by the oxidative stress and local inflammation associated with diabetes. However, the possibilities of other mechanisms by which Byrsonina A and B exerts effects were not excluded.

In conclusion, dimeric guaianolides sesquiterpene lactone Byrsonina A and Byrsonina B from hexane extracts of *Byrsonima crassifolia* seeds possessed antioxidant, hypoglycemic, and hypolipidemic activities and have an important role in blood glucose level in STZ induced hyperglycemia by improving the function of pancreatic islets and increasing glycolysis and decreasing gluconeogenesis. The mechanism of antidiabetic activity may involve an antioxidant effect, improvement in insulin resistance, and an effect on pancreatic β -cells to secrete insulin. The precise mechanism of action is worthy of further study.

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