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Anti-diabetic activity of an hexane extract of *Prosthechea michuacana* in streptozotocin-induced diabetic rats

[Actividad antidiabética de un extracto de hexane de *Prosthechea michuacana* en ratas diabéticas inducidas por estreptozotocina]

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Abstract

In this study we investigated the antihyperglycaemic, antihyperlipidaemic and antiglycation effects of some extracts of *Prosthechea michuacana* bulbs in normoglycemic and diabetic rats induced by streptozocin (STZ). Hexane, chloroform and methanol extracts of *P. michuacana* were screened for hypoglycemic activity, and biochemical parameters as serum triglycerides, total cholesterol, lipid peroxidation, liver glycogen, skeletal muscle glycogen levels, superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase activity in diabetic rats. Additionally we determined Glucose 6 Phosphatase and glucokinase activities in liver, inhibition of insulin and protein glycation. Glucose levels in blood plasma were determined by using GOD-POD method. Administration of chloroform and methanol extracts showed no effect on STZ induced diabetic rats (SD). On the other hand, treatment with hexane extract at 200 and 400 mg/kg doses, resulted in a reversal of diabetes and its complications. Both doses significantly brought down blood glucose concentration (35.75 and 47.78% in diabetic rats, 50.64 and 57.10% in nondiabetic rats), increased glycogenesis and decreased glyconeogenesis bringing the glucose metabolism toward normalcy. These doses also reversed the hyperlipidemia by reducing cholesterol (41.56%, 46.08%) and triglycerides (37.5%, 46.27%) and improved hepatic antioxidant enzyme activities. Its effect was compared with that of glibenclamide and tolbutamide, as reference antidiabetic drugs. The hexane extract decreased the hyperinsulinemia by 24% in SD and showed a significant change on AGEs formation *in vitro* with IC_{50} values of 48.3 µg/ml comparable to inhibitory effect of aminoguanidine with IC_{50} values of 27.2 µg/ml. It reduced HbA_{1C} levels by 6.4% in chronic STZ-diabetic rats. It is concluded that hexane extract of *Prosthechea michuacana* bulbs possesses anti-hyperglycemic and antihyperlipemic activity, improves the hyperinsulinemia and produces a significant change on AGEs formation, which may prove to be of importance in the management of diabetes and its complications.

Keywords: *Prosthechea michuacana*, antihyperglycaemic, antihyperlipidaemic, antiglycation effects.

Resumen

En este estudio se determinaron los efectos antidiabéticos, antihiperlipidemico y glicación (AGEs) de algunos extractos de *Prosthechea michuacana* (PM) en ratas normoglucémicas y con diabetes inducida por estreptozotocina (STZ). Se probó el efecto de los extractos de hexano, cloroformo, metanol de PM sobre la actividad hipoglucemiante, la carga de glucosa, los parámetros bioquímicos tales como triglicéridos, niveles de colesterol total, peroxidación lipídica, glucógeno del hígado, los niveles de glucógeno muscular, niveles de superoxide dismutase, catalasa, glutation reductasa and glutation peroxidasa en ratas normales y diabéticas. También se determinó la glucosa 6 Phosphatasa y las actividades de GK en el hígado, la inhibición de la insulina y la glicosilación de las proteínas. Los niveles de glucosa sanguínea se determinaron por el método de GOD-POD. La administración de los extractos de cloroformo y metanol no presentaron ningún efecto sobre la SD, en cambio el tratamiento con el extracto de hexano (PM) a dosis de 200 y 400 mg/kg, inhibió la diabetes y sus complicaciones. Ambas dosis redujeron significativamente los niveles de glucosa sanguínea (35.75 y 47.78% en las ratas diabéticas, 50.64 y 57.10% en las ratas diabéticas), el aumento de la glucogénesis y la disminución de la gluconeogénesis conduce el metabolismo de la glucosa hacia la normalidad. Estas dosis disminuyeron la hiperlipidemia reduciendo el colesterol (41.56%, 46.08%) y los triglicéridos (37.5%, 46.27%) así como también mejoran las actividades antioxidantes de las enzimas hepáticas. Su efecto se comparó con la glibenclamida y tolbutamida, fármacos usados como antidiabeticos. El extracto de hexano disminuyo la hiperinsulinemia en un 24% en SD. PM mostró un cambio significativo *in vitro* sobre la formación de los AGEs con valores de IC_{50} de 48.3 mg/ml comparable al efecto inhibidor de la aminoguanidina con valores de IC_{50} de 27.2 mg/ml. Se redujo en las ratas con diabetes crónica los niveles de HbA1C en un 6.4%. Por lo que se concluye que el extracto hexánico de los bulbos de *Prosthechea michuacana* posee actividad anti-hiperglucémica, antihiperlipemica, mejora la hiperinsulinemia mostrando un cambio significativo en la formación de AGEs, lo que puede llegar a ser de importancia en el tratamiento de la diabetes y sus complicaciones.

Palabras Clave: *Prosthechea michuacana*, antihiperglicaemico, antihiperlipidaemico, efecto antiglicación.

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INTRODUCTION

Diabetes is a serious metabolic disorder with micro and macro vascular complications that result in significant morbidity and mortality. The increasing number of ageing population, changes in life style such as preference for rich calories food and sedentary, obesity, have led to an increase in the number of diabetic patients world wide. A special diet and exercise are the first steps toward achieving an improvement in the condition of patients; still 90% of them are unable to maintain long-term glycemia (Modi, 2007). Thus hypoglycemic agents are necessary for the treatment of diabetes. The currently available ones, exhibit several undesirable side effects as hypoglycemia, hyponatremia, lactic acidosis and vitamin B12-malabsorption (Berger,1985) to mention just a few. Therefore, there is a need for more effective oral antihyperglycemic agents, particularly those that can normalize both insulin and glucose levels. The plant kingdom represents a largely unexplored reservoir of biologically active compounds (Valavala *et al*., 2011). A wide array of plants and their active principles, provide an alternative therapy for diabetes. Experience in traditional medicine has shown that very often this natural extracts produce minimal side effects. Diabetes is characterized by hyperglycemia that causes protein glycation and considerable pathogenesis of long-term complications; In particular, oxidative stress and advanced glycation end-products formation (AGEs) induced by hyperglycemia that are known to influence diabetic renal changes and nephropathy (Comelli *et al*., 2009). AGEs are the final products of the nonenzymatic reaction of reducing sugars and amino groups in proteins, lipoproteins and nucleic acids (Singh *et al*., 2001). They are a group of complex and heterogeneous compounds that are known as brown and fluorescent cross-linking substances such as pentoside, no fluorescent cross-linking products like methylglyoxal-lysine dimers, non-cross-linking adducts such as carboxymethyllysine and pyrraline, a pyrrole aldehyde. Recently, AGEs accumulation *in vivo* has shown to play a major role in the pathogenic process of diabetes and its complications, including neuropathy, nephropathy, retinopathy, cataract formation and in other health disorder such as atherosclerosis, Alzheimer´s disease, and normal aging (Rahbar and Figarola, 2002). Thus, the discovery and investigation of compounds with an AGEs inhibitor activity, would certainly offer a potential therapeutic approach for the prevention of diabetes and its pathogenic complications.

Prosthechea michuacana (Lex.) WE Higgins belongs to the family Orchidaceae. Mixteco and zapoteco natives of Oaxaca Mexico, give to this plant several uses: As food they collect the bulbs to eat them raw, to eliminate thirst or liquefy them with water to prepare a beverage, that is also used as anti-inflammatory, to depurate the circulatory system, and treat renal disease and diabetes. During the months of november and december, this beautiful orchid is used for religious purposes as a decorative element in the traditional "Birth" (Téllez-Velasco, 2001). We have previously reported the relaxant and antispasmodic effect of this plant in isolated guinea pig ileum (Vargas and Perez, 2009), its hepatoprotective activity, its ability to inhibit oxidative stress in liver (Perez and Vargas, 2009), its anti-inflammatory and wound healing properties (Perez and Vargas, 2009), its nephroprotective activity (Perez *et al*., 2010a) and its antioxidant activity (Perez and Neira, 2010b). The aim of the present study was to explore the utility of the orchid *P. michuacana* bulbs for correction of hyperglycemia and hyperlipidemia using a model of STZ-induced diabetic rats and non-diabetic rats.

MATERIALS AND METHODS

Plant material

Prosthechea michuacana (Lex.) WE Higgins belong to the family Orchidaceae, bulbs were collected in the Mexican state of Oaxaca. Plants were taxonomically authenticated in the Instituto Politécnico Nacional and a voucher specimen of the plant is stored for reference (Nº 6478) in the Herbarium of the Metropolitan autonomous university campus Xochimilco (UAM) for further reference. 300 g of the aerial parts and bulbs were dried and powdered in a mechanical grinder. The powdered material was extracted with 900 ml of hexane, chloroform and methanol consecutively using a soxhlet apparatus. These extracts were filtered and concentrated in a rotary vacuum evaporator and kept in a vacuum desiccator for complete removal of solvent. An aqueous suspension of each extract was prepared using 2% (v/v) Tween-80 and used for administration.

Animals

The study was conducted in male Wistar albino rats, weighing about 180 - 225 g procured by the UAM bioterium. Before and during the experiment, animals

were fed with normal laboratory diet (Mouse Chow 5015) and water ad libitium. Animals were kept in microlon boxes and were acclimatized for a period of 3 days in the new environment (temperature 25 ± 2º C) before initiation of experiment. The litter in their cages was renewed three times a week to ensure hygiene and maximum comfort for the animals. Ethical clearance for handling animals (NIH publication No. 85-23 revised 1985) was observed.

Acute toxicity studies

Healthy adult Wistar albino rats weighing between 180 - 230g of either sex, fasted overnight were divided into four groups (n=6). They were orally fed with the aqueous extract of *P. michuacana* at increasing doses of 1000, 2000, 3000, mg/kg body weight (Ghosh, 1984). The animals were observed continuously for 2 h for behavioral, neurological and autonomic profiles and later after a period of 24 and 72 h for any lethality (Sarkhail *et al*., 2010).

Streptozotocin (STZ)-induced diabetic model

Severe diabetes was induced in over night fasted male rats by a single intraperitoneal injection of streptozotocin, with a dose of 65 mg/kg body weight dissolved in cold citrate buffer (Nirmala *et al*., 2011). Diabetes was confirmed in the STZ-treated rats by measuring the fasting blood glucose concentration 1 day after injecting the STZ. Rats with permanent high fasting blood glucose level > 250 mg/dl were included for the panel of tests of our experimental design.

Effects of a single dose of the hexane, chloroform and methanol extracts in normal and diabetic rats

In the experiment a total 72 rats (36 diabetic rats and 36 non-diabetic rats) were used. The rats were divided into twelve groups with six animals in each group. The treatment was scheduled as follows:

- Group I: non-diabetic control received only 2% Tween-80 Solution
- Group II: non-diabetic rats treated with *P. Michuacana* extract 100 mg/kg body weight
- Group III: non-diabetic rats treated with *P. Michuacana* extract 200 mg/kg body weight
- Group IV: non-diabetic rats treated with *P. Michuacana* extract 400 mg/kg body weight.
- Group V: non-diabetic rats treated with tolbutamide at 40 mg/kg body weight.
- Group VI: Diabetic control received only 2% Tween-80 Solution.
- Group VII: Diabetic rats treated with *P. Michuacana* extract 100 mg/kg body weight
- Group VIII: Diabetic rats treated with *P. Michuacana* extract 200 mg/kg body weight.
- Group IX: Diabetic rats treated with *P. Michuacana* extract 400 mg/kg body weight.
- Group X: Diabetic rats treated with glibenclamide at 0.5 mg/kg body weight.
- Group XI: Diabetic rats treated with tolbutamide at 40 mg/kg body weight.

Drugs solutions or vehicle were administered orally by gastric intubations daily at 9:00 am for a period of 28 days. Blood glucose levels were determined in fasting animals before initiation of the experiment and from then, every third day. On the 28th day, animals were sacrificed by an ether over-dose. Different biochemical parameters like serum triglycerides and total cholesterol, liver and skeletal muscle glycogen content (Kiyici *et al*., 2010) were essayed in the groups.

Estimation of oral glucose tolerance test

Rats of each group were orally administered 400 mg/kg of extracts for 28 days. At the end of the period, the oral glucose tolerance test (OGTT) was performed to assess the sensitivity to a high glucose load. For this purpose, overnight-fasted rats were fed orally 2 g glucose/kg b.w. Blood samples were collected from the caudal vein through a small incision at the end of the tail at 0 min (immediately after glucose load), 30, 60, 90 and 120 min after glucose administration.

Measurement of glucose, total cholesterol, triglyceride and lipid peroxidation in serum

Rats were fasted for 18 h and blood samples were immediately collected by puncture of retro-orbital plexus with a capillary tube under ether anesthesia into glass vials containing potassium oxalate and sodium fluoride as anticoagulant at 0 h (before treatment) and 2, 4, 6, 8, 12 h after treatment. Plasma glucose levels were determined by using GOD-POD method. This colorimetric method is based on the enzymatic reactions between invertase and glucose oxidase-peroxidase (GOD-POD) (Sang-Eun *et al*., 2007).

The content of triglycerides and total cholesterol levels in serum were estimated with the respective Diagnostic Reagent Kit by Genzyme Diagnostics. The cholesterol kit is based on the cholesterol oxidase-DAOS method, wheatear the triglycerides kit is based on the GPO-*p*-chlorophenol method. The total lipids level were measured as a thiobarbituric acid (TBARS) by a LPO test (Wako

Pure Chemical Industries Ltd.), based on 3-α-hydroxysteroid dehydrogenase (Fraga *et al*., 1988).

Liver and skeletal muscle glycogen content and G6Pase, GK activities in liver

Rats of each group were administered orally 400 mg/kg of extracts for a period of 28 days, after that period of time, animals were sacrificed and livers were extracted. The glycogen content of liver and skeletal muscles was determined by using anthrone reagent (Davis and Granner, 1996). 100 mg of tissue was centrifuged in a tube containing 1 ml of KOH at 30%. The tube was kept 20 minutes in a boiling water bath, the digest was cooled, and 1.25 ml of 95% ethanol was added. The tube was boiled in a hot water bath and cooled again before a centrifugation of 15 min at 3000 rpm. The supernatant was decanted and the residue was redissolved in 1 ml of distilled water and treated as before until decanted again. Sedimented glycogen was dissolved exactly in 5 ml of water. The tube was submerged in ice-cold water and 10 ml of anthrone was added, all was mixed by swirling the tubes. The cold tubes were covered with glass marbles and heated for 10 min in a boiling water bath. Once cooled, the content was measured in a spectrophotometer at 620 nm. Results are expressed in mg/g of tissue. A standard curve of glycogen type III from rabbit liver (Sigma) was simultaneously analyzed to determine the final liver glycogen concentrations.

The hepatic G6Pase activity was assayed by the method of Baginski *et al*., (1969). In brief, the glucose-6-phosphate in the liver extract was converted into glucose and inorganic phosphate. The inorganic phosphate liberated was determined with ammonium molybdate; ascorbic acid was used as reducing agent. Any molybdate excess was removed by the arsenite-citrate reagent, so it could no longer react with other phosphate esters or with inorganic phosphate formed by the acid hydrolysis of the substrate. The amount of liberated phosphate per unit time, determined as the blue phosphor-molybdous complex at 700 nm, is a measure of the glucose-6-phosphatase activity. The glucose-6-phosphatase activity (mU) was expressed as mmol of phosphate released/min/ mg of protein. The standard curve was elaborated with a standard solution of inorganic phosphate in water. The protein content of the liver extract was quantified with Bradford reagent (Bradford, 1976).

GK (glucokinase) activity was measured by spectrophotometry as previously described by Panserat *et al*., (2001). Briefly, liver tissues were homogenized and centrifuged, the supernatant was supplemented with 1 mM NADP, 5 mM ATP, and 100 mM or 0.5 mM glucose at pH 7.5. The enzymatic reaction was started by the addition of 0.2 units of glucose-6-phosphate dehydrogenase (E.C.l.l.l.49) and incubated for 5 min at 37º C. NADPH generated by GK was measured using a spectrophotometer at 340 nm. GK activity was estimated by the standard method, i.e. subtracting the rate of NADPH formation in the presence of 0.5 mM glucose from that obtained in the presence of 100 mM glucose. Gk activity was estimated as the difference in activity when samples were assayed at 100 mmol/l (GK plus hexokinase activity) and 0.5 mmol/l glucose (hexokinase activity). Protein concentration was quantified with Bradford reagent (1976) and one unit of enzyme activity (mU) was defined as mmol of substrate molecules converted by 1 mg protein per minute.

Measurements of reduced GSH, SOD, CAT and GPx

The livers were homogenized with Ultra Turrax T25 homogenizer (Rose Scientific Ltd., Edmonton, Canada) in 10 volumes of a 50 mM sodium phosphate buffer (PH 7.4) at 4º C. Homogenates were centrifuged (Beckman, USA) at 15000Xg for 10 min, and the supernatant obtained was used for antioxidant enzyme measurements. Antioxidant enzyme activities in the liver were assayed using commercial kits for superoxide dismutase (SOD) (Bioxytech kit), SOD-525 for SOD activity (Oxis International), catalase (CAT) assay kit (Cayman Chemical), glutathione peroxidase assay kit Bioxytech GPx-340 (Oxis International), and glutathione reductase assay kit Bioxytech GR-340 (Oxis International).

Determination of insulin

Serum and pancreatic insulin was measured by a Glazyme Insulin-EIA Test (Zamami *et al*., 2008). 400 mg/kg of hexane and chloroform *P. michuacana* bulbs extracts were orally administered to the rats for three weeks, after that time, blood samples were taken for insulin determination. Insulin concentration in serum is expressed in µIU/ml. The pancreas was rapidly excised, and homogenized in acidified ethanol. Samples were incubated at 4º C for 48 h with continuous shaking before the insulin test.

Formation of AGE in the BSA/glucose

P. michuacana extracts were evaluated for their potential glycation inhibitory activity, we performed this evaluation as previously described (Kim and Kim, 2003). Bovine serum albumin (Sigma, USA)

(10mg/mL) was incubated with 250 mM D-fructose in the presence or absence of test materials for 21 days in 0.2 M potassium phosphate buffer (pH 7.4) at 37º C. After incubation, the fluorescence was measured at an excitation wavelenght of 370 nm and an emission wavelenght of 440 nm. Both incubations and measurements were carried out by triplicate. Aminoguanidine (Sigma, USA) (AG) dissolved in distilled water was used as a positive control. The concentration of each test sample giving 50% inhibition of the activities (IC_{50}) was estimated from the least-squares regression line of the logarithmic concentration plotted against the remaining activity.

Statistical analysis

Statistical calculations were done using one-way analysis of variance (ANOVA). Individual differences among groups were anayzed by Dunnett´s test using SPSS software. P values less than 0.05 were considered as statistically significant. Data a expressed as mean ± SEM for 6 rats in each group.

RESULTS

Toxicity evaluation

The extracts did not show any mortality up to a dose of 3 g/kg body weight or any toxic reactions were found at any doses selected until the end of the study period.

Effects of hexane, chloroform and methanol extracts on diabetic and nondiabetic rats

Table 1 shows the level of blood glucose in STZ induced diabetic rats and no-diabetic rats at different time intervals. There was a significant increase in blood glucose level ($p < 0.01$) in diabetic rats after 24 h of the STZ injection compared to the control group. Treatment with hexane extract of PM at 100, 200 and 400 mg/kg, tolbutamide 40 mg/kg and glibenclamide 0.5 mg/kg significantly decreased blood glucose in diabetic rats. However, chloroform and methanol extracts have no effect on blood glucose levels. The percentage blood glucose reduction with 200 and 400 mg/kg doses at 6 h was 35.75% and 47.78% respectively. Tolbutamide 40 mg/kg and gibenclamide 0.5 mg/kg produced 46.74% and 39.82% respectively of blood glucose reduction after 6 h. Changes in the plasma glucose concentration are shown in Table 1.

Maximum percentage blood glucose reduction to normoglycemic rats at 6 h with 400 mg/kg doses of hexane extract of *P. michuacana* was 57.10%. Tolbutamide 40 mg/kg dose produced 43.47% blood glucose reduction at 6 h in nondiabetic rats (Table 1). However, chloroform and methanol extracts to the same doses did not produced hypoglycemic activity.

Oral Glucose Tolerance Test

The postprandial blood glucose levels in rats showed a significant change after glucose loading. Oral administration of a glucose load (2 g/kg) rapidly increased blood glucose levels in all groups of diabetic rats within 30 min and remained high over the next 120 min in diabetic control rats (Table 2), and at the end of the period, it did not come back to the initial value (0 min level). In both groups treated with the hexane extract, blood glucose level decreased significantly and after 60 minutes, this parameter was resettled to control level.

Serum lipid profile

Serum triglycerides, total cholesterol and TBARS levels were highly elevated in liver and kidney of diabetic rats while LDL-cholesterol was decreased (Table 3). There were also noticeable changes in serum lipids levels in the diabetic treated rats when compared with the untreated diabetic group. Daily administration of the hexane extract to diabetic and non-diabetic rats at a dose of 400 mg/kg for 28 days significantly reduced serum triglycerides and total cholesterol by 51% and 46% respectively. TBARS level in diabetic rats liver and kidney decreased upon administration of the hexane extract by 27.5% and 25% in non-diabetic rats. Decreased LDL-cholesterol observed in diabetic animals was found to increase upon treatment.

Table 1
Effect of hexane extract of bulbs of *Prosthechea michuacana (*PM) on fasting blood glucose level in diabetic rats

Group	Dose (mg/kg)	Blood glucose levels (mg/dl) at different time intervals (hours)					
		At the time of grouping	2 h	4 h	6 h	8 h	12 h
NDC	----	103.41 ± 0.91[a]	102.30 ± 0.95	100.21 ± 1.38	101.67 ± 0.98	99.65 ± 1.75	99.59 ± 0.85
ND + PM	100	102.24 ± 0.97[a]	95.98 ± 4.83[b]	88.23 ± 2.57[b]	81.31 ± 2.54[a,]	86.24 ± 3.35[a]	89.53 ± 1.65[a]
	200	101.52 ± 2.02[a]	86.59 ± 5.04[a,c]	54.26 ± 1.35[a,c]	50.11 ± 1.13[a,b]	54.44 ± 2.06[a,c]	63.17 ± 0.84[b]
	400	105.10 ± 0.89[a]	82.25 ± 5.12[a,c]	48.97 ± 1.46[a,c]	45.08 ± 0.87[b,c]	51.03 ± 2.46[a,c]	62.94 ± 0.48[b]
ND + TB	40	99.34 ± 1.69[b]	76.12 ± 3.47[a,c]	58.34 ± 1.06[a,c]	55.23 ± 1.65[a,b]	58.10 ± 2.44[a,c]	70.10 ± 1.23[b,c]
DC	--	362.51 ± 1.89	349.47 ± 2.23[a]	358.08 ± 3.76[b]	339.32 ± 5.16	351.91 ± 2.32	341.56 ± 4.51
D + PM	100	345.43 ± 2.32[a]	334.21 ± 3.16[a]	322.41 ± 3.30[a]	313.46 ± 3.76[a]	316.43 ± 2.48[a]	320.43 ± 3.12[a]
	200	358.19 ± 3.61[a]	320.21 ± 5.25[b]	263.20 ± 1.80[c]	230.08 ± 4.35[b]	237.14 ± 3.70[c]	251.31 ± 3.97[b]
	400	362.17 ± 3.80[a]	300.95 ± 3.64[b]	210.71 ± 4.04[a]	189.12 ± 5.21[c]	202.19 ± 1.79[a]	227.36 ± 2.78[b]
D + TB	40	348.43 ± 4.07[a]	275.32 ± 6.80[c]	213.23 ± 4.61[a]	185.60 ± 1.78[c]	209.50 ± 2.57[a]	244.27± 2.50[b]
D + GB	0.5	336.62 ± 6.14[a]	269. 51 ± 7.20[c]	194.48 ± 1.61[a]	202.6 ± 2.38[c]	214.43± 2.12[a]	259.22 ± 2.43[b]

Each values represent Mean ± SD (n = 6), ANOVA followed by multiple two tail "*t*" test. In each column, mean with different superscripts (a, b, c) differ from each other significantly, $p > 0.05$. Those with the same letter are not significantly different at $p > 0.05$. Nomenclature of table: NDC: Non-diabetic Control; ND (Non-diabetic) + PM (Hexane extract from P. michuacana); ND + TB (Tolbutamide); DC: Diabetic Control; D + PM: Diabetic + PM; D (Diabetic) + TB: D + TB; D + GB (Gibenclamide).

Table 2
Effect of hexane extract of bulbs of *Prosthechea michuacana* on oral glucose tolerance test in diabetic rats

Groups	Blood glucose levels (mg/dl)				
	At the time of grouping	30 min	60 min	90 min	120 min
No-diabetic	90.34 ± 1.65[b]	178.53 ± 2.06[a]	159.16 ± 1.47[a]	126.79 ± 1.76[a]	96.75 ± 0.96[a]
Diabetic control	289.61 ± 3.48[c]	365.08 ± 1.37[b]	421.23 ± 4.63[c]	394.52 ± 4.83[c]	360.27 ± 2.72[c]
Diabetic + PM 200	239.29 ± 2.74[a]	301.47 ± 1.68[c]	246.52 ± 3.63[a]	189.31 ± 2.20[a]	142.39 ± 1.26[a]
Diabetic + PM 400	221.07 ± 3.14[a]	299.86 ± 2.31[b]	217.58 ± 3.54[c]	167.73 ± 2.86[c]	130.29 ± 1.47[c]

Values are mean ± SD (n = 6). ANOVA followed by multiple two tail "*t*" test. Line with (a, b, c) within fixed duration of measurement differ from each other significantly, $p > 0.05$.

Glycogen level, GK and Glucose 6-phosphatase activities

Glycogen levels were significantly decreased in STZ induced diabetic rats compared to control group. After a 28 days period of administration of *P. michuacana* hexane extract there was a considerable elevation in the level of glycogen in liver and skeletal muscle compared to diabetic control rats and these parameters were resettled toward those of the control group (Table 3).

G6Pase and GK activities were increased in STZ induced diabetic rats compared to control (Table 4). After the same 28 days treatment with the hexane extract, there was a significant diminution in these parameters by 55.0% and 52% respectively, and these parameters resettled toward those of the control group as well.

Table 3
Effect hexane extract bulbs of *Prosthechea michuacana (*PM) on lipid profile in STZ-induced diabetic rats

Group (mg/kg)	Mean Concentration (mg/g) ± SEM Triglycerides (mg/dl)	Total cholesterol (mg/dl)	HDL-cholesterol (mg/dl)	TBARS (µM/g) Liver	Kidney
Normal	90.21 ± 3.43	128.71 ± 4.76	68.43 ± 1.75	0.98 ± 0.080	1.5 ± 0.096
Diabetic control	185.64 ± 5.11[a]	245.09 ± 2.84 [a]	34.61 ± 1.30 [a]	1.60 ± 0.042 [a]	2.3 ± 0.043 [a]
PM 200	129.56 ± 2.43[ab]	153.21 ± 3.48[ab]	50.18 ± 2.41[ab]	1.40 ± 0.024[ab]	1.9 ± 0.017 [ab]
PM 400	90.34 ± 6.21[ab]	132.15 ± 3.96[ab]	56.37 ± 1.84[ab]	1.00 ± 0.056[ab]	1.6 ± 0.045 [ab]

All values are expressed as Mean ± SEM, n = 6 Values. [a]P < 0.05 when compared to normal control group, [b]P < 0.01when compared to diabetic control group, where the significance was performed by Oneway ANOVA followed by post hoc Dunnett´s test.

Table 4
Effect of hexane extract of Prosthechea *michuacana (*PM) on hepatic glucose regulation enzyme activities profile and glycogen content of liver and skeletal muscle in STZ induced diabetic rats

Treatment (mg/kg)	Glucose 6-phosphatase activity (mU)	Glucokinase activity (mU)	Mean Concentration (mg/g) ± SEM Liver glycogen	Skeletal muscle
Normal control	0.38 ± 0.18	3.23 ± 0.08	18.56 ± 0.26	12.24 ± 0.32
Diabetic Control	0.69 ± 0.69[a]	1.19 ± 2.04[a]	8.24 ± 1.37[a]	3.98 ± 3.25 [a]
Diabetic+PM 200	0.41 ± 0.38[b]	1.94 ± 2.03[b]	14.83 ± 0.25 [b]	9.35 ± 0.57 [b]
Diabetic+PM 400	0.33 ± 0.74[b]	2.91 ± 1.71[b]	16.98 ± 0.47 [b]	11.27 ± 0.38 [b]
Glibenclamide (4 mg/kg)	0.36 ± 0.49[b]	3.10 ± 0.51	17.67 ± 0.29 [b]	12.01 ± 0.41 [b]

Each values represent Mean ± SD, (n = 6); ANOVA followed by multiple two tail "*t*" test. In each vertical column, mean with different superscripts (a, b) differ from each other.All values are expressed as Mean ± SD, n = 6 Values. Significant difference of diabetic control from normal control [a]P < 0.001.Significant difference of treated groups from diabetic control [b]P < 0.01, [c]P < 0.05. [d]P < 0.01 when compared with glibenclamide 4 mg/kg treated group.

Effect of P. michuacana hexane extract on SOD, CAT, GSH and GPx in hepatic and renal tissues

The antioxidant effect of the extract on tissue antioxidant markers was studied (Table 5). The diabetic rats showed an important reduction in SOD, CAT, GSH and GPx in hepatic and renal tissues. The decreased levels of SOD, CAT, GSH and GPx in diabetic rats were found to be reverted to near normal levels after treatment with bulbs extract. The levels of these enzymes quantified in treated groups were comparable to those attained with the standard drug glibenclamide.

Administration of *P. michuacana* to diabetic rats showed a significant effect, and restored the activities of liver and kidney regarding these parameters.

Table 5
Effect hexane extract bulbs of *Prosthechea michuacana (*PM) on antioxidant enzyme activities in liver and kidney in diabetic rats

Parameters	Normal Control	Diabetic control	Diabetic+PM	Diabetic+PM	Diabetic+ GB
			(200 mg/kg)	(400 mg/kg)	(5 mg/kg)
SOD-Liver	7.12 ± 3.42	3.76 ± 0.25[a]	5.87 ± 1.06[c]	6.59 ± 1.65[b]	6.78 ± 0.21[c]
SOD-Kidney	13.97 ± 2.65	7.02 ± 1.86[a]	9.78 ± 0.65[b]	12.45 ± 1.67[b]	12.99 ± 0.16[b]
CAT-Liver	81.25 ± 4.09	44.36 ± 1.34[a]	59.85 ± 0.96[c]	69.87 ± 5.17[b]	70.09 ± 0.78[b]
CAT-Kidney	35.21 ± 3.77	20.10 ± 0.95[a]	30.04 ± 0.78[a]	33.87 ± 3.21[c]	34.12 ± 1.44[b]
GSH –Liver	46.31 ± 1.16	22.35 ± 1.92[a]	35.41 ± 1.83[b]	42.05 ± 1.67[b]	41.38 ± 2.65[b]
GSH –Kidney	23.62 ± 2.31	5.53 ± 0.79[a]	15.61 ± 0.89[b]	19.15 ± 2.43[b]	19.46 ± 0.83[b]
GPx –Liver	7.56 ± 3.26	4.68 ± 0.87[a]	5.25 ± 1.38[c]	5.67 ± 4.30[b]	5.89 ± 1.52[b]
GPx –Kidney	5.89 ± 1.32	3.49 ± 1.36[a]	4.11 ± 0.43[b]	4.67 ± 2.36[b]	4.53 ± 2.16[b]

All values are expressed as Mean ± SEM, n = 6 Values. [a]P < 0.01 when compared to normal control group; [b]P < 0.01; [c]P < 0.05 compared to diabetic control group; where the significance was performed by Oneway ANOVA followed by post hoc Dunnett´s test. Glibenclamide (GB). The values are given in U/mg of protein

Effect of P. michuacana on serum insulin level and pancreatic insulin content

Serum insulin level and pancreatic insulin content were significantly decreased in STZ-induced diabetic rats compared to control. After three weeks of administration of the hexane extract of *P. michuacana* there was a noticeable elevation in serum and pancreatic insulin level. This is shown in Table 6.

Table 6
Effect of hexane extract from seeds of *Prosthechea michuacana (*PM) on plasma insulin level and pancreatic insulin content in diabetic rats

Groups	Plasma insulin	Pancreatic insulin
	(µU/ml)	(mU/g protein)
Nodiabetic control	3.52 ± 0.42	25.12 ± 1.65
Diabetic control	1.20 ± 0.31[a]	[d]14.53 ± 3.65 [a]
PM 200	2.60 ± 0.47[c]	[d]20.42 ± 2.78 [b]
PM 400	3.20 ± 0.31[b]	[d]24.15 ± 1.98[b]
Glibenclamide 4 mg/kg	3.38 ± 0.19 [b]	15.03 ± 3.62[b]

All values are expressed as Mean ± SD, n = 6 Values. Significant difference of diabetic control from normal control [a]P < 0.001. Significant difference of treated groups from diabetic control [b]P < 0.01, [c]P < 0.05. [d]P < 0.01when compared with glibenclamide 4 mg/kg treated group.

Effect of PM on AGEs and hemoglobin lycosylation

Table 7 shows the effect of the hexane extract on hemoglobin glycosylation and inhibition of AGEs. The BSA-glucose *in vitro* model adopted in this study provides a useful tool for assessing the effect of extracts on nonoenzymatic glycation process. Hexane extract of *P. michuacana* bulbs, reduced AGEs formation with IC_{50} values of 48.3 µg/ml comparable to the inhibitory effect of aminoguanidine with IC_{50} values of 27.2 µg/ml. Aminoguanidine is an AGE inhibitor tested in clinical trials for the treatment of diabetic complications. The concentration of carbohydrate bound to hemoglobin was increased in the diabetic group. However, chronic treatment with *P. michuacana* extract caused a significant ($p < 0.05$) reduction in the absorbance of 5-hydraxyrnethyl furfural (5-HMF) generated from hemoglobin-bound sugar in diabetic rats.

Table 7
Effect of hexane extract of bulbs of *Prosthechea michuacana (PM)* on plasma levels of HbA_{1c} in chronic diabetic rats

Treatment	Baseline (%)	HbA_{1c} (%)
No-diabetic	3.8	3.9
Diabetic Control	4.1[a]	8.5 [a]
Diabetic + insulin	4.3 [b]	7.1 [b]
Diabetic+ PM (400 mg/kg)	4.2 [b]	[b]6.9 [b]
Glibenclamide 4 mg/kg	4.4 [b]	7.3 [b]

All values are expressed as Mean ± SD, n = 6 Values.
Significant difference of diabetic control from normal control [a]$P < 0.001$.
Significant difference of treated groups from diabetic control [b]$P < 0.01$, [c]$P < 0.05$. [d]$P < 0.01$ when compared with glibenclamide 4 mg/kg treated group.

DISCUSSION AND CONCLUSION

In the present study we wanted to test the efficacy of an hexane extract of bulbs of *P. michuacana* in correcting physiological disorders caused by STZ-induced diabetes in rats, where β-cell degeneration is dramatic (diabetes type 1). Diabetes is associated with hyperlipidemia, here we show the antihyperlipidemic efficacy and potency of the extracts in STZ-induced diabetic rats evidenced by the decrease of total cholesterol, and triglycerides levels, after the extract administration. Furthermore, the attenuating effect of this extract on experimental STZ-induced diabetes has been confirmed here by the study of glucose-6-phosphatase activity in liver, as well as the quantification of glycogen in liver and skeletal muscle, which are important indicators of diabetes mellitus (Boby *et al*., 2003). These effects may be due to low activity of cholesterol biosynthesis enzymes and/ or low level of lipolysis which are under the control of insulin (Huang *et al*., 2000).

The possible mechanism of antihyperglycemic action of this extract appears to be both pancreatic and extra pancreatic, which have been supported here by the serum insulin assay in STZ-induced diabetic rats. The extra pancreatic effect of this extract was evaluated by the significant recovery of glucose-6-phosphatase activity in liver in STZ-induced diabetic rats. The extra pancreatic effect may be due to the sensitization of insulin receptor in target organ or by inhibition of insulinase activity in both liver and kidney. From the glucose tolerance test we have learned that the hexane extract did not execute the antihyperglycemic effect by modulating the absorption of glucose in the intestine.

Hyperglycemia induced oxidative stress may also cause liver cell damage. The administration of extracts improves impairments of SOD, GSH, GSSG and CAT activities in the STZ-induced diabetic group. These results suggest that the hexane extract prevents oxidative stress, acting as a suppressor against liver cell damage and inhibiting the progression of liver dysfunction induced by chronic hyperglycemia (Narvaez-Mastache *et al*., 2007).

Chronic hyperglycemia leads to the auto-oxidation of glucose and causes nonenzymatic glycation of proteins through Maillard´s reaction (Goh and Cooper, 2008). In these processes, reactive oxygen species are produced. To avoid oxidative stress,

antioxidant enzymes, such as catalase, superoxide dismutase, and glutathione peroxidase, play an important role against oxidative stress. However, hyperglycemia also causes nonenzymatic glycation of these antioxidant enzymes (Jagtap and Patil, 2010). Furthermore, AGE formation, one of the metabolic disorders caused by hyperglycemia, has been focused on as a marker of long-term glycemic control in body tissues. Our study found that hexane extract of *P. michuacana* could inhibit the formation of AGEs, which have been implicated in the pathogenic process of diabetic complications. The inhibition of AGEs formation was comparable to that obtained by using the standard antiglycation agent aminoguanidine. The glycosylated serum hemoglobin of diabetic control rats was significantly increased relative to the control levels. Treatment of *P. michuacana* decreased formation of glycated hemoglobin. Therefore, we considered that *P. michuacana*, might have influence on AGEs formation. The possible mechanisms of this must be clarified in future studies.

CONCLUSION

Our study demonstrated that *P. michuacana* exhibited hypoglycemic activity through improving hyperlipidemia, insulin resistance, and improving the antioxidant defense system. Our findings strongly suggest that the inhibition of oxidative stress and AGE formation would be also very helpful preventing diabetes complications. Therefore, *P. michuacana* can be considered as a plant with an enormous potential as an anti-diabetic agent; however the investigation of its major active constituents is still in progress.

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