Effect of the hexane extract of *Piper auritum* on insulin release from β-cell and oxidative stress in streptozotocin-induced diabetic rat

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ABSTRACT

Background: The large-leafed perennial plant *Piper auritum* known as Hoja Santa, is used for its leaves that because of their spicy aromatic scent and flavor have an important presence in Mexican cuisine, and in many regions, this plant is known for its therapeutic properties. **Materials and Methods:** In the present study, we investigated the effect of hexane, chloroform and methanol extracts from *Piper auritum* on cell culture system and the effect in streptozotocin-induced type 1 diabetic rats treated by 28 days on the physiological, metabolic parameters and oxidative stress. **Results:** The hexane extract of *P. auritum* (HS) treatment significantly reduced the intake of both food, water and body weight loss as well as levels of blood glucose, serum cholesterol, triglycerides and increase HDL-cholesterol. After 4-week administration of HS antioxidant enzyme as SOD, CAT, GSH, GPx in pancreas were determined. These enzyme increased significantly compared with those of the diabetic rats control and normal animals. For all estimated, the results of HS treated groups leading to a restoration of the defense mechanism. The treatment also improves pancreatic TBARS–reactive substance level and serum NO and iNOS. To determine the insulin releasing activity, after extract treatment the serum and pancreatic sections were processed for examination of insulin-releasing activity using an immunocytochemistry kit. The results showed that administration of the hexane extract (200 and 400 mg/kg) exhibited a significant increase in serum and pancreas tissue insulin. Administration of streptozotocin decreased the insulin secretory activity in comparison with intact rats, but treatment with the HS extract increased significantly the activity of the beta cells in comparison with the diabetic control rats. The extract decreased serum glucose in streptozotocin-induced diabetic rats and increased insulin release from the beta cells of the pancreas. In cultured RIN-5F cells, we examined whether hexane extract of *P. auritum* would protect the pancreas-derived β-cells from oxidative stress. Moreover, HS could protect pancreatic β-cells from advanced glycation end products-induced oxidative stress. **Conclusion:** From these results, HS is suggested to show anti-diabetic effect by stimulating insulin-dependent and by protecting pancreatic β-cells from advanced glycation end products-induced oxidative stress.

Key words: Antioxidant enzyme, glycation, piper auritum, pancreatic β-cells

INTRODUCTION

Diabetes is a serious metabolic disorder with micro and macro vascular complications resulting in significant morbidity and mortality. Diabetes is characterized by hyperglycemia, abnormal lipid and protein metabolism, along with specific long-term complications affecting the retina, the kidney and the nervous system mainly.[1] Oxidative stress and advanced glycation endproducts (AGEs) formation induced by hyperglycemia are known to influence diabetic renal changes and nephropathy.[2] The number of people with diabetes is increasing worldwide due to population growth, aging, urbanization, increasing prevalence of obesity, calorie rich diet and physical inactivity. Current treatments, although provide a good glycemic control do little preventing complications.[3] Besides, most of the prescribed hypoglycemic drugs or insulin are associated with unwanted side effects. Because of this there is an increasing demand to research for natural products with antidiabetic

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activity.[4] Herbal medicines are an option because of their comparably therapeutic effects and nontoxic side effects.[5]

The large-leafed perennial plant *Piper auritum* known by the name of Hoja Santa, is used for their spicy aromatic scent and flavor have an important presence in Mexican cuisine, this plant is known for its therapeutic properties. It has been used traditionally as an emollient, antirheumatic, diuretic, stimulant and abortifacient, anti-inflammatory, antibacterial, antifungal and antidermatophytic.[6] Phytochemicals, the main components of the essential oil of leaves of *P. auritum* are safrol, and myristycin[6] and it has been reported to possess pharmacological properties like antioxidant.[7] A search of the literature revealed that no studies of the potential hypoglycemic effects of this plant have ever made. The aim of this investigation was to test the effect of *P. auritum* on hypoglycemic, oxidative stress, insulin level in diabetic rodent model and AGEs formation in pancreatic β-cells in culture.

MATERIALS AND METHODS

Plant material

Fresh plants of *Piper auritum* were collected in Mexico State. A voucher specimen (No. 7345) was deposited in the Herbarium of the UAM-Xochimilco, for further reference.

Animals

Study was conducted in male Wistar rats, provided by the bioterium of the National School of Biological Sciences. Rats weighted from 180 to 225 g. Animals were housed in microlon boxes in a controlled environment (temperature 25 ± 2°C) with standard laboratory diet (Mouse Chow 5015, Purina) and water *ad libitum*. Litter was renewed three times a week to ensure hygiene and maximum comfort for animals. Rats were acclimatized for a period of three days in their new environment before the initiation of the experimental procedures. Ethical clearance for handling animals (NIH publication No. 85-23 revised 1985) was observed at all times.

Preparation of plant extracts

A total of 300 g of the aerial parts of *P. auritum* were dried and powdered in a mechanical grinder. The grinded material was extracted with 900 ml of hexane, chloroform and methanol consecutively using a soxhlet apparatus. These extracts were filtered and concentrated by a rotary vacuum evaporator and kept in a vacuum desiccator for complete removal of solvent. An aqueous suspension was prepared using 2% (v/v) Tween-80 and then used for oral administration.

Induction of experimental diabetes

Severe diabetes mellitus was induced in overnight fasted male rats by a single intraperitoneal injection of streptozotocin, at a dose of 50 mg/kg body weight dissolved in cold citrate buffer (pH 4.5).[8] Hyperglycemia was confirmed by measuring glucose 72h after the streptozotocin shot and 7 days after injection, confirming a high glucose level. Rats with permanent high fasting blood glucose level > 300 mg/dl were included for the experiments.

Experimental design

In the experiment a total 40 rats for each extract were divided into four groups (n =10 per group): 1 normal control, 2 diabetes control, groups 3 to 4 correspond to diabetic rats treated with *P. auritum* aqueous reconstituted solution from organic solvent extract at dose of 200 and 400 mg/kg body weight on a daily basis for 28 days. All the drugs solutions or vehicle were administered orally by gastric intubations once daily at 9:00 am for 28 days. At the end of the experiment rats were fasted overnight and were euthanized by anesthesia. Each pancreas was quickly removed from the sacrificed rat, placed in ice cold saline solution, trimmed of adipose tissue and weighed. Part of each pancreas was finely minced and homogenized in 50 mM phosphate buffer, pH 7.4 and centrifuged at 2000 rpm for 10min at 4° C. The supernatant was used for all the assays.

Body weight

Body weights of rats and the intake of food and water and were taken prior to the induction of hyperglycemia, at day 0 of *Piper auritum* treatment, and on a daily basis thereafter, for 4 weeks.

Serum lipid profile and glucose levels

The serum determination of total cholesterol (TC), triglycerides (TG) and HDL-cholesterol, using a commercial Diagnostic Kit (Genzyme Diagnostics, MA. USA). Blood glucose levels were measured employing the glucose oxidase-peroxidase (GOD-POD) method.[9]

Termination of treatment

In each treatment group, animals were killed at 28 days of the completion of treatment under pentobarbital (60 mg/ kg bw). Laparotomy was performed and the pancreas was excised, trimmed free of fat, rinsed in PBS. Portions of the pancreas were also stored at 80 ºC for analysis of oxidative stress markers.

Lipid peroxidation and oxidative stress markers

The tissue homogenate was used for the determination of lipid peroxidation and antioxidant enzyme. Pancreas homogenate was obtained by centrifugation (3000Xg) in 50 mM phosphate buffer, pH 7.4 at 4ºC according to the method of Johnson and Lardy.[10] The concentration of TBA-reactive substance was determined using

malonaldehyde level by Oxis international, CA, USA, and expressed as mol/mg of protein.

Antioxidant enzyme activities in the pancreas were assayed using commercial kits: superoxide dismutase (SOD) assay kit Bioxytech SOD-525 for SOD activity (Oxis International, CA, USA), catalase assay kit for catalase activity (CAT) (Cayman Chemical), and glutathione reductase (GSH**)** assay kit Bioxytech GR-340 for GR activity, (Oxis International) and glutathione peroxidase (GPx**)** assay kit GPx-340 for GPx (Oxis International).

Determination of insulin

Serum insulin was measured by enzyme linked inmunosorbent assay (ELISA) using the kit (Boehringer Mannheim Diagnostic, Mannheim, Germany). The intra assay variation was 4.9%. As the samples were run at a time, so there is no inter assay variation. The level of insulin in serum was expressed in μIU/ml.

Syntheses of advanced glycation end products

Advanced glycation end products (AGEs) were generated from co-incubation of BSA with either D-glucose (AGE1) or D-glyceraldhyde (AGE2) according to the method of Kume *et al.*[11] AGE1 and AGE2 were incubated at 37°C for 8 weeks and 2 weeks, respectively. BSA alone was incubated at 37°C for 2 weeks under conditions without any carbohydrates, and employed as the control for AGE1 and AGE2.

Protective effect onpancreatic β-cells from oxidative stress

β-cells are vulnerable to oxidative stress. Thus, in cultured RIN-5F cells, (American Type Culture Collection; number: CRL-2058) we examined whether *P. auritum* would protect the pancreas-derived β-cells from oxidative stress. RIN-5F cells that had been adhered and pre-cultured with medium alone for 72 h were treated with HS or medium alone for another 3 h. At the end of the culture, the intracellular peroxide level was measured with 2',7'-dichloro-dihydrofluorescein diacetate (DCFH-DA) fluorescent probe.[12]

Statistical analysis

The effect of *Piper auritum* aqueous extracts on each parameter was examined using one-way analysis of variance (ANOVA). Individual differences among groups were analyzed by Dunnett´s test using SPSS software. *P* values less than 0.05 were considered as statistically significant. Data are expressed as mean ± S.E.M. for 6 rats in each group.

RESULTS

Effect on blood glucose

STZ (50mg/kg) injection resulted in a significant elevation of blood glucose level. Daily oral administration of the hexane extract of leaves of *P. auritum* to diabetic rats for 14 and 28 days produced significant improvements in glycemic control as shown in Table 1. In contrast with body weight loss, diabetic control rats showed an increase in blood glucose from initial value from 306.2 to final 356.7 mg/dl. One the other hand, 400 mg of *P. auritum*-treated diabetic rats showed a significant decrease compared with diabetic control rats (from 325.4 mg/ dl to 100.2 mg/dl) resulted a significant recovery of glucose level and resettled to the control level. However, chloroform and methanol extracts to the same doses did not produced hypoglycemic activity.

Body weight and intakes of food, water

The body weight and food, water intakes in control and experimental animals are shown in Table 2; the induction of STZ-diabetes resulted in elevated intake of both. A significant decrease in body weight during the 28 days was observed in the diabetic control rats compared with control rats, showing no difference between initial and final values; however, administration of *P. auritum* at doses of 400 mg to diabetic rats increased body weight gain significantly (from 192.4g to 225.1g). Diabetic rats showed increase in food and water intakes as compared to normal control rats but the administration of 200 and 400mg/kg of hexane extract led to a decrease in water and food intake in experimental groups.

Serum constituents

Table 3 shows the results of lipids in the serum. Total cholesterol, triglycerides levels of diabetic control rats were significantly increased to the control levels. These parameters were decreased significantly in the hexane extract supplemented groups in respect to the corresponding diabetic groups, and were resettled towards the control level. HDL-cholesterol, a friendly lipoprotein, was decreased in diabetic groups in respect to the control. After 28d of hexane extract of leaves of *P. auritum* supplementation, there was a significant elevation of

Table 1: Effect of hexane extract *Piper auritum* (HS) after 28 days treatment on blood glucose level in STZ-induced diabetes

Group (mg/kg)	Fasting blood glucose level (mg/dl) Days of PA supplement		
	0	14	28
No-diabetic control	92.3 ± 2.4^{a}	93.5 ± 1.6^{a}	91.9 ± 2.3^{a}
Diabetic control	306.2 ± 3.6^{c}	332.7 ± 4.4^{c}	356.7 ± 3.9^{c}
HS 200	365 ± 5.7^{c}	180.9 ± 3.5^{b}	119.4 ± 2.3^{b}
HS 400	325.4 ± 4.0^{c}	163.8 ± 2.9^{b}	100.2 ± 1.8^{a}

Each value represents mean ± S.E.M. *(n=10),* ANOVA followed by multiple two-tail "*t*" test. In each vertical column, mean with different superscripts (a, b, c) differ from "*t*' each other significantly, <0.05

this lipoprotein level in serum to and was resettled to the control level.

Effect on pancreatic oxidative status and serum nitric oxide level

In the pancreatic function of the diabetic rats, lipid peroxidation and antioxidant defense system capabilities were evaluated. Table 4 shows the level of lipid peroxidation marker TBARS, antioxidant defense system components as CAT, SOD, GSH, GPx and serum NO, iNOS in normal and experimental rats. There was a significant elevation in TBARS concentration and serum NO level, while the activity of catalase and SOD, and glutathione (GSH and GPx) content decreased in diabetes when compared with normal control group. Treatment of the rats with HS significantly decreased lipid peroxidation TBARS pancreatic and serum NO. In addition, SOD and catalase activities and the glutathione content in pancreatic tissue significantly increased compared to diabetic control group.

Insulin releasing activity

Serum insulin level was significantly decreased in diabetic rats in respect to control. After 28 days of hexane extract of leaves of *P. auritum* supplementation to the diabetic rats, there was a significant elevation in serum insulin level in respect to diabetic-control group though the level of this hormone was significantly low than the control [Table 5]. In pancreas insulin level with ex- tract supplementation (200 and 400mg/kg) resulted also in a significant variation in insulin level in respect to only diabetic-control. Treatment group increased active β cells.

Effect on pancreatic AGEs

The fluorescence intensity of the HS-treated group was significantly lower than that of the control (0μg HS) group [Table 6]. This result suggested that HS might reduce oxidative stress in RIN-5F cells. To verify this hypothesis, RIN-5F cells were given oxidative stress by adding AGE1 and AGE2, BSA conjugated with glucose and glyceraldehyde,

Table 2: Effect on physico-metabolic symptoms of hexane extract *Piper auritum* (HS)

Group mg/kg)	Body weight (g)			Intake (g/d)	
	Initial	Final	Gain	Food	water
No-diabetic	232.6 ± 3.9[a]	305.4 ± 7.4[a]	72.8 ± 4.8[a]	20.7 ± 1.8[a]	40.6 ± 3.1[a]
Diabetic	189.4 ± 5.2[c]	198.7 ± 6.3[c]	9.3 ± 2.6[c]	31.4 ± 1.2[c]	139.8 ± 5.7[c]
HS 200	177.3 ± 4.2[c]	196.4 ± 6.7[b]	19.1 ± 1.9[b]	27.8 ± 1.2[c]	117.3 ± 3.6[b]
HS 400	192.4 ± 6.5[c]	225.1 ± 8.7[b]	32.7 ± 3.0[a]	24.7 ± 0.7[c]	101.3 ± 2.4[a]

Each value represents mean ± S.E.M. *(n=10)*, ANOVA followed by multiple two-tail "*t*" test. In each vertical column, mean with different superscripts (a, b, c) differ from *t'* each other significantly, *P*<0.05

Table 3: Effect hexane extract leaves of *Piper auritum* (HS) on lipid profile in STZ- induced diabetic rats

Group (mg/kg)	Mean Concentration (mg/g) ± SEM		
	Triglycerides (mg/dl)	Total cholesterol	HDL-cholesterol (mg/dl)
Normal control	91.12 ± 5.23	129.46 ± 3.29	69.11 ± 2.19
Diabetic control	187.45 ± 4.35[a]	247.23± 1.67[a]	35.36 ± 1.57[a]
HS 200	127.25 ± 4.12[ab]	152.36 ± 2.98[ab]	52.40 ± 2.80[ab]
HS 400	91.78 ± 6.03[ab]	130.21 ± 3.17[ab]	58.12 ± 1.95[ab]

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

Table 4: Effect hexane extract leaves of *Piper auritum* (HS) on antioxidant enzyme, TBARS in pancreas and NO and inducible iNOS in serum

Parameters	Normal Control	Diabetic control	Diabetic+HS (200 mg/kg)	Diabetic+HS (400 mg/kg)
SOD	54.14 ± 2.43	37.41 ± 1.64[a]	44.21 ± 2.98[b]	49.76 ± 1.38[b]
CAT	59.98 ± 1.65	25.73 ± 0.76[a]	46.20 ± 2.54[a]	51.45 ± 2.51[c]
GSH	12.16 ± 0.62	5.78 ± 1.38[a]	8.91 ± 1.41[b]	11.10 ± 2.03[b]
GPx	4.28 ± 1.42	2.51 ± 1.84[a]	3.16 ± 1.21[b]	3.89 ± 1.46[b]
TBARS (mM/mg protein)	1.93 ± 0.18	2.84 ± 0.17[a]	1.99 ± 0.13[ab]	1.96 ± 0.15[ab]
NO (μM)	25.76 ± 3.18	40.38 ± 2.94[a]	35.59 ± 3.78[ab]	30.12 ± 1.98[ab]
iNOS (U/ml)	10.98 ± 2.6	20.31 ± 4.32[a]	16.27 ± 1.76[b]	13.05 ± 1.90[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. The activities of antioxidant enzyme are given in U/mg of protein SOD: Superoxide dismutase, CAT: Catalase assay kit for catalase activity, GSH: Glutathione reductase, GPx: Glutathione peroxidase, TBARS: Thiobarbituric acid reactive substances, NO: Nitric Oxide, iNOS: Nitric oxide synthase

Table 5: Effect of HS on pancreatic insulin levels in serum and pancreas

Groups (mg/kg)	Serum insulin (mIU/ml)	Pancreatic insulin (mU/g protein)
Normal control	14.10 ± 4.5[a]	27.12 ± 2.1[a]
Diabetic control	11.23 ± 3.3[b]	17.23 ± 4.8[b]
HS 200	12.15 ± 3.5[b]	20.45 ± 1.9[c]
HS 400	13.08 ± 3.1[c]	25.89 ± 2.6[d]

Data are expressed as mean: ± S.E.M.: *n=10*. ANOVA followed by multiple two tail "*t*"test. Different superscripts (a, b, c, d) differ from each other significantly (*P*<0.05)

Table 6: Effect of HS on advanced glycation end products-induced oxidative stress in pancreatic culture RIN-5F cells

Fluorescence Groups	Intracellular ROS level
BSA	12.1 ± 3.48[a]
BSA+ HS + RIN-5F	5.3 ± 1.76[c]
AGE1 + RIN-5F	19.45 ± 3.21[a]
AGE1+ RIN-5F + HS	7.82 ± 1.63[b]
AGE2 + RIN-5F	30.45 ± 4.50[a]
AGE2 + RIN-5F + HS	14.67 ± 3.24[d]

Data are expressed as mean: ± S.E.M. Different superscripts (a, b, c, d) differ from each other significantly (*P*<0.05)

respectively, to experimental media. Control cells were incubated with BSA alone. After 3h treatment with AGEs, fluorescence intensity of AGE1 and AGE2-treated cells was significantly higher than that of the BSA-treated cells. In the same experiments with AGEs, treatment of RIN-5F cells with HS for 3 h resulted in dramatic reductions in oxidative stress with significant differences. These results clearly defined that *P. auritum* was capable to protect pancreatic β-cells from AGEs-induced oxidative stress.

DISCUSSION

When rats are injected with streptozotocin they provide an animal model of non-insulin-dependent diabetes mellitus. In this model, the destruction of β-cells and disorder of insulin secretion in the diabetic state causes physic-metabolic abnormalities such as higher levels of fasting and fed glucose levels, lower body weight, increase in food and water intakes as compared to normal control rats.

Treatment with *P. auritum* significantly reduced body weight loss, food and water intakes. Those observations could be due to an improvement of elevated blood glucose and the effect of the plant extract on lipolysis. Administration of HS at doses of 200 and 400 mg/kg significantly decreased serum glucose in streptozotocin-induced diabetic fasted animals and also increased the activity of beta cells in comparison with the diabetic control rats.

Hyperglycemia-induced oxidative stress plays a key role in the development of diabetes or its complications. On the other hand, reactive oxygen species are well known as important risk factors for pancreas diseases. These facts indicate that hyperglycemia-induced oxidative stress may also cause pancreas cell damage. It is possible that the antioxidant defense system against oxidative stress induced by chronic hyperglycemia plays an important role in the pathogenesis of pancreas disease.

Our results showed that the chronic administration of HS leaves improves impairments of SOD activity and glutathione homeostasis in STZ-induced diabetic rat pancreas with lowering the blood glucose level. In particular, the diabetes-associated free radical injury, accumulation of lipid peroxidation products, depletion of GSH, decrease in GSH/GPx ratio and down-regulation of key antioxidant enzymes, have been detected not only in the liver, but also in pancreas. Accordingly, there was a decrease in GSH in the pancreas of diabetic rats, probably due to increased utilization following the diabetes-induced oxidative stress. Previous studies have reported that there was an increased lipid peroxidation in the pancreas of diabetic rats[13] that that could be due to increased oxidative stress in the cell as a result of depletion of antioxidant scavenger systems.

iNOS, as a source of hydroxyl radicals in diabetes, leads to lipid peroxidation and its protein expression correlated with increased free radical production in the serum examined.[14] Treatment with HS strongly decreased serum NO level and iNOS activity to those of normal ones.

A significant increase in TBA-reactive substance, an index of endogenous lipid peroxidation, has been shown under diabetic conditions. Therefore, the measurement of TBA-reactive substance is frequently used to determine the level of oxidative stress in diabetic patients. In addition, the increased lipid peroxidation in the pancreas implies the level of susceptibility of diabetic oxidative stress. Lipid peroxide-mediated tissue damage has been observed in the development of type 1 and type II diabetes.[15]

The repeated administration of HS increased the GSH content in the pancreas leading to a restoration of the defense mechanism and significantly decreased the pancreas lipid peroxidation. HS may help to control free radicals and offered protection to cells against oxidative stress by scavenging free radicals. These results suggest that *P. auritum* may act as a suppressor against pancreas cell damage and inhibit the progression of pancreas dysfunction induced by chronic hyperglycemia.[16,17]

It is well known that chronic hyperglycemia leads to the auto-oxidation of glucose and causes the nonenzymatic glycation of proteins through Maillard's reaction which

produces Schiff base, Amadori product, and finally AGEs.[18] In these processes, reactive oxygen species are produced. To avoid oxidative stress, antioxidant enzymes play an important role against oxidative stress. However, hyperglycemia also causes nonenzymatic glycation of these antioxidant enzymes. In the diabetic state, glycation reaction is observed in various tissues and organs, and various kinds of glycated proteins such as glycosylated hemoglobin, albumin, and lens crystalline are produced in a nonenzymatical manner through the glycation reaction.[19] In the present study, *P. auritum* lowered the level of blood glucose significantly as compared with that of the diabetic control group. Therefore, our data demonstrated that HS protected RIN-5F cells from AGEs-induced oxidative stress which is followed by decreasing insulin gene expression and secretion.[20] Although, the serum insulin level of the HS group tended to increase as compared with that of the diabetic control suggesting that HS might partially rescue exhausted pancreatic β-cells of rats from further AGEs-induced oxidative stress. AGEs might deteriorate function of pancreatic beta cells in patients with long-term hyperglycemia.

CONCLUSION

P. auritum leaves prevents hyperglycemia-induced oxidative stress in the pancreas using STZ-induced type-I diabetic rat models. As a result, we found that the chronic administration of HS mitigates the pancreas dysfunction in STZ-diabetic rat *via* the improvement of the antioxidant defense system. However, we did not try to determine which ingredient(s) of this leaves exerts this beneficial effect. Further study will be needed.

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