Full Length Research Paper

Anti-diabetic properties of *Tinospora cordifolia* stem extracts on streptozotocin- induced diabetic rats

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The oral administration of various extracts (hexane, ethyl acetate and methanol) of *Tinospora cordifolia* stem (TCS) were found to have potent antidiabetic activity that reduces blood sugar level in streptozotocin-(STZ) induced diabetic rats. In this study, the chronic (100 days) antihyperglycemic effect of the extracts at a dose of 250 mg/kg b.w.p.d of TCS were investigated. Insulin was used as a reference drug at a dose of 3 I.U/kg.b.w.p.d. Fasting blood glucose, glycosylated hemoglobin (HBA\(_1\)C), serum insulin, C-peptide and liver enzymes levels were evaluated in normal, diabetic and treated rats. Supplementation of methanol extract significantly reduces the fasting blood glucose level when compared to other 2 extracts. Moreover this supplementation significantly decreases the glycosylated hemoglobin level as compare to diabetic control (p < 0.001), reduced glucokinase and increased glucose-6-phoaphatase activity were reversed significantly by the treatment of TCS methanol extract in respect to diabetic group. In the TCS treated groups, the insulin and C-peptide levels were improved which shows the regeneration of β-cell which secretes insulin, histopathological studies of pancreas of TCS methanol extract treated groups substantiate the regenerating capacity of extract.

Key words: *Tinospora cordifolia*, streptozotocin, hypoglycemic, glycosylated hemoglobin (HBA\(_1\)C), serum insulin, C-peptide, glucokinase and glucose-6-phoaphatase.

INTRODUCTION

Diabetes mellitus (DM) currently is a major health problem for the people of the world and is a chronic metabolic disorder/syndrome resulting from a variable interaction of hereditary and environmental factors and is characterized by abnormal insulin secretion or insulin receptor or post receptor events affecting metabolism involving carbohydrates, proteins and fats in addition to damaging liver, kidney and β-cells of pancreas (Baynes, 1991). The number of people suffering from the disease worldwide is increasing at an alarming rate with a projected 366 million peoples likely to be diabetic by the year 2030 as against 191 million estimated in 2000 (Wild et al., 2004). From literature review it has been revealed that 15 - 20% of diabetic patients are suffering from insulin-dependent diabetes mellitus (IDDM) or type-I (Urger and Foster, 1998). The IDDM is noted both in adult and child hood (Urger and Foster, 1998; Muhlhauser et al., 2000). It is characterized by elevation of both fasting and post-prandial blood sugar levels. Chronic hyperglycemia during diabetes causes glycation of body proteins that in turn leads to secondary complications affecting eyes, kidneys, nerves and arteries (Sharma et al., 1993). These may be delayed, lessened or prevented by maintaining blood glucose values close to normal.

In modern medicine, no satisfactory effective therapy is still available to cure the diabetes mellitus. Though insulin therapy is also used for the management of diabetes mellitus, but there are several drawbacks like insulin resistance (Piedrola et al., 2001), anorexia nervosa, brain atrophy and fatty liver (Yaryura-Tobias et al., 2001) after chronic treatment. Besides the use of insulin for the treatment of insulin dependent diabetes mellitus (IDDM), other approaches for the control of hyperglycemia include the use of amylin analogues which regulate gastric emptying and inhibitors of intestinal alpha glucosidases like acarbose, miglitol and voglibiose which delay postprandial hyperglycemia. Sulphonylureas, the most widely used class of drugs act by closure of ATP dependent channel. Metformin, a biguanide oral antibiotic limits intestinalglucose absorption. These drugs have certain effects like causing hypoglycemia at higher doses, liver problems, lactic acidosis and diarrhea. It is apparent that due to the side effects of...
the currently used drugs, there is a need for a safe agent with minimal adverse effects, which can be taken for long durations. Though biguanides and sulfonylureas are valuable in treatment of diabetes mellitus, their use is restricted by their limited action, pharmacokinetic properties, secondary failure rates and accompanying side effects (Bailey et al., 1989). Moreover, these therapies only partially compensate for metabolic derangements seen in diabetics and do not necessarily correct the fundamental biochemical lesion (Taylor and Agius, 1988).

Recently, there has been increasing interest in the use of medicinal plants. The use of medicinal plants in modern medicine suffers from the fact that though hundreds of plants are used in the world to prevent or to cure diseases, scientific evidence in terms of modern medicine is lacking in most cases. However today it is necessary to provide scientific proof as whether to justified the use of plant or its active principles (Singh et al., 2000).

Throughout the world many traditional plant treatments for diabetes exist. The influence Costus speciosus (Koen) (Daisy et al., 2008), Costunolide isolated from C. speciosus (Koen) (James Eliza et al., 2008), Elephantopus scaber (Daisy et al., 2008) and Cassia fistula stem bark extract (Nirmala et al., 2008) were studied on streptozotocin-induced diabetic wister albino rats. However, few have received scientific or medical scrutiny and the world health organization (WHO) has recommended that traditional plant treatments for diabetes warrant further evaluation (world health organization, 1980). Recently, search for appropriate antihyperglycemic agents has been focused on plants used in traditional medicine because of leads provided by natural products that may be better treatment than currently used drugs (Hu et al., 2003).

The herb Tinospora cordifolia (TC) known to the Menispermacae family and is commonly known as Guancha or Tinospora in English and Giloya or Ambervel in Hindi. It has a long history of use in Ayurvedic medicine (the traditional medicine of India). Evidence hints that Tinospora may have anti-cancer (Singh et al., 2005; Singh et al., 2004), immune stimulating (Rawal et al., 2004), anti-diabetic (Stanely et al., 2007; Rathi et al., 2002), cholesterol-lowering (Stanely et al., 2003) and liver-protective (Bishayi et al., 2002) actions. T. cordifolia has also shown some promising speed in healing the diabetic foot ulcers (Purandare et al., 2007). However, all these findings are far too preliminary to be relied upon.

In the present study, we investigated the hypoglycemic effect of T. cordifolia stem (TCS) extracts (methanol, hexane and ethyl acetate) on healthy and streptozotocin-induced diabetic rats for 100 days to find its regenerative efficacy.

MATERIALS AND METHODS

Plant material and extraction

During summer the bark of matured T. cordifolia was freshly collected from Tirunelveli, Tamil Nadu, India, chopped, shade dried and coarsely powdered. An authenticated voucher specimen (No.HC-15) of the plant has been preserved in our department for future reference. The powder was defatted with petroleum ether (60 - 80°C) then extracted with hexane using soxhlet extractor. The extracts were dried under reduced pressure using a rotary vacuum evaporator. The % yield was 7% w/w and the extracts were kept in refrigerator for further use. A dose determination process was carried out by administering 100 mg/kg b.w.p.d, 150 mg/kg b.w.p.d, 200 mg/kg b.w.p.d, 250 mg/kg b.w.p.d, 300 mg/kg b.w.p.d and 350 mg/kg b.w.p.d dissolved in 1 ml 0.3% carboxy maethyl cellulose (CMC) solution. 250 mg/kg b.w.p.d and above were found to have similar and better hypoglycemic activity. Thus 250 mg/kg b.w.p.d was chosen for the treatment.

Chemical used

Streptozotocin was purchased from Sigma-Aldrich, St. Louis, USA. All other commercial reagents used were of analytical grade.

Animals

To demonstrate the antidiabetic property of TCS and its effect on blood glucose levels, male albino wister rats aged 7 to 8 weeks (180 - 200 g) bred in the animal division of King's institute, Chennai were used. Animals were kept in animal house at an ambient temperature of 25 - 30°C and 45 - 55% relative humidity with a 12 h each of dark and light cycle. Animals were fed pellet diet (Sai Durga Feeds and Foods, Bangalore, India,) and water ad libitum. The experimental protocol has been approved by the institutional animals ethics committee and by the regulatory body of the government (Reg.No 585/05/A/CPCSEA).

Acute toxicity tests

A separate experiment was performed to know whether any toxic effect was produced by T. cordifolia on liver and kidney. Normal healthy male rats fasted for 12 h were randomly divided into drug-treated 'test' groups and vehicle-treated 'control' groups, totally making up 6 groups of 6 rats per cage T. cordifolia (0.25, 0.5, 1.0 and 2.0 g) was separately administered orally to the rats in each of the test groups. Each of the rats in the control group was treated with vehicle alone (CMC 0.5%, 1 ml/kg b.w.p.d). Then the rats in both the test and control groups were allowed access to food and water and behavioral changes were observed over a period of 24 h for sign of acute toxicity. The mortality caused by the extract within this period of time was observed.

Induction of experimental diabetes

The animals were fasted overnight and diabetes was induced by a single intraperitoneal injection of a freshly prepared solution of streptozotocin (55 mg/kg b.w.p.d) in 0.1 M citrate buffer (pH 4.5) (Sekar et al., 1990). Control rats were injected with citrate buffer alone. On the third day of STZ-injection, the rats were fasted for 6 h and blood glucose was taken from tail artery of the rats (Burcelin et al., 1995). Rats with moderate diabetes having hyperglycemia (that is, with blood glucose of 250 - 400 mg/dl) were taken for the experiment. The blood was collected from sinocular puncture. The rats were kept for 15 days to stabilize the diabetic condition (Jyoti et al., 2002).

In the experiment, a total of 36 rats (30 diabetic surviving rats and 6 normal rats) were used. The rats were divided into 6 groups comprising of 6 animals in each group as follows:

Group I: Normal untreated rats.
Group II: Normal rats given TCS Hexane Extract (250 mg/kg b.w.p.d) in 0.3% CMC solution, daily using intragastric tube for 100 days.

Group III: Normal rats given TCS Ethyl Acetate extract (250 mg/kg b.w.p.d) in 1ml of 0.3% CMC solution, daily using intragastric tube for 100 days.

Group IV: Normal rats given TCS Methanol extract (250 mg/kg b.w.p.d) in 1ml of 0.3% CMC solution, daily using intragastric tube for 100 days.

Group V: Diabetic control rats given 1ml of 0.3% CMC solution using an intragastric tube.

Group VI: Diabetic rats given TCS Hexane Extract (250 mg/kg b.w.p.d) in 0.3% CMC solution, daily using intragastric tube for 100 days.

Group VII: Diabetic rats given TCS Ethyl Acetate extract (250 mg/kg b.w.p.d) in 1ml of 0.3% CMC solution, daily using intragastric tube for 100 days.

Group VIII: Diabetic rats given TCS Methanol extract (250 mg/kg b.w.p.d) in 1ml of 0.3% CMC solution, daily using intragastric tube for 100 days.

Group IX: Diabetic rats given Insulin (3 I.U/kg.b.w.p.d) daily using Insulin Syringe for 100 days.

Sample collection
This experiment was carried out for 100 days, with oral administration of TCS extracts. At the end of 100 days, the animals were deprived of food overnight and sacrificed by decapitation. Blood was collected in 2 different tubes, one with anticoagulant, potassium oxalate and sodium fluoride for plasma and another without anticoagulant for serum separation. The blood was then centrifuged at 3000 rpm for 20 min using refrigerated centrifuge at 4°C to separate the plasma and serum. Pancreas was immediately dissected, washed in ice cold saline, patted dry and weighed. The tissues were fixed in 10% formalin immediately after removal from the animal to avoid decomposition. Embedding in paraffin wax was carried out by removal of water using alcohol dehydration and infiltration of chloroform as a solvent for the wax.

Biochemical analysis
Fasting blood glucose was estimated by the oxidase/peroxidase method (Trinder, 1969). Glycosylated hemoglobin was estimated using the diagnostic kit from Biosystems, Spain. Plasma insulin level was assayed by the Radio Immuno Assay (RIA) kit (Diasorin, Saluggia, Italy), using human insulin as standard. C-peptide level was assayed by the chemiluminescence immunoassay method. Hexokinase and glucose-6-phosphatase were assayed by standard protocols (Brandstrup et al., 1959; Koida et al., 1959).

Histopathological studies

Light Microscopic studies
The pancreas form the untreated and the experimental groups were blotted free of mucus, washed in physiological saline, cut into pieces of desired size and fixed in Bouin- Hollande fixative for 72 h. After fixation, the tissues were washed in 70% alcohol for 2 or 3 days to remove the excess picric acid and dehydrated in graded series of alcohol. The tissues were cleared using xylene. The cleared tissues were infiltrated with molten paraffin at 58 - 60°C through 3 changes (20 - 30 min) and finally embedded in paraffin. 3 - 5 µm thick sections of all the tissues were obtained using rotary microtome and stained in Ehrlich's hematoxylin with eosin as the counter stain. The slides were mounted using DPX mountant. (Paraffin Method- Humason, 1979)

Transmission electron microscope
The pancreas was cut into 1 mm cubes and immersed in 2.5% glutaraldehyde (primary fixative) overnight. Then the tissues were rinsed in washing buffer and post-fixed in 1% osmium tetroxide (OsO4) (secondary fixative) for 2 - 3 h. Subsequently, the tissues were washed thoroughly in washing buffer to remove excess OsO4. Then the tissues were dehydrated gradually in ethyl alcohol and de-alcoholized using propylene oxide. Infiltration was carried out with propylene oxide and Spurr's mixture (Sigma, USA) at increasing concentrations at room temperature, using a slow speed rotary shaker. Embedding was done in a flat embedding mold with tissues oriented to get cross sections. Semithin sections were obtained with Reichert Jung (Austria) ultra microtome and stained in TBO. Area was chosen to obtain ultra-thin sections (silver to gray, 60 - 90 nm) using LKB-Bromma ultracut (Germany). Sections were picked in copper grids and stained with uranyl acetate and lead citrate. Leitz diapalm microscope (Leica, Germany) was used to obtain light micrographs of TBO-stained semithin sections, while Philips 200 (Holland) transmission electron microscope (TEM) was used to obtain electron micrographs of 2000 to 70,000 X magnifications.

Statistical analysis
All values were expressed as the mean obtained from a number of experiments (n). Data from all the tables of normal, diabetic control, reference drug treated and Tinospora stem extracts treated animals were compared by ANOVA followed by Duncan's multiple range test (DMRT).

RESULTS AND DISCUSSION

Results
Acute toxicity studies revealed the non-toxic nature of the methanol extract of T. cordifolia. Experiment was carried out on normal healthy male rats. No mortality was observed in the extract-treated rats and behavior of the treated rats also appeared normal. There was no lethality or toxic reaction found at any dose selected until the end of the study. Administration of extracts to normal animals does not alter the blood glucose level which is evidenced from Figure 1. The increased blood glucose in the diabetic condition is gradually reduced and almost nearer to normal after 90 days by the administration of TCS extracts (Figure 1) in which methanol extract shows better reduction. The glycosylated hemoglobin level in diabetic control is 5.11 ± 0.68%, which is reduced to 2.58 ± 0.65% in diabetic TCS methanol extract treated rats (Figure 2). Figures 3 and 4 shows the mean ± SD values of serum insulin and C-peptide. The insulin and C-peptide levels of untreated rats are 39.05 ± 6.51 µmol/ml and 243.07 ± 10.4 pM/ml, which is decreased to 8.13 ± 2.04 µmol/ml. The insulin and C-peptide levels are considerably increased after the treatment with the extracts of
Figure 1. Mean ± S.D (n=6) of the decrease in Serum Glucose level (mg/dl) in the different groups of control and diabetic rats which have been treated by different extracts of *T. Cordifolia* (Hexane, Ethyl Acetate and Methanol) at a dosage of 250 mg/kg.b.w.p.d Experimental groups compared with Diabetic control.* Significance at *P < 0.001.

Figure 2. Mean ± S.D (n=6) of the decrease in HBA1C (%) in the different groups of control and diabetic rats which have been treated by different extracts of *T. Cordifolia* (Hexane, Ethyl Acetate and Methanol) at a dosage of 250 mg/kg.b.w.p.d.

TCS where methanol extract has given a better result. The levels of glucokinase and glucose-6-phosphatase are given in Figures 5 and 6. The table reveals that the glucoki-nase level is high in untreated rats and reduced to about 50% in STZ rats. But the status has reversed in the *T. cordifolia* treated rats where the activity of glucokinase is appreciably increased. Figure 4 reveals that the glucose-6-phosphatase level is low in untreated rats and
increased in STZ rats. But in the TCS treated rats the activity of glucose-6-phosphatase is significantly decreased. Hematoxylin and eosin sections of pancreas of untreated rats reveals that each of islets of langerhans is formed of numerous compactly arranged cells occurring as dense cords.

The islets stained with toluidine blue O, shows them to be islands of lightly stained cells surrounded by a thin layer of reticular fibers. Transmission electron microscope (TEM) of β cells shows that they contain abundant secretion granules (Picture 1).

Islets from the pancreas of the diabetic control rats
Figure 5. Mean ± S.D (n = 6) of the glucokinase (μmol of Glu.6,PO4/hr/mg of protein) in the different groups of control and diabetic rats which have been treated by different extracts of T. Cordifolia (hexane, ethyl acetate and methanol) at a dosage of 250 mg/kg.b.w.p.d.

Figure 6. Mean ± S.D (n = 6) of glucose-6-phosphatase level (μmol of Pi liberate/hr/mg of protein) in the different groups of control and diabetic rats which have been treated by different extracts of T. Cordifolia (hexane, ethyl acetate and methanol) at a dosage of 250 mg/kg.b.w.p.d.

possessed pyknotic nuclei, whereas some cells contain dark nuclei and few cells at the peri-phery had round or ovoid nuclei. Semithin sections re-veal that the nuclei of the islets are shrunken and spin-dle-shaped In TEM in nucleus of β-cells appears to be dark and shrunken and to possess irregular outline (Picture 2).  

Hematoxylin and eosin sections of pancreas of the T. cordifolia hexane and ethyl acetate treated diabetic rats show islet cells similar to diabetic control. The islets stained with toludine blue O, reveals the islet cells to possess
fewer ovoid nuclei. TEM of the islets form the treated rats shows the β-cells to possess less secretion granules. (Pictures 3 and 4). Hematoxylin and eosin sections of pancreas of the *T. cordifolia* methanol treated diabetic rats show islet cells with normal architecture. The islets stained with toludine blue O, reveals the islet cells to possess ovoid nuclei. TEM of the islets form the treated rats shows the β-cells to possess dense secretion granules nearly equal to the untreated rats (Picture 5).

**DISCUSSION**

Streptozotocin is well known for its selective pancreatic islet β-cell cytotoxicity and has been extensively used to induce diabetes mellitus in animals. It interferes with cellular metabolic oxidative mechanisms (Papaccio et al., 2000).

In all diabetic patients, treatment should aim to lower blood glucose to near normal level (ADA, 1997). The present investigation fulfills this statement by producing a significant fall in blood glucose levels in methanol extract administered STZ diabetic rats than the hexane and ethyl acetate extracts treated rats. Earlier report states that insulin deficiency occurs in STZ induced diabetic rats leading to alterations in the carbohydrate metabolism such as elevated blood glucose and reduced level of insulin (Levine et al., 1949).

In our study, it was observed that methanol extract of TCS reversed these effects and tends to bring the parameters significantly towards the normal indicating a better
trend compared to the hypoglycemic drug (insulin) in diabetic animals. The possible mechanism by which methanol extract brings about its hypoglycemic action may be by induction of pancreatic insulin secretion from β-cells of islets of langerhans or due to enhanced transport of blood glucose to peripheral tissue. This is a clear evidenced from the data with significant (p- value < 0.05) increase in plasma insulin level of diabetic rats treated with methanol extract.

The increase in serum C-peptide levels of diabetic rats shows that some regeneration of pancreatic β-cells has occurred with the use of methanol extract of TCS. This regeneration of pancreatic β-cells has occurred slowly and was maximum after a period of 100 days. C-peptide, a cleavage product of the proinsulin molecule, has long been regarded as biologically inert, serving merely as a surrogate marker for insulin release. But the level of insulin is not proportionate to C-peptide because insulin is metabolized by the liver and has a short half-life of 4 - 5 min. Although the stimulation of insulin secretion might have contributed to the effects of TCS, the other extra pancreatic effects are also additional possibilities for the large effects seen with TCS. Especially, when the high dose level of streptozotocin (55 mg/kg) used in the present study can effectively destroy the β-cells, the effectiveness of drugs to treat this condition might rely on the actions other than pancreatic cells insulin release (Muruganandan et al., 2005).

The histopathological studies revealed that alterations occurred in the architecture of pancreatic tissue in STZ-induced diabetic rats. It is interesting to note that these alterations are corrected apparently near normal by
methanol extract of TCS treatment. This trend in the pancreatic β-cells regeneration substantiates the increased in the C-Peptide level of diabetic rats treated with methanol extract of TCS. In long standing diabetes, there is an increased glycation of a number of proteins including hemoglobin. This has been shown to be associated with the induction of oxidative stress and subsequent tissue damage to major organs such as heart and kidneys that may lead to other diabetic complications (Muruganandan et al., 2002). Hemoglobin is highly susceptible to non-enzymatic glycation. Glycated hemoglobin is an effective means to screen for diabetes (Peters et al., 1996; Tanaka et al., 2001). In diabetic condition, the excess of glucose present in the blood reacts with hemoglobin to form glycated hemoglobin, which has altered affinity for oxygen and this may be a factor in tissue anoxia. Glycated hemoglobin was significantly increased in diabetic rats, and this increase was directly proportional to fasting blood glucose. Administration of methanol extract of TCS to STZ diabetic rats significantly increased the level of hemoglobin and significantly decreased the levels of glycated hemoglobin than the other two extracts. The activity of glucokinase in liver decreased markedly, whereas the activity of glucose-6-phosphatase increased significantly in diabetic control rats. Treatment with methanol extract of TCS in diabetic rats increased the Glucokinase activity and decreased the glucose-6-phosphatase activity nearly equal to normal whereas the other two extracts did comparatively lower alteration than the methanol extract. Accordingly, it can be concluded that methanol extract of TCS regenerate the damaged endocrine pancreas and thereby stimulation of insulin secretion in β-cells as revealed by insulin and C-peptide assays, LM and TEM staining. Further studies are needed to isolate the active principles of the plant extract.

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REFERENCES


