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Tinospora Cordifolia Promotes Expression of Insulin Receptor and Proglucagon Genes in Streptozotocin Induced Diabetic Rats

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Abstract The aim of the present study was to determine the effect of aqueous extract of *Tinospora cordifolia* (AQTC) on antihyperglycemia, *in vitro* and *in vivo* antioxidant, intestinal proglucagon and pancreatic insulin gene expression levels in streptozotocin induced diabetic rats. The AQTC (100, 200 and 400 mg/kg body weight) was administered orally once a daily for 28 days in STZ induced diabetic rats. Serum blood glucose levels, glycosylated hemoglobin (HbA1c), insulin were estimated. Antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT) and malondialdehyde (MDA) were estimated in the pancreas and liver homogenates. Finally the expression levels of pancreatic insulin receptor and intestinal proglucagon gene expression were estimated at the end of 28 days treatment period. The results of the study indicates a significant reduction in blood glucose, HbA1c levels with significant increase in serum insulin and total protein levels with AQTC treatment in diabetic rats and a significant reduction in MDA with elevated levels of SOD, CAT in the pancreas and liver homogenates of diabetic rats compared to normal rats. The expression levels of genes of pancreatic insulin receptor and intestinal proglucagon genes were increased significantly with AQTC treatment. This indicates AQTC has protective effects on expression of intestinal proglucagon, which is precursor for GLP- 1 synthesis and also shown to increase the effects of insulin receptor expression. The protective role of *Tinospora cordifolia* might be due to the presence of active principles.

Keywords: *diabetes, Tinospora cordifolia, Proglucagon, insulin*

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1. Introduction

Incretins, hormones produced by the gastrointestinal tract in response to nutrient entry, stimulate insulin secretion [1]. Incretins were first defined in the 1930's but the little work was done in the period between 1930 and 1960 because incretin were not believed to be present or have significant physiologic effects. There are many incretin hormones, but the 2 major incretin hormones belong to the glucagon peptide super family. They are gastric inhibitory peptide also known as GIP and GLP-1. GLP-1 may also mediate its effects on glucose-induced stimulation of insulin biosynthesis and secretion, inhibition of glucagon secretion, β-cell proliferation and survival [2]. Further important effects of GLP-1 include inhibition of gastrointestinal secretion and motility and food intake [3]. However, the half life of GLP-1 is very short (1-2 min) due to the cleavage and inactivation of these proteins by dipeptidyl peptidase IV (DPP IV). Hence, the use of DPP-IV inhibitors increases the GLP-1 time of action [4].

There are several GLP-1 Analogues available in market such as exenatide liraglutide. But the long term use of these drugs produces several side effects as pancreatitis, renal failure, thyroid tumors and GI disturbances such as nausea, diarrhoea [5]. Now a day, potential use of herbal drugs was found to be benefit over the use of allopathic medications because of its fewer side effects. *Avena sativa* [6], *Berberis arista* [7], *Bridella ndellensis* [8], *Curcuma longa* [9], *Cymbopogon citrates* [10] and Resveratrol [11] are plants reported to have effect on incretins. The activity was reported due to the presence of antioxidant principles. Therefore, studies with plant extracts are useful to understand their efficacy, mechanism of action and safety for treatment and management of diabetes.

Tinospora cordifolia belongs to the family *Menispermaceae* and is known as Gulancha in English, Guduchi in Sanskrit, and Giloya in Hindi. T. *cardifolia* may have anti-cancer [12,13], immune stimulating [14], anti-diabetic [15,16], cholesterol-lowering [17] and liver-protective [18] actions.

T. *cordifolia* has also shown some promising speed in healing the diabetic foot ulcers [19].

Hence the present work was undertaken with the objective of studying the effect of *Tinospora cordifolia* for *in vitro* and *in vivo* antioxidant activity and their beneficial effect on incretins in STZ induced diabetic rats.

2. Materials and Methods:

2.1. Plant Extract

Aqueous stem extract of *Tinospora cordifolia* (AQTC) was obtained from Lalia Implex, Vijayawada.

2.2. Chemicals and Drugs

2, 2-diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma Chemical Co. (St.Louis, MO, USA). TRIzol reagent was purchased from GeNei, Bangalore, India. Taq DNA polymerase was acquired from Invitrogen (Carlsbad, CA, USA). Sitagliptin obtained as a gift sample from Sun Pharma Pvt., Ltd., Bangalore. All other chemicals and reagents used were of analytical grade.

2.3. *In Vitro* Antioxidant Activities

The DPPH scavenging activity of AQTC was measured according to the method of Liu and Zhao., 2006 [20]. The ability of the AQTC to scavenge hydrogen peroxide was determined according to the method of Ruch *et al*., 1989 [21]. According to Winter bourn and Sutton, 1984 [22] hydroxyl radical scavenging activity was calculated. Scavenging activity of superoxide anion radical was determined by the method of Stewart and Bewley.,1980 [23]. The nitric oxide scavenging activity was calculated according to the method of Marcocci et al., 1994 [24].The reducing power of the extracts was determined by the method of Oyaizu., 1986 [25].

2.4. Animals

Adult albino Wistar rats (180 ± 10 g bd.wt), Swiss albino mice (18-20 g bd.wt) were obtained from the Mahaveer Enterprizes, Hyderabad, India. They were kept under temperature of (23± 2) ºC, humidity of 50 % and 12 h: 12 h of light and dark cycles, respectively. They were fed with a Commercial pellet diet (Rayon's Biotechnology Pvt Ltd, India) and water was provided *ad libitum*. The prior approval for conducting the experiments in rats was obtained from our Institutional Animal Ethics Committee and our lab is approved by CPCSEA, Government of India (Regd. No. 516/01/A/ CPCSEA), AU College of pharmaceutical sciences, Andhra University.

2.5. Acute Toxicity Studies

Healthy adult albino mice of either sex, starved overnight were divided into five groups (n=6). They were orally fed with the AQTC in increasing dose levels of 100, 500, 1000, 1500, 2000 mg/kg bd.wt [26]. The animals were observed continuously for 72 h for any signs of behavioral, neurological and autonomic profile, toxicity and mortality.

2.6. Experimental Design

Rats were acclimatized to the environment for 15 days prior to the experiment; animals were divided into five groups. Each group contains 6 rats. Fasted animals were deprived of food for at least 16 hr but allowed free access to water. Fasting blood was collected for blood glucose estimation before starting the treatment on the first day. The first group was used as control and received water as vehicle. The second group received a single dose of STZ (60 mg/kg bd.wt) and dissolved in citrate buffer and was divided into four subgroups after establishing of the diabetes for 1 week [27]. The first subgroup was kept as a diabetic control while the second, third, fourth and fifth subgroups received orally 1.0 ml of Sitagliptin (5mg/kg), AQTC (100 mg/ kg), AQTC (200 mg/ kg) and AQTC (400 mg /kg) respectively by gastric intubation daily for 28 days. Blood was collected from retro orbital plexus. All five groups were sacrificed on the 28th day in fasting condition by cervical dislocation and then blood was collected for various biochemical estimations.

2.7. Biochemical Estimation

At the end of 28 days the serum blood glucose levels, HbA1C and insulin levels were estimated with kits. The pancreas was isolated and cut into small pieces, place in chilled 0.25 M sucrose solution and blotted on a filter paper. The tissues were then homogenized in 10 % chilled Tris hydrochloride buffer (10 mM, pH 7.4) by tissue Homogenizer (Remi Motors, Mumbai, India) and centrifuged at 12000rpm for 15 min at 0°C using cooling centrifuge (R-247, Refrigerated Centrifuge, Mumbai, India) Lipid peroxidation (LPx) was estimated in terms of malondialdehyde (MDA) content and determined by using the thiobarbituric acid (TBA) by the method of Hiroshi et al., 1979 [28]. The activity was expressed as µmol of malondialdehyde formed/g wet weight of tissue. Superoxide dismutase (SOD) was assayed for its ability to inhibit the autooxidation of epinephrine in alkaline medium [29]. The SOD activity levels were expressed in units per mg protein per min. Catalase was assayed by the method of Maehly and Chance., 1954 [30] by determining the decrease in the concentration of hydrogen peroxide [H_2O_2]. The activity of the enzyme was expressed in µmol of H_2O_2 metabolized/mg protein/min. The protein concentration was determined by using bovine serum albumin as the standard [31].

2.8. Total RNA Extraction and Reverse Transcription and Polymerase Chain Reaction

Total RNA was isolated from the intestinal samples using TRIzol reagent (GeNei, Bangalore, India) according to manufactures instructions. The quality of RNA was confirmed by formaldehyde gel in comparison with 28S and 18S RNA. The RNA concentration was measured at 260 nm using a UV spectrophotometer (UV-1800 UV-Vis Spectrophotometer, Japan). The RNA pellet was dissolved in diethylpyrocarbonate (DPEC)-treated water. cDNA was synthesized using 9µL of total RNA and reverse transcriptase (Invitrogen, Carlsbad, CA, USA).

Polymerase chain reaction (PCR) was performed on a Thermal Cycler PCR Machine (Model No.LT- 240) using Taq DNA polymerase with the following thermal cycle profiling: initial denaturation at 95°c for 5 min followed by 30 cycles (Denaturation at 95°c for 5 min, annealing at 60°c for 30 Sec, renaturation at 72°c for 30 Sec, and extension at 72°c for 10 min).

The following primer sets were used to amplify proglucagon and insulin gene expression. All the primers were ordered from GeNei, Bangalore, India.

Proglucagon:

Forward:

PLG-F_R: GTAATGCTGGTACAAGGCAG

Reverse:

PLG-R_R: TTGATGAAGTCTCTGGTGGCA

Insulin:

Forward:

INSULIN-F_384: CCCTAAGTGACCAGCTACA

Reverse:

INSULIN-R_384: TTGCAGTAGTTCTCCAGTTG

3. Results

3.1. Effect of AQTC on *in vitro* Anti Oxidant Activity

Table 1. Effect of AQTC on *In Vitro* antioxidant activity

In Vitro Antioxidant Activity	IC_{50}(µg)	
	Ascorbic acid	AQTC
DPPH radical	22.65± 1.0	53.91±0.8
H_2O_2 radical	02.59± 0.9	02.91± 0.5
Nitric Oxide radical	265.71± 1.9	159.44± 1.2
Reducing Power	16.82± 0.8	21.46± 0.4
Phosphomolybdenum	61.34± 0.5	106.41± 0.9
Hydroxyl radical	301.32± 1.2	261.8± 1.6

The *in vitro* antioxidant activity of AQTC was shown in Table 1. The various concentrations of AQTC and standard ascorbic acid showed antioxidant activity in a dose dependent manner. The IC50 values of AQTC for scavenging of DPPH, hydrogen peroxide, nitric oxide, reducing power, phosphomolybdinum and hydroxyl radical scavenging activity of AQTC extract were 53.91 ± 0.8, 2.91 ± 0.5, 159.4 ±1.2, 21.46 ± 0.4, 106.41 ± 0.9 and 261.8 ± 1.6 µg/ml respectively.

3.2. Effect of AQTC on Blood Glucose, HbA1c and Insulin

STZ induced diabetic rats showed significant increase in blood glucose and HbA1c levels when compared with controls (Table 2). Whereas AQTC and sitagliptin treated diabetic rats showed significant ($p<0.05$) reduction of blood glucose and HbA1c levels. In diabetic rats insulin levels were decreased significantly ($p<0.05$) when compared to normal rats, whereas in AQTC treated diabetic rats showed significantly ($p<0.05$) increase the insulin levels when compared to diabetic rats.

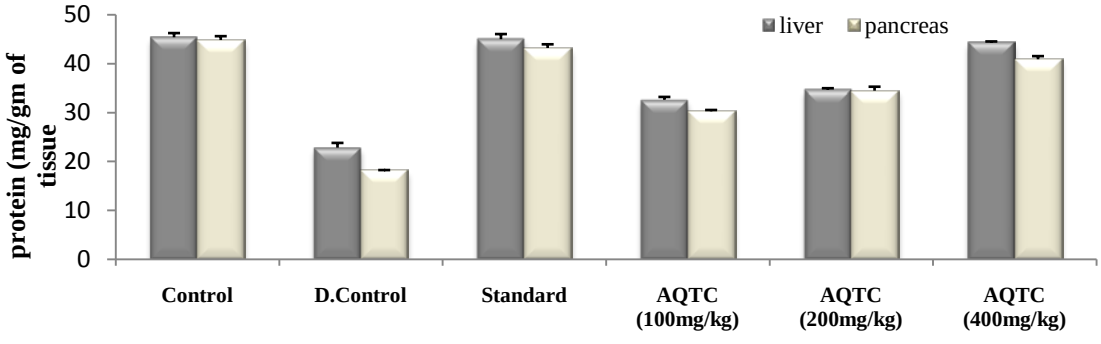
3.3. Effect of AQTC on Protein, Lipid Peroxidation and Antioxidant Enzyme Levels in Pancreas and Intestine

The total protein levels were decreased in diabetic liver and pancreas where as treatment with AQTC showed increased levels of protein levels (Figure 1). The antioxidant enzymes like SOD and catalase levels were significantly ($p<0.05$) decrease in both pancreas and intestine of diabetic rats. Furthermore, AQTC extract and Sitagliptin when compared to diabetic rats (Figure 2, Figure 3). The MDA contents were significantly ($p<0.05$) increased in both of the pancreas and liver of diabetic rats when compared to that of normal control rats (Figure IV). However, AQTC and Sitagliptin treated diabetic rats showed significant ($p<0.05$) decline in MDA contents when compared to diabetic rats.

Table 2. Effect of AQTC of blood glucose, %HbA1c and insulin levels in STZ induced diabetic rats

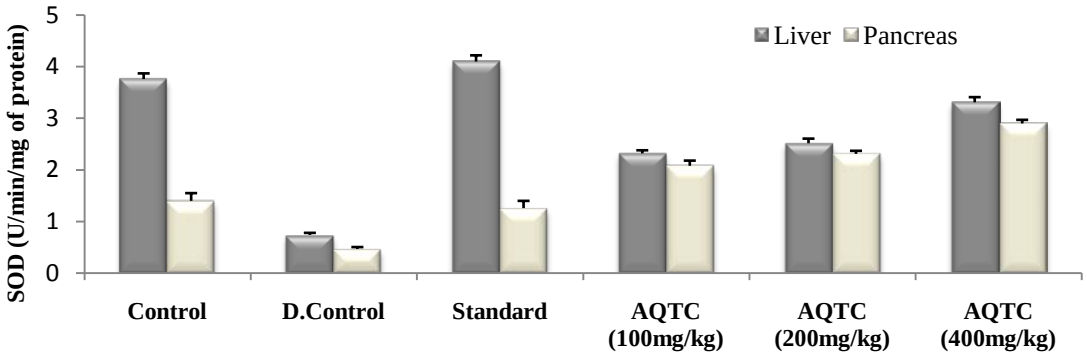
Groups	Blood Glucose (mg/dL)	%HbA1c	Insulin (uIU/ml)
Normal	88.05±1.8*	2.57±0.10*	5.04±0.62*
Diabetic control	359.2±7.5	11.12±0.6	3.45±0.99
Standard	95.1±3.5*	3.16±0.09*	4.65±0.45*
AQTC(100mg/kg)	128.8±2.5*	8.30±0.16*	4.06±0.66*
AQTC(200mg/kg)	102.4±4.2*	5.46±0.07*	4.33±1.66*
AQTC(400mg/kg)	84.12±5.7*	3.86±0.20*	4.96±0.86*

The data represents Mean±SEM values, n=6, $P<0.05^*$ significance followed by two way ANOVA followed by Bonferroni's post test when compared with toxicant group.



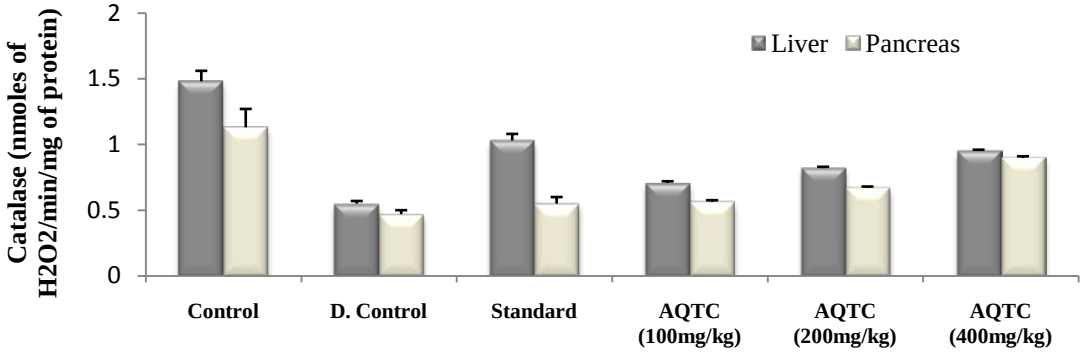
The data represents Mean±SEM values, n=6, $P<0.05^*$ significance followed by two way ANOVA followed by Bonferroni's post test when compared with toxicant group.

Figure 1. Effect of AQTC on protein levels in liver and pancreas of STZ induced diabetic rats



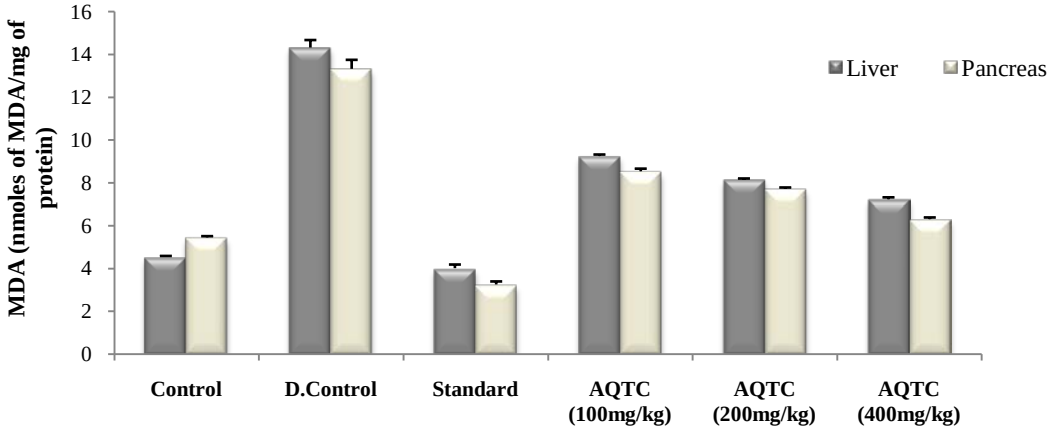
The data represents Mean±SEM values, n=6, P<0.05* significance followed by two way ANOVA followed by Bonferroni's post test when compared with toxicant group.

Figure 2. Effect of AQTC on SOD levels in liver and pancreas of STZ induced diabetic rats



The data represents Mean±SEM values, n=6, P<0.05* significance followed by two way ANOVA followed by Bonferroni's post test when compared with toxicant group.

Figure 3. Effect of AQTC on CAT levels in liver and pancreas of STZ induced diabetic rats

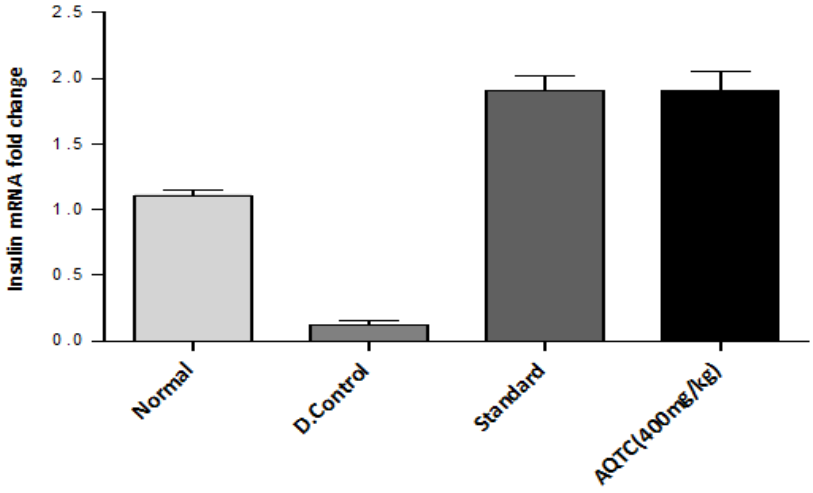


The data represents Mean±SEM values, n=6, P<0.05* significance followed by two way ANOVA followed by Bonferroni's post test when compared with toxicant group.

Figure 4. Effect of AQTC on MDA levels in liver and pancreas of STZ induced diabetic rats

3.4. Effect of AQTC on Pancreatic Insulin and Intestinal Proglucagon Gene Expression Levels:

The pancreatic insulin gene expression levels were significantly ($p< 0.05$) decreased in diabetic rats when compared to control rats (Figure 6). However, in AQTC and sitagliptin treated diabetic rats insulin gene expression levels and expression fold change were significantly ($p< 0.05$) increased when compared with diabetic rats (Figure VIII, Figure V). Figure III showed the STZ induced diabetic rats significantly ($p<0.05$) decreased in intestinal proglucagon gene expression levels as compared to control rats. Whereas, in AQTC and sitagliptin treated diabetic rats, proglucagon gene expression levels and expression fold change were significantly increased (Figure 4, Figure 7) when compared with diabetic rats.



L-Marker, M-Control, D.Control, 2-Standard, 3-AQTC (400mg/kg)

Figure 5. Effect of AQTC on insulin expression fold change in pancreas of STZ induced diabetic rats**.**

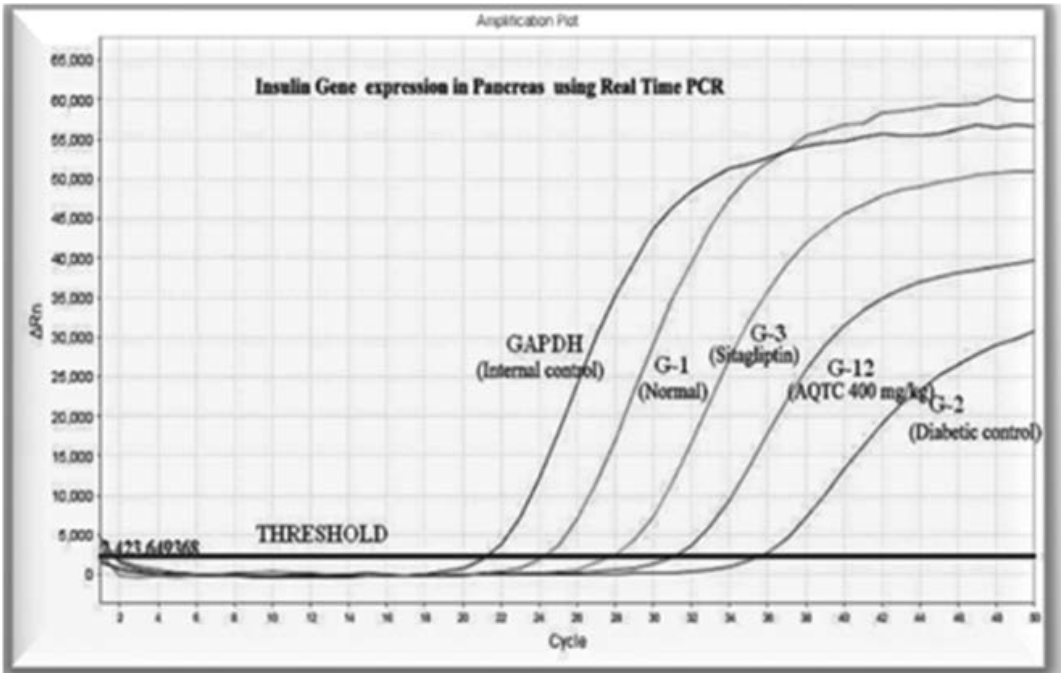


Figure 6. Effect of AQTC on insulin gene expression in pancreas of STZ induced diabetic rats

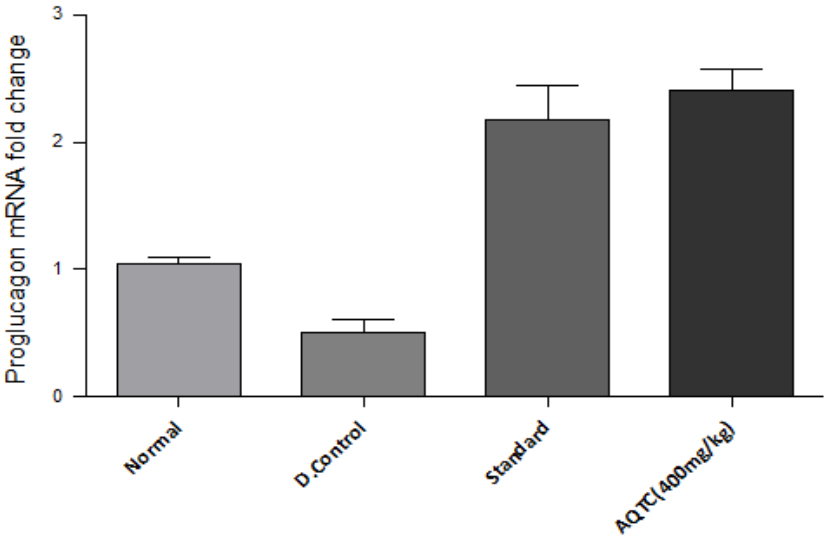


Figure 7. Effect of AQTC on Proglucagon expression fold change in ileum of STZ induced diabetic rats

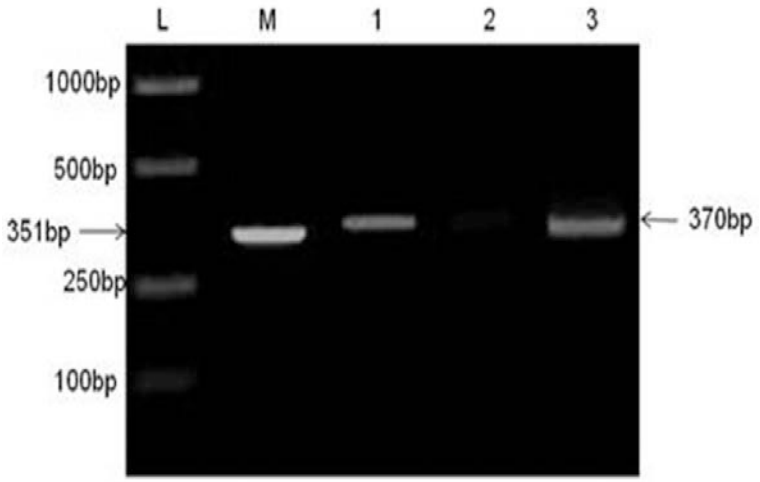
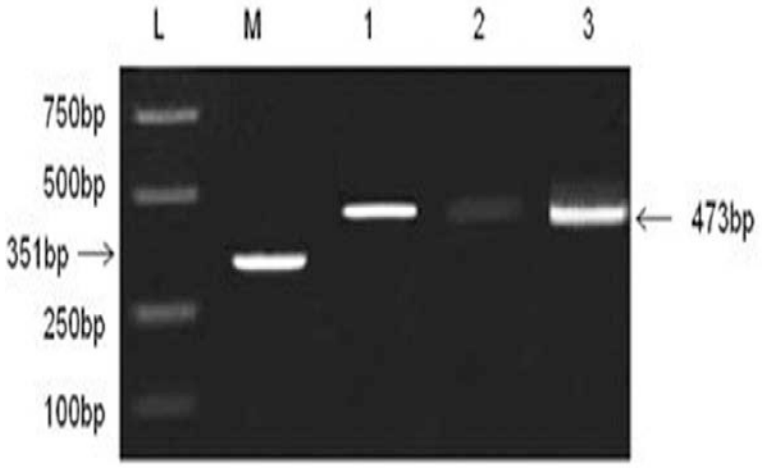


Figure 8. Effect of AQTC on insulin gene expression fold change in pancreas of STZ induced diabetic rats by using RT-PCR.



L-Marker, M-Control, 1-D.Control, 2-Standard, 3-AQTC (400mg/kg)

Figure 9. Effect of AQTC on Proglucagon gene expression in ileum of STZ induced diabetic rats

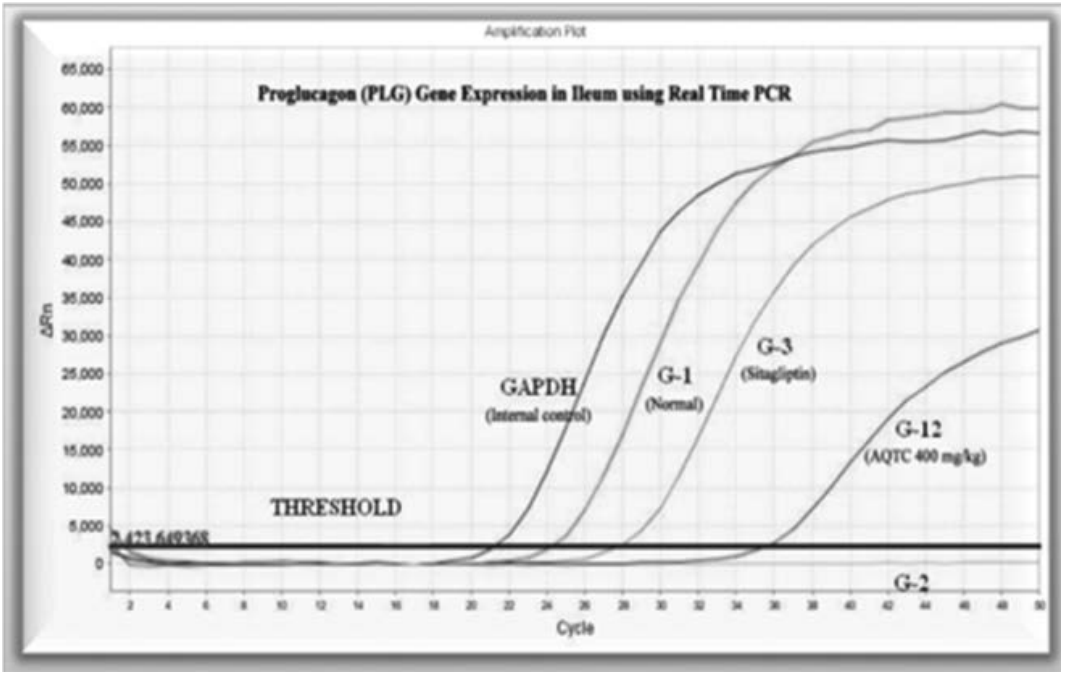


Figure 10. Effect of AQTC on proglucagon gene expression fold change in ileum of STZ induced diabetic rats by using RT-PCR.

4. Discussion

Diabetes mellitus is associated with increased formation of free radicals and decreased antioxidant potential. An imbalance of oxidant/antioxidant defense systems results in alteration in the antioxidant enzyme activity [32]. Hyperglycemia causes generation of reactive oxygen species which in turn causes lipid peroxidation. AQTC extract possesses significant antioxidant activity in various *in vitro* models, it may be due to AQTC extract contains compounds that are electron donors, which can react with free radicals to convert them to more stable products and terminate radical chain reaction. In the present study AQTC extract shows significant *in vitro* antioxidant activity when compared to ascorbic acid (Table 1).

In this study, Streptozotocin (STZ) is used to induce diabetes mellitus in albino Wistar rats. The treatment with the aqueous extract of *Tinospora cordifolia* (AQTC) significantly ($p<0.05$) reduced the elevated plasma glucose levels in STZ induced diabetic rats (Table II). The AQTC was shown delayed absorption in diabetic animals when compared to normoglycemic animals, which might be due to delayed gastric absorption, motility and nervous control of the entire system of gastrointestinal tract during diabetic condition. The AQTC was shown to have elevated insulin levels in STZ induced diabetic rats might be due to the controlled blood glucose levels (Table 2).

Glycosylated hemoglobin was an indicator of irreversible condensation of glucose with the N terminal residue of the β-chain of hemoglobin [33]. In our study, the diabetic rats had higher levels of glycosylated haemoglobin, the significant decrease in glycosylated hemoglobin was observed in diabetic rats after treatment with AQTC extract indicates that the overall blood glucose levels were controlled which might be due to an improvement in insulin secretion (Table 2).

The estimation of total protein is useful for measuring gross changes in protein levels caused by various disease states. The protein concentrations in liver and pancreas of the diabetic groups were found to be decreased. These decreased protein concentration in STZ induced diabetic rats was found to raised back to normal with AQTC treatment (Figure 1).

The SOD plays a pivotal role in oxygen defense metabolism by reducing superoxide to hydrogen peroxide [34]. The decrease in SOD activity in diabetic rats could result from inactivation of H_2O_2 or by glycosylation of the enzyme which have been reported to occur in diabetes [35]. The AQTC showed to elevate the SOD levels in both liver and pancreas as shown in Figure 2. The enzymes CAT and GPx are involved in the elimination of H_2O_2 [36]. The CAT activity in liver and pancreas was found to decrease due to inactivation by superoxide radical and glycation of the enzyme by the treatment with AQTC extract (Figure 2, Figure 3). Also, CAT is involved in detoxification of high H_2O_2 concentration [37]. AQTC extract treated diabetic rats pancreatic antioxidant enzymes were significantly increased which could be attributable to strong antioxidative properties [38]. In the present study in STZ induced diabetic rats, MDA content was increased. Treatment with AQTC extract for 28 days significantly reduced the liver and pancreatic MDA content (Figure 4).

GLP-1 is produced by differential posttranslational processing of proglucagon in the gut and brain [39]. Proglucagon is cleaved to glucagon by prohormone convertase 2 (PC2) in pancreatic α-cells, but is cleaved to glucagon-like peptide-1 (GLP-1) by PC1 in intestinal L-cells [40]. The aim of this study was to identify mechanisms which switch processing of proglucagon to generate GLP-1 in the pancreas; given that GLP-1 can increase insulin secretion and β-cell mass. GLP-1 slows gastric emptying, inhibits gut motility, and suppresses appetite while it increases β cells proliferation, enhances β cells function, and stimulates pancreatic insulin secretion [41]. The insulin stimulating effect of GLP-1 is tightly regulated by the blood glucose concentration. In Figure 6 and Figure IX showed that the AQTC normalize the blood glucose levels and insulin levels as well as increase the expression of pancreatic insulin expression and intestinal proglucagon expression to enhance the GLP-1 secretion in STZ induced diabetic rats, this may be attributed the presence of photochemicals such as alkaloids, glycosides and steroids [42].

5. Conclusions

The anti-diabetic properties, *in vitro* and *in vivo* antioxidant activity and enhanced expression of insulin and proglucagon due to enhancing GLP-1 expression levels in STZ induced diabetic rats. The activities are attributed due to the presence of alkaloids, tannins, cardiac glycosides, flavonoids, saponins, steroids in *Tinospora cordifolia*.

Acknowledgements

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