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Research Article

PROTECTIVE EFFECT OF *CISSUS QUADRANGULARIS* IN STREPTOZOTOCIN INDUCED TYPE-2 DIABETES RAT MODEL AND ON SHSY5Y NEURONAL CELLS

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ABSTRACT

Type 2 diabetes is a metabolic disorder with decreased insulin secretion and insulin resistance, mainly affecting the metabolism of carbohydrates, protein, and fat. Chronic diabetes affects major organs like kidney, liver, and cardiovascular system. Brain is more sensitive to changes in glucose levels, hyperglycemia induces reactive oxygen species (ROS) and cause neuronal damage. In the present study ethanolic extract of *Cissus quadrangularis* (EECQ) was evaluated for its hypoglycemic activity in Streptozotocin induced diabetes in rats and hyperglycemia induced ROS generation in SHSY5Y neuronal cells. STZ (40 mg/kg, i.p) treated rats significantly altered the blood glucose levels in Oral Glucose Tolerance Test (OGTT) and serum, liver glycogen content, SGOT, SGPT levels, change in body weight, lipid profile and histology of pancreas. Oral administration of EECQ (100, 200, & 400 mg/kg, p.o) significantly reverted the effects of STZ and restored the changes induced by STZ to normal. Cytoprotective effect of EECQ (5, 50,100µM) on SHSY5Y neuronal cells were studied using MTT assay and ROS produced by hyperglycemia was evaluated using dye carboxy-H_2DCFDA. EECQ treated cells showed protective effect against STZ induced cytotoxicity and reduced the ROS generated by hyperglycemia. The present study suggests that the ethanolic extract of *Cissus quadrangularis* possess hypoglycemic and neuroprotective activity.

Key words: Streptozotocin, *Cissus quadrangularis,* Carboxy-H_2DCFDA dye, Hypoglycemia, SHSY5Y neuronal cells.

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INTRODUCTION

Diabetes, is a heterogeneous disorder characterized by hyperglycemia due to decreased insulin secretion and insulin resistance which results in long term complications such as atherosclerosis, retinopathy, hypertension, hyperinsulinemia, hyperlipidemia and fatty liver (Herman-Edelstein *et al.*, 2014; Tag *et al.*, 2012).

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The conventional drugs used in diabetes mellitus showed unwanted side effects like hypoglycemia, GI disturbances, weight gain, liver, and heart problems (Hung *et al.*, 2012). This lead to the focus of research on plant derived products for the treatment of diabetes mellitus and several studies have reported that the plant sources were found to be beneficial in treating several diseases with minimal side effects and less toxic than the synthetic drugs (Elvin-Lewis, 2001; Kumar *et al.*, 2015).
The role of insulin receptor signaling in brain and its regulation on hippocampal synaptic plasticity and cognitive function is the emerging area of research (Park, 2001; Figlewicz and Benoit, 2009). JK Morris *et al.*, 2016, have reported that insulin resistance was commonly observed in patients with cognitive impairment. Neuronal cells are more vulnerable to the changes in glucose

homeostasis. Hyperglycemia induces free radicals that damage the neuronal cells and alters the functions of insulin resulting in cognitive impairment. (Nishikawa *et al.,* 2000; Bonnefont-Rousselot 2002; Evans *et al.,* 2002). *Cissus quadrangularis* (CQ) is a climbing shrub, belongs to the family Vitaceae widely seen in various states of India, and other countries like Malaysia, Sri Lanka, and West Africa. In literature survey it was found that the plant commonly used for bone setting but numerous studies have reported that it possess plenty of medicinal uses (Fernandes and Banu, 2012). The present work was designed to study the protective effect of ethanolic extract of *Cissus quadrangularis* in STZ induced diabetes in rats and hyperglycemia-induced damage in SHSY5Y neuronal cells.

MATERIALS AND METHODS

Plant material and extract preparation:

Cissus quadrangularis was collected from local areas of Tirupati, shade dried and coarsely powdered. The powder was extracted with ethanol by simple soxhlation process after maceration with petroleum ether for 72 hrs. The obtained extract was concentrated and stored in desiccator.

Preliminary phytochemical studies:

The extract was tested for the presence of phytochemical constituents according to the standard procedures (Kokate, 2001).

α- glucosidase and α- amylase Inhibitory Assay:

α-glucosidase inhibitory activity of EECQ was carried out according to the method described by (Yao Yang *et al.,* 2009; Kim *et al.,* 2005), using multimode plate reader at 405nm. α -amylase inhibitory activity of EECQ was carried out according to the method (Bernfeld and Peter, 1955; Misbah, *et al.,* 2013) and the absorbance was measured at 540 nm using multiplate reader. All experiment procedures were performed in triplicates and IC50 values were determined.

Total phenol and flavonoid content:

The total phenolic content in the EECQ was determined according to Ovais *et al.,* 2014, by using gallic acid as standard. The total flavonoid content of the EECQ was also carried out according to Ovais *et al.,* 2014, by using Quercetin as standard. All procedures were carried out in triplicates.

Animals:

Male wistar rats weighing (160-180g) were obtained from Sree Venkateswara Enterprises, Bangalore, and were kept in animal house at temperature 22 ± 3^0 C with relative humidity 45-75% and maintained light/dark cycle (12/12 h). The animals were acclimatized to laboratory conditions for one week prior to the study. Food and water was provided *ad libitum* throughout the experimental period. The study was carried out in accordance and with prior approval from Institutional Animal Ethics Committee (IAEC) of SVCP, Tirupati.

EXPERIMENTAL DESIGN

Acute toxicity study:

Acute toxicity study of ethanolic extract of *Cissus quadrangularis* was performed as per OECD -423 guidelines (Acute toxic class method), and was found to be safe up to dose 2000mg/kg p.o.

Oral Glucose Tolerance Test (OGTT):

OGTT was performed in overnight fasted rats and the animals were divide into five groups (n=6). They were treated with vehicle (control group), Glibenclamide 5 mg/kg (standard group), and EECQ 100, 200, and 400 mg/kg/p.o respectively. After one hour, glucose 2g/kg was administered orally to all the groups and the blood was withdrawn from the tail vein at regular intervals 0, 30, 60, & 120 minutes. The blood glucose levels were measured with glucose strips using one touch glucometer (Tahara *et al.,* 2011).

Induction of diabetes in rats:

Diabetes mellitus was induced in rats by administration of freshly prepared STZ (40mg/kg/i.p) in 0.1 M citrate buffer at pH 4.5. After 4 days of STZ administration, blood glucose levels were estimated. Rats with fasting glucose levels ≥ 200 mg/dl were considered diabetes and included in the experimental study (Mohammed *et al.,* 2016; Li Zhang *et al.,* 2010).

Experimental design:

Rats were divided into six groups (n=6) and were treated with the extract suspended in 0.5% CMC (vehicle) for 2 weeks.

Group I: Normal control (vehicle 0.5% CMC)
Group II: Diabetic control (vehicle 0.5% CMC)
Group III: Glibenclamide (5mg/kg/p.o)
Group IV, V & VI received 100,200, & 400 mg/kg EECQ respectively.

Biochemical estimation:

After two weeks of treatment with EECQ, the blood was collected from the retroorbital plexus in overnight fasted rats under mild anesthesia. Blood samples were centrifuged at 4^0C at 10,000 rpm for 15 minutes and the serum was separated for estimation of blood glucose, liver enzyme levels (SGOT & SGPT), lipid profile (TC, HDL, TG) by using Erba Mannheim diagnostic kits. LDL and VLDL were calculated by Friedewald formula. After collection of blood, rats were sacrificed by cervical dislocation method, liver and pancreas were isolated for measuring the glycogen content and histopathological studies respectively.

Liver glycogen estimation:

According to Sam Seifter *et al.,* 1949, the tissue liver glycogen content was estimated by using anthrone reagent.

Histopathological studies:

The isolated pancreatic tissues were kept in 10 % formalin after washing with normal saline and tissues were sectioned to 4µm using microtome and stained with eosin and hematoxylin for histopathological analyses.

Cell viability analysis:

SH-SY5Y cells were kept in Dulbecos Modified Eagles Medium (DMEM) (Himedia) at 37^0C in a humidified 5% CO_2 incubator. Cell viability was evaluated using MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The different concentrations (5µM, 50µM, & 100µM) of EECQ were prepared and added into 96 well plate and incubated for 30 minutes. After incubation, streptozotocin (30mM) was added to these cells, later 15 mg of MTT was added to it. The cell viability was evaluated by direct observation of cells and by recording the absorbance at a wavelength of 570 nm in a microplate reader (Haider and Annie, 2012).

Hyperglycemia induced ROS:

Numerous studies have shown that hyperglycemia produces Reactive Oxygen Species (ROS) in brain that causes neuronal damage leading to cognitive impairment. ROS was measured spectrofluorimetrically by using dye carboxy-H_2DCFDA and the fluorescence was detected by using excitation and emission wavelengths 430nm and 580 nm respectively by TECAN multimode reader (Infinite 200 pro), (Rabii and Corinne, 2013).

Statistical analysis:

All data are presented as mean ± standard error mean of 6 animals. Data were analyzed statistically by graph pad prism 5 using Dunnett's 't' test, p values were considered significant at $p < 0.05$.

RESULTS

Effect of EECQ on phytochemical analysis:

EECQ showed the presence of Alkaloids, Flavonoids, Tannins, Sterols, Terpenoids, Carbohydrates, and Glycosides.

Acute Toxicity study:

The acute toxicity studies revealed that EECQ did not show any mortality and was safe up to 2000 mg/kg in rats. General behavior and appearance of rats were normal during the whole experimental period.

Total flavonoid and phenol content:

The total flavonoid content present in the EECQ was 2.19 ± 0.09 mg of quercetin equivalents /g dry material. The total phenolic content present in EECQ was 0.27 ± 0.21 mg of gallic acid equivalents /g dry material (Table-1).

α-glucosidase and α-amylase Inhibition Assay:

α-glucosidase and α-amylase inhibition assay was determined using acarbose as standard (positive control). The addition of different concentrations of EECQ decreased the function of α-amylase and showed IC_{50} at 175.66 ± 4.65 while acarbose showed IC_{50} value at 89.71 ± 0.65. Similarly EECQ reduced the activity of α-glucosidase at IC_{50} value of 12.01 ± 3.36 and acarbose showed IC50 at 2.04 ± 0.03 (Table-2).

Effect of EECQ on OGTT:

The figure-1 shows the OGTT of EECQ, the elevated blood glucose levels were significantly ($p < 0.05$) lowered by EECQ at 60 & 90 minutes in overnight fasted rats after glucose load.

Effect of EECQ on blood glucose, liver enzymes, liver glycogen content and body weight:

Administration of EECQ in diabetic rats reduced the blood glucose levels and significant ($p < 0.05$) effect was observed at dose 400mg/kg compared to diabetic rats. Diabetic rats treated with standard drug glibenclamide also significantly ($p < 0.05$) lowered the elevated blood glucose levels. Diabetic rats showed elevated levels of SGOT and SGPT, which was significantly decreased in rats treated with EECQ in dose dependent manner and in glibenclamide treated group. The significant effect was observed at dose 400 mg/kg with EECQ. The decreased liver glycogen content was restored to normal in glibenclamide and EECQ treated groups. Diabetic rats showed marked decrease in body weight, rats treated with EECQ restored the decrease in body weight to normal (Table - 3).

Effect of EECQ on lipid profile:

STZ treated rats showed increased levels of TC, TG, LDL, VLDL, and decreased levels of HDL cholesterol. Diabetic rats treated with glibenclamide and EECQ significantly ($p < 0.05$) reversed the elevated levels of TC, TG, LDL, VLDL, and increased the levels of HDL cholesterol. The EECQ at dose 400mg/kg showed significant ($p < 0.05$) effect similar to glibenclamide group (Table - 4).

Effect of EECQ on pancreas:

Histopathological studies showed that STZ significantly reduced the number and size of pancreatic beta islet cells. The treatment groups administered with glibenclamide and EECQ at 400 mg/kg significantly

improved the number of beta islet cells and their size by restoring the pancreas to normal (Figure - 5).

Effect of EECQ on SHSY5Y Neuronal Cells MTT assay and ROS:

The EECQ at different concentrations (5, 50,100 µM) were tested for its protective effect on SHSY5Y neuronal cells against STZ (30mM) induced neuronal damage. The EECQ at 100 µM significantly improved the cell viability and decreased the ROS generated by hyperglycemia (Figure-2, 3 & 4).

DISCUSSION

In the present study the ethanolic extract of whole plant *Cissus quadrangularis* was evaluated for its antihyperglycemic effect in STZ induced diabetic rats and for its neuroprotective activity in SHSY5Y neuronal cells against hyperglycemia induced ROS. STZ at dose 40 mg/kg/i.p induces insulin deficiency by partial alkylation of DNA in pancreatic beta cells producing diabetes in rats similar to type-2 diabetes in humans (Nishigak *et al.,* 1989).

The elevated levels of blood glucose in OGTT and in STZ induced diabetic rats were significantly reversed to normal in rats treated with ethanolic extact of *Cissus quadrangularis*. Moreover the EECQ showed potent inhibitory effect on α-glucosidase and α-amylase to that of standard acarbose. Several studies have reported that the polyphenols and flavonoids have potent antioxidant activity and also lower the post prandial glucose by inhibiting the enzymes α-glucosidase and α-amylase (Adisakwattana *et al.,* 2012). The EECQ showed potent inhibitory effect on α-glucosidase and α-amylase to that of standard acarbose.

Liver plays an important pivotal role in storage of excess glucose in the form of glycogen, decreased insulin secretion depletes the glycogen storage and also leads to dysfunction of lipid metabolism causing increase in TG, TC, LDL, VLDL and decreased HDL levels (Zhang *et al.,* 2003). Diabetic rats treated with EECQ improved the levels of HDL and decreased the levels of TG, TC, LDL, and VLDL.

The body weight of animals was drastically decreased in STZ induced rats. Diabetic rats treated with EECQ prevented the weight loss and improved the weight gain. The glycogen content in the liver greatly depends upon the uptake of glucose by liver. Decreased insulin levels diminished the liver glycogen storage which was reported in later stages of type-2 diabetes mellitus (Irimia *et al.,* 2010). The rats treated with EECQ showed increased glycogen content comparable to glibenclamide group which indirectly shows that EECQ improves the insulin levels in the blood.

Pancreatic tissue in STZ treated rats showed severe damage to the β- cells with decreased number of pancreatic islets. The EECQ treated groups restored pancreatic β- cell histology on par with glibenclamide.

To further investigate the protective effect of EECQ in brain, MTT assay was performed with STZ on SHSY5Y neuronal cells. MTT analysis revealed that EECQ treated cells showed increase in cell viability in a dose dependent manner and the morphology of SHSY5Y neuronal cells were reverted to normal. Hyperglycemia induced ROS was studied by using H_2DCFDA, an indicator for oxidative stress and EECQ treated cells showed reduced fluorescence suggesting its protective role in CNS.

RESULTS

Table 1. Effect of EECQ on total phenol and flavonoid content

Concentration of extract	Phenolic content (mg of gallic acid equivalent/ g dry material)	Flavonoid content (mg of quercetin equivalent/ g dry material)
EECQ (100µg/ml)	0.27 ± 0.21	2.19 ± 0.09

Values are mean ± SEM, n=3.

Table 2. Effect of EECQ on α-glucosidase and α-amylase Inhibition Assay

	α-glucosidase inhibition (IC_{50} µg/ml)	α-amylase inhibition (IC_{50} µg/ml)
Acarbose	2.04 ± 0.03	89.71 ± 0 .65
EECQ	12.01 ± 3.36*	175.66 ± 4.65*

Values are mean ± SEM, n=3. *p < 0.05 compared with control.

Table 3. Effect of EECQ on Liver enzymes, Liver glycogen Content, Blood glucose, and Change in Body Weight in STZ Induced Diabetic Rats:

Groups	SGOT (mg/dl)	SGPT (mg/dl)	Liver glycogen content (mg/100 mg wet wt.)	Blood Glucose (mg/dl)	Body weight (gm)	
					Initial	Final
Control	58.81 ± 1.47	36.75 ± 1.32	99.17 ± 2.56	112.7 ± 2.25	191.11 ± 0.25	198.81 ± 277
Diabetic Control	104.2 ± 1.86*	82.63 ± 2.31*	55.67 ± 3.21*	305.5 ± 3.81*	184.64 ± 1.90	162.51 ± 2.16
Glibenclamide (5mg/kg)	69.67 ± 2.28#	44.71 ± 1.92#	91.42 ± 1.76#	185.5 ± 3.24#	190.8 ± 3.74	199.2 ± 2.37
EECQ-100	96.33 ± 1.59#	74.67 ± 1.25#	66.14 ± 2.17#	238.7 ± 2.86#	189.72 ± 2.91	196.7 ± 2.13
EECQ-200	83.44 ± 2.23#	67.01 ± 2.14#	76.52 ± 1.89#	200.41 ± 3.04#	193.5 ± 2.38	198.01 ± 2.08
EECQ-400	75.91 ± 1.42#	54.68 ± 1.84#	92.74 ± 1.51#	154.07 ± 2.31#	192.2 ± 2.59	196.5 ± 2.36

All values are expressed as mean ± SEM for n=6 animals. Significance was determined by one way ANOVA followed by Dunnett's 't' test. *p < 0.05 compared with control, # p < 0.05 compared with diabetic control.

Table 4. Effect of EECQ on lipid profile:

Groups	TC (mg/dl)	TG (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	VLDL (mg/dl)
Control	60.4 ± 2.01	58.33 ± 1.93	42.67 ± 2.10	34.56 ± 2.24	18.4 ± 2.26
Diabetic Control	102.13 ± 2.52*	135.5 ± 2.61*	21.6 ± 0.99*	84.34 ± 2.81*	49.6 ± 2.65*
Glibenclamide (5mg/kg)	69.21 ± 1.32#	77.41 ± 1.68#	39.26 ± 2.34#	41.25 ± 2.51#	25.18 ± 1.98#
EECQ-100	90.8 ± 3.67#	104.14 ± 1.89#	26.6 ± 1.52#	70.47 ± 2.01#	40.72 ± 2.39#
EECQ-200	78.67 ± 3.99#	96.83 ± 2.91#	29.68 ± 1.78#	60.16 ± 3.26#	38.02 ± 2.12#
EECQ-400	70.33 ±0.92#	83.96 ± 1.01#	32.92 ± 1.63#	54.31 ± 1.59#	33.09 ± 1.62#

All values are expressed as mean ± SEM for n=6 animals. Significance was determined by one way ANOVA followed by Dunnett's 't' test. *p < 0.05 compared with control, # p < 0.05 compared with diabetic control.

Fig 1: Oral glucose tolerance test of EECQ in rats loaded with glucose 2gm /kg p. o.

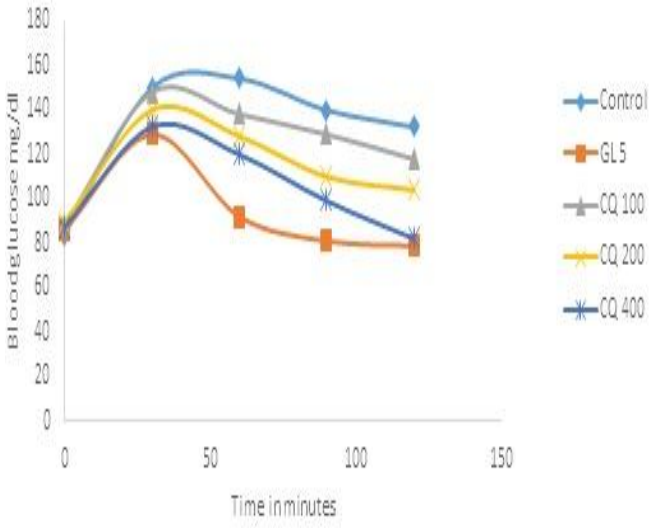


Fig 2: Effect of STZ (30mM) on SHSY5Y Neuronal cell

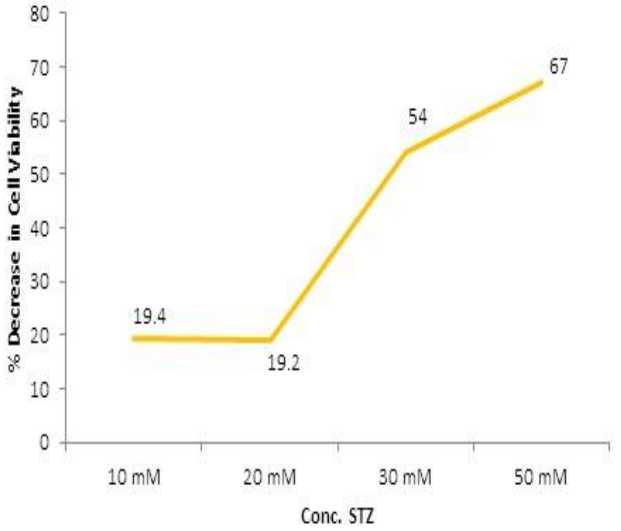


Fig 3. Effect of EECQ on SHSY5Y Neuronal cell

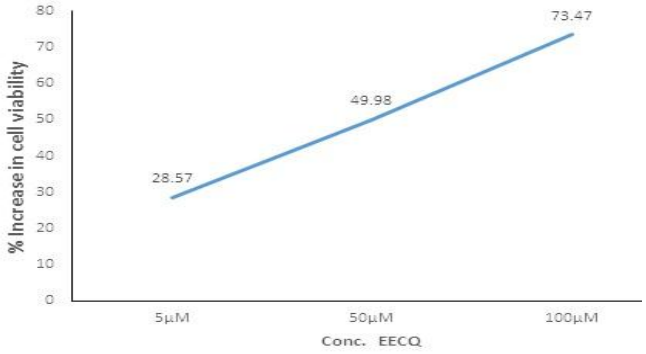
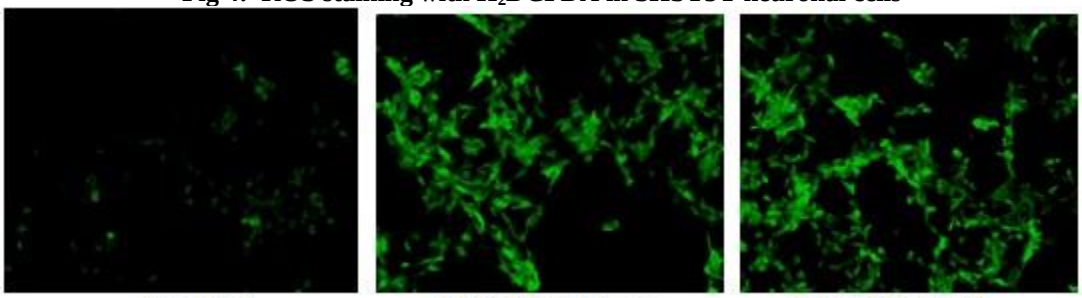


Fig 4: ROS staining with H_2DCFDA in SHSY5Y neuronal cells



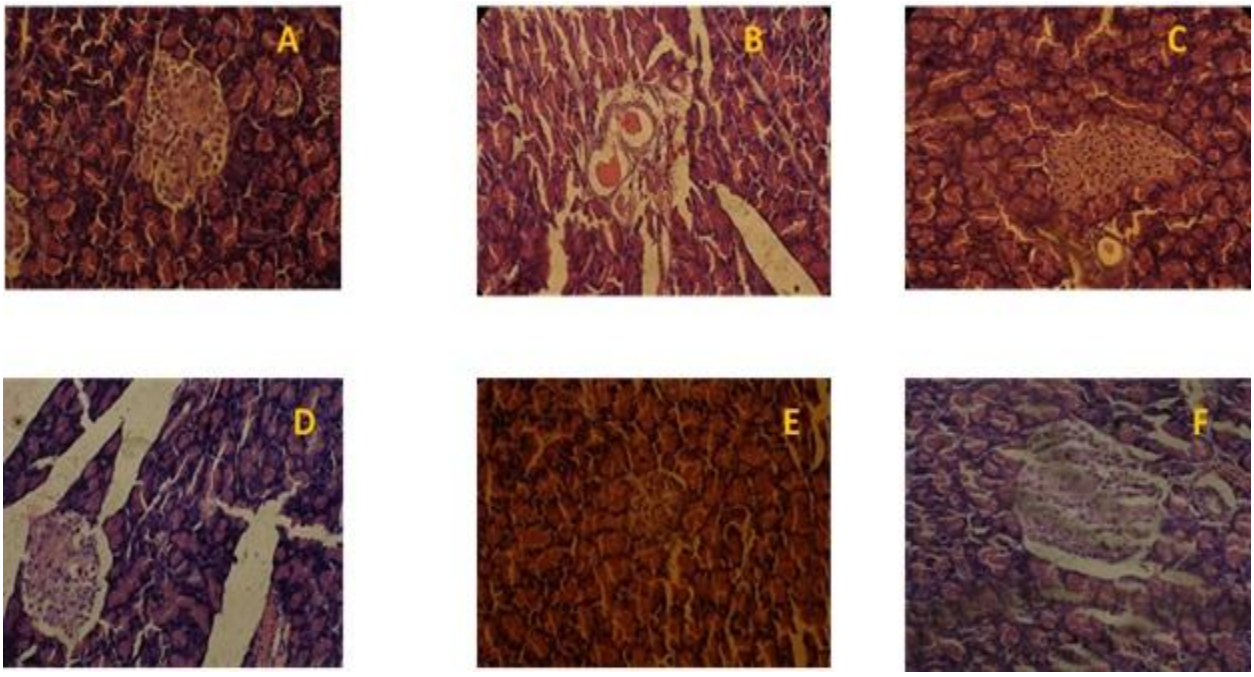


Fig 5: Histology of pancreas: Normal control (A), shows the intact beta islet cells and acinar cells. DC group (B), pancreatic cells were degenerated with decreased number of beta islets. Glibenclamide group (C), shows restored beta islet cells with increase in number. EECQ 100 (D), EECQ 200 (E), and EECQ 400 (F) treated rats dose dependently regenerated the pancreas and increased the number beta islet cells.

CONCLUSION

From the data obtained it was suggested that the ethanolic extract of *Cissus quadrangularis* have demonstrated its hypoglycemic activity by restoring the normal blood glucose, SGOT, and SGPT levels, improving weight gain in animals, reverting histology of beta cells, increasing the liver glycogen content, reducing hyperglycemia and dyslipidemia in diabetic rats. In SHSY5Y neuronal cells, STZ induced toxicity and ROS generation was ameliorated by the *Cissus quadrangularis* extract. This might due to the presence of polyphenols and flavonoids in the extract which has to be further supported by its mechanism of action and efficacy in human subjects.

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