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Studies on histopathological and immunohistochemical evaluation of *Trigonella Foenum-graecum* efficacy in streptozotocin induced diabetic wister rats

Shesharao, Suguna Rao, Nagaraja K and Manjunatha KP

Abstract
The present study was taken up to evaluate the efficacy of *Trigonella foenum graecum* individualy and in combination along with glibenclamide in streptozotocin induced diabetic rats for a period of 90 days. The various groups in this study included normal control (Group-I), diabetic control (Group-II), diabetic rats treated with glibenclamide (Group-III), diabetic rats treated with *Trigonella foenum graecum* (Group-IV), *Trigonella foenum graecum* and glibenclamide (Group-V), respectively. There was significant variation in histopathological and immunohistopathological parameters of diabetic rats when compared to normal control rats. The alleviation of the diabetic and its complications induced by streptozotocin was obseved in all the treatment groups with variable degree of improvement. *Trigonella foenum graecum* extracts was effective in alleviating streptozotocin induced diabetes and were comparable with glibenclamide. Combination of *Trigonella foenum graecum* with glibenclamide showed better improvement compared to individual extracts alone and improvement was statistically significant. However the combined treatment of *Trigonella foenum graecum* and glibenclamide half dose revealed a very good antidiabetic effect with reference to improvement in insulin level and beta cell number which indicated a synergistic effect between *Trigonella foenum graecum* and glibenclamide half dose.

Keywords: diabetes, *Trigonella foenum gaecum*, glibenclamide, streptozotocin

Introduction
Diabetes is a very complex disease in people and equally so in dogs and cats. It is one of the most frequently diagnosed endocrinopathies in cats and dogs and the incidence is increasing due to an increase in the frequency of predisposing factors such as obesity and physical inactivity in these animals. Type 1 diabetes mellitus is the most common type reported in dogs, whereas type 2 in cats especially in males. Diabetes has also been reported in many other species of animals but only rarely such as equines, bovines, ovine, swine, primates as well as birds.

To understand the pathogenesis and testing of therapeutic agents, appropriate experimental models are required. Diabetes animal models could be obtained through chemical, dietary or surgical manipulations. Recently, large numbers of new genetically modified animal models comprising transgenic, generalized knockout and tissue specific knockout mice have been extensively used for screening anti diabetic drugs (Frode *et al*., 2008) [9].

Currently available oral treatment includes sulfonylureas, biguanides, alpha – glucosidase inhibitors and glinides which are used alone or in combination with other drugs to result in better effect. Sulphonylureas are useful in the treatment of diabetes which cannot be controlled by diet or other available therapy. Tolbutamide, chlorpropamide, glibenclamide, tolazamide etc. are some of the important sulphanylureas which are absorbed rapidly from the intestine. Biguanides control all types of diabetes and reduce glucose absorption from the intestine and also can be used to treat mild diabetes associated with pregnancy. However, many of these oral antidiabetic agents have a number of serious side effects. Long-term treatment with sulphonylurea may desensitize the beta cells of the pancreas and high concentrations of sulphonylurea inhibit insulin biosynthesis *in vitro* and perhaps also *in vivo* (Anderson and Borg, 1980; Melander *et al*., 1987) [3, 15].

The limitations of currently available oral antidiabetic agents have encouraged a concerted effort to discover new drugs to manage type 2 diabetes more efficiently. Thus discovery and development of novel therapeutic agents and alternate strategy has become prime in the recent years.

Currently the focus is more on development of indigenous, inexpensive plant derived traditional antidiabetic treatment with no side effects. World health organization expert committee on diabetes has also recommended that traditional medicinal herbs be further investigated (Modak *et al*., 2007) [17].

Herbal medication has been used for the treatment of variety of ailments and a huge number of populations in the world are still entirely dependent upon traditional medicines. A number of medicinal plants and their formulations are being used for treating diabetes in Ayurvedic medicine system as well as in ethnomedicinal practices (Pareek *et al*. 2009) [19]. Since the time of Charaka and Sushrutha indigenous remedies have been used in the treatment of diabetes. From the ethnobotanical information, more than 1200 species of plants have been screened for antidiabetic activity on the basis of ethnomedicinal uses (Singh *et al*., 2011) [9] and about 800 plants with antidiabetic potential have been reported (Venkatesh *et al*., 2010; Singh *et al*., 2011 and Patel *et al*., 2012) [30, 27, 21]. The hypoglycaemic effect of some of the herbal extracts has been confirmed in human and animal models. However, the major drawback in usage of herbal medicine in modern medical practices is the lack enough of scientific and clinical data proving their efficacy and safety. There is a need for conducting clinical research, experimental evaluation in various animal models for their efficacy and safety, pharmacological and toxicological evaluation and long term studies. Also, there is a need for studies on replacement of oral antidiabetic treatment with herbal medicines by experimental research in animal models. Hence, the present study was conducted with *Trigonella foenum graecum* commonly known as methi which is reported to possess hypoglycaemic effect (Raju *et al*., 2001; Srinivasan, 2006; Khalki *et al*., 2010) [22, 28, 12]. to evaluate their antidiabetic effect individually and comparison with an oral antidiabetic drug glibenclamide.

1. To evaluate the antidiabetic effect of *Trigonella foenum graecum* in induced diabetes in rats.
2. To study histopathological and immunohistopathological changes in induced and treated diabetic rats.
3. To compare the hypoglycemic effects of *Trigonella foenum graecum* with an oral hypoglycemic agent glibenclamide.

Martials and Method

Experimental animals: Healthy female albino Wistar rats weighing 190±20g obtained from R.L Instrument and lab animal supplier, Yeswanthpur, Bangalore, were used for the present investigation. The animals were maintained under standard laboratory conditions, with provision of standard laboratory animal feed (Amruth Feeds, Banglore) and clean drinking water *ad libitum*. The animals were acclimatized to the experimental conditions for two weeks after procurement. After acclimatization, animals were grouped and housed in polypropylene rat cages during the experimental period. The experiment was carried out for a period of 90 days. The study was carried out with a prior approval from the Institutional Animal Ethical Committee, Veterinary College, Bangalore-560024.

Drugs and chemicals: To induce experimental diabetes in rats, streptozotocin was used which was procured from Sigma Chemicals, St. Louis, USA. All the other chemicals used for the study were of analytical grade.

Preparation of STZ solution: Fresh 0.1 M citrate buffer having pH 4.5 was prepared and the same was maintained at 4-8 ^{0}C. The STZ of required quantity was dissolved in ice-cold citrate buffer and injected intraperitoneally to rats immediately to avoid degradation.

***Trigonella foenum graecum*:** The alcoholic seed extract of *Trigonella foenum graecum* used in the present study was obtained from Plantex Herbal Drug Company, Vijaywada. The powdered extract was weighed according to body weight and dissolved in distilled water to make the final concentration and given to the expimental animals.

Glibenclamide solution: Glibenclamide (Daonil®, 5 mg), an oral hypoglycaemic drug was dissolved in distilled water (82.33 ml) to give a concentration of 60 µg/ml. This was used as a stock solution and administered orally at a dose of 600 µg/ kg using clean and dry gavaging needles (Ramalingam *et al*., 2004) [23].

Administration of plant extracts and glibenclamide: Throughout the period of the experiment the plant extracts and glibenclamide were administered orally for their respective groups using clean gavaging needle attached to an appropriate disposable syringe during morning hours of the day for a period of 90 days.

Experimental design: Sixty female albino Wistar rats were weighed and randomly distributed into five groups of twelve rats each. Care was taken to maintain the intra-group weight variation to be less than 25 g and inter-group weight variation by 35 g.

The groups and treatments used were as follows:

Group I (NC)	Normal control:- Used for studying baseline values of the parameters
Group II (DC)	Diabetic control:- Streptozotocin induced diabetic rats
Group III (GC)	Diabetic rats supplemented with glibenclamide at a dose of 600 µg / kg
Group IV (TFG)	Diabetic rats supplemented with extract *of Trigonella foenum graecum* at the dose rate of 1g/kg body weight.
Group V (TFG +G)	Diabetic rats supplemented with extract *of Trigonella foenum graecum* and Glibenclamide at the dose rate of 1g/kg, and 300 µg /kg body weight respectively.

Experimental induction of diabetes: The animals were fasted overnight and diabetes was induced in Groups II to IX by a single intra peritonial injection of a freshly prepared solution of streptozotocin (45 mg/kg body weight) in 0.1 M cold citrate buffer having a pH of 4.5 (Babu and Prince, 2004). The normal control animals received citrate buffer alone.

Confirmation of diabetes: The diabetic state was confirmed by estimating the blood glucose levels after 72 hours of STZ injection using ready to use Span diagnostic kit with semi-automatic biochemical analyzer. The animals that showed the blood glucose level above 200 mg/dl were considered as diabetic. After confirmation of diabetic state, the treatment was commenced.

The rats of group I and II were gavaged only with normal saline and the rats of all other groups with their respective treatments daily for 90 days. The animals were observed daily for recording treatment effect.

Sacrifice of animals: To study the progressive effects of the treatments given to different groups, two rats from each group were sacrificed under light ether anaesthesia on Day 15 and 30, 6 animals on 45th day and remaining two rats on Day 90 of the experiment. Sacrificed animals were subjected for detailed post mortem examination and gross changes, if any were recorded. Further, representative tissue samples from pancreas, were collected in 10 per cent neutral buffered formalin (NBF) for histopathology and immunohistochemistry.

Histopathological studies: The pancreas were instantly dissected out, excised and rinsed in ice cold saline solution. The tissues were 10% formalin was used as a fixative and paraffin wax was used for embedding the tissues. Paraffin section were taken (5µm thick) and stained with Haematoxylin and Eosin (H & E) for histopathological examination. After dewaxing, all slides were examined under a light microscope for pathological studies.

Immunohistochemical detection of insulin in the pancreatic islets: Sections of pancreas were subjected for immunohistochemistry to demonstrate insulin in the β-cells of islets of Langerhans using polyclonal antibody raised against insulin antigen.

Immunochemicals: Primary antibody: Ready to use Flex Polyclonal Guinea Pig Anti-Insulin (Code No. IS002) shown to react with insulin antigen was procured from Dako Cytomation, Denmark. It was stored at -20 ^{0}C until used.

Secondary antibody: Polyclonal Rabbit Anti-Guinea Pig Immunoglobulins conjugated with HRP (Horse Raddish Peroxidase) known to detect guinea pig immunoglobulins bound to antigen in tissue sections was procured from Dako Cytomation, Denmark and was used at a dilution of 1:75. It was stored at -20 ^{0}C until end.

Section adhesive 3-aminopropyltriethoxy-silane (APES): Procured from Sigma chemicals, USA.

Hydrogen peroxide (H_2O_2) in methanol (3%): Three per cent H_2O_2 in methanol was prepared by adding one ml of 30 per cent H_2O_2 in 9 ml of methanol.

Citrate buffer (pH-6): 50 ml of 0.1M citric acid solution was prepared by dissolving 1.051 g of citric acid (MW =210.14) in 50 ml of distilled water. 100 ml of 0.1M sodium citrate solution was prepared by dissolving 2.941 g of sodium citrate in 100 ml of distilled water.1000 ml of 0.01M citrate buffer was prepared by adding 18 ml of 0.1M citrate solution and 82 ml of 0.1M sodium citrate solution to 900 ml of distilled water. The pH was adjusted to 6.0 with 1N NaOH.All the solutions were prepared freshly just before the use.

DAB plus substrate: 3,3-diamine benzidine tetrahydrochloride substrate was prepared freshly at the time of use by addition of 1 mg of 3,3-diamine benzidine tetrahydrochloride (Sigma Chemicals, USA) in 1 ml of 0.01 M PBS to which 12 µl of 3 per cent H_2O_2 was added.

0.01M phosphate buffer saline (pH-7.2): 10X concentration of 500 ml PBS was prepared by adding the following chemicals. Sodium chloride (MW 58.44) 40 g. Potassium Chloride (MW 74.56) 1 g. Disodium hydrogen orthophosphate (MW 141.96) 7.2 g. Potassium dihydrogen orthophosphate anhydrous (MW 136.09) 1 g. Distilled water 500 g.1X concentration of wash buffer was prepared using 10X PBS by adding 25ml of 10X PBS to 225ml of distilled water. To this 125µl of Tween 20 was added and the pH was adjusted to 7.2.

Harris haematoxylin for nuclear staining (Luna, 1968) [14]: Harris haematoxylin was used for nuclear staining. Counter staining was carried out for 45 seconds.

Preparation of organosilane (Apes) treated slides for IHC: The slides were placed on racks, washed thoroughly in soap water, rinsed in tap water and finally rinsed in distilled water. The slides were allowed to dry completely. A 2% solution of 3-aminopropyltriethoxy-silane (APES) in acetone in a dry staining dish was prepared. The slides were immersed in the APES solution for 5-15 minutes. The slides were rinsed in acetone and then rinsed in two changes of distilled water. Slides were allowed to dry at 37°C for two hours and then stored at room temperature until used.

Method: Tissue sections were mounted on 3-aminopropyltriethoxy-silane (APES) coated slides and dried at 37 °C for three hours. Later stored at 40°C until its usage. The paraffin tissue sections were deparaffinized using xylene and rehydrated using descending grades of ethanol. Endogenous peroxidise was blocked by covering the whole section with 3 per cent of H_2O_2 in methanol (100 µl) and incubated at room temperature for fifteen minutes. Later washed in three changes of wash buffer. Heat induced epitope retrieval (HIER) was carried out by immersing tissue sections in a cooker containing citrate buffer (pH 6.0) and was cooked for 6 minutes after maximum pressure was attained. Sections were allowed to cool down to room temperature for approximately 30 minutes. Later washed in three changes of wash buffer.

Addition of primary antibody: Ready to use Flex Polyclonal Guinea Pig Anti-Insulin was added to cover the sections. Subsequently the sections were incubated at room temperature in humidified chamber for one hour and washed with wash buffer as mentioned earlier.

Addition of secondary antibody (Polyclonal Rabbit Anti-Guinea Pig Immunoglobulins conjugated with HRP): The whole section was covered with secondary antibody and incubated at room temperature in humidified chamber for 30 minutes. After incubation sections were washed with PBS as mentioned earlier.

Addition of DAB plus substrate: Freshly prepared 3,3-diamine benzidine tetrahydrochloride (DAB) with 3 per cent H_2O_2 was poured to cover the section and incubated for 15-20 minutes or until the desired stain intensity was achieved. Later the sections were washed again with three changes of distilled water. Nuclear counter staining with Harris haematoxylin was carried out for 45 seconds. The sections were washed in distilled water, dehydrated with ascending grades of ethanol and cleared with xylene and cover slipped with DPX mounting media. Pancreas of non-diabetic control rats was used as a positive control for standardization of the technique.

Percentage positivity of insulin secreting cells: To determine the percentage positivity for insulin production, the number of insulin positive cells immunohistochemically in 1000 β-cells (approximately 10-12 islets) were counted under high magnification and was expressed in percentage.

Result and Discussion

The present study was conducted to evaluate the antidiabetic efficacy of *Trigonella foenum graecum* extracts in STZ induced diabetes in rats and to compare the hypoglycemic effects with an oral hypoglycemic agent glibenclamide.

Histopathology

Normal control group (Group-I): The animals belonging to control group remained healthy throughout the experimental period. All the values of various parameters analysed were within the normal range and indicated their healthy status (Plate 1 and 3).

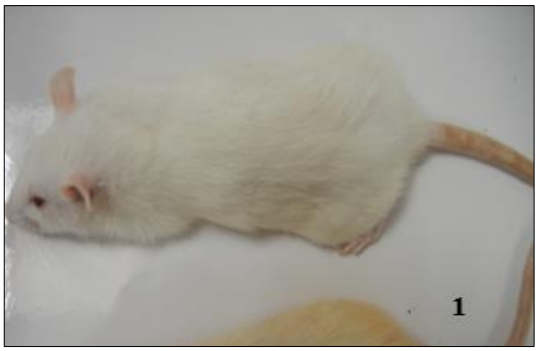

Plate 1: Normal control animal showing good body condition and shiny hair coat on Day 90 of the study.

Plate 2: Diabetic induced by Streptozotocin treated group rat on 15th day of the experiment. Note the variation in body size, condition and hair coat.

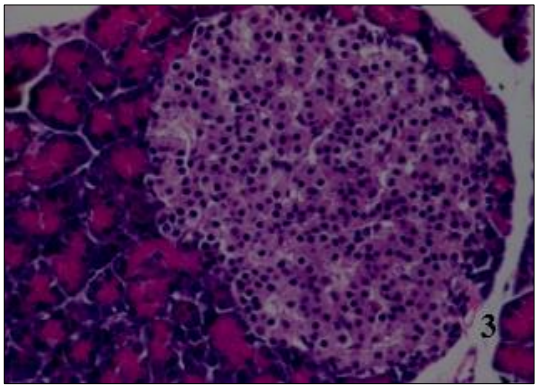

Plate 3: Section of pancreas of normal control showing a normal islet with round to oval shape and compact arrangement of beta cells at the centre and alpha cells at the periphery. H&E X 200

Group-II (Diabetic control): In the present study, on 15th day of post STZ injection, affection of both exocrine and endocrine components of pancreas was observed. The exocrine portion revealed loss of normal lobular architecture characterized by reduction in the lobular size and widely separated out lobules. The widened interlobular space revealed presence of edema fluid, haemorrhage and mild infiltration of mononuclear infiltration perivascularly. The blood vessels were severely congested. The acinar epithelial cells were highly vacuolated, degenerated and necrotic with loss of zymogen granules. In some lobules complete loss of acinar cells was observed (plate 2 and 4).

In the endocrine component, pancreas revealed reduced number of islets per lobule with loss of normal architecture. There was loss of demarcation between islets and the surrounding exocrine portion and the islet cells were observed to be infiltrating into exocrine portion.

On 30th day, the lesions were similar to those of 15th day with disorganized exocrine component and islets. The β cells were highly vacuolated and necrotic and there was mild α cell hyperplasia in some of the islets. In addition mild ductular hyperplasia was also observed.

On 45th day, damage to the exocrine portion persisted with slight improvement in the number and size of islets. Some of the islets were hyper cellular with increase in the number of α-cells. The islets showed a 'star fish' appearance with infiltration of the islet cells into the surrounding exocrine portion in different directions. There was absence of cells with normal β-cell morphology and showed persistence of occasional highly vacuolated β-cells. Apoptotic cells were observed both in endocrine and exocrine portion of the pancreas. The histopathology of islets of Langerhans in diabetic rats indicated progressive destruction of β-cells from 15th to 45th day of investigation.

On 90th day of the experiment, there was an improvement in the architecture of exocrine portion with regeneration of acinar cells. There was complete absence of cellular damage and revealed formation of compact lobules comprising of hyperplastic exocrine tissue characterized by densely packed exocrine glands with hyperchromatic nucleus, basophilic scanty cytoplasm and absence of zymogen granule formation. Only the centrally placed exocrine glands in the lobules revealed varying amount of zymogen granule formation (Plate 5).

The morphological appearance of islets was also improved and appeared larger in size as well as increased in number. The islets were irregular, compact and hyper cellular. In addition there was mild to moderate degree of hyperplasia of ductular epithelial cells traversing into the exocrine portion with formation of small cluster of cells and ducts. Small sized newly formed islets were also observed adjacent to the hyperplastic ducts.

The diabetic control rats in the present study microscopically revealed affection of both exocrine and endocrine components of pancreas. The exocrine portion showed loss of normal lobular architecture characterized by reduction in the lobular size and wide separation of lobules with presence of edema fluid, haemorrhage and mild infiltration of mononuclear cells. The blood vessels were severely congested. The acinar epithelial cells were highly vacuolated, degenerated and necrotic with loss of zymogen granules.

Injury to the endocrine component of pancreas in the present study could be attributed to STZ which causes direct irreversible damage to β-cells of pancreatic islets of

Langerhans, resulting in degranulation and loss of insulin secretion, as also reported by Li *et al*., 2000; Babu and Prince, 2004; Jelodar *et al*., 2005; Mir *et al*., 2008; Selvan *et al*., 2008; Dhanush, 2009; Atangwho *et al*., 2010 and Mudasir, 2011[13, 5, 11, 16, 25, 7, 4, 18].

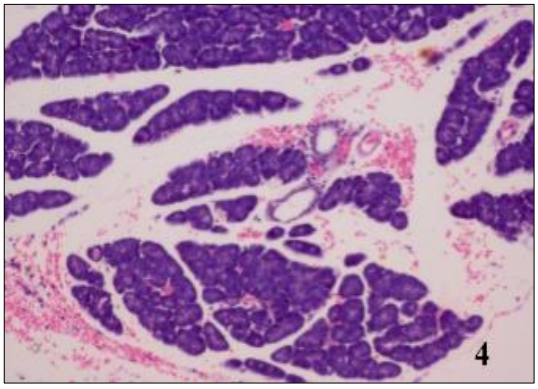

Plate 4: Pancreas of diabetic animal showing loss of normal architecture, atropic and vacuolated acinar cells with vacuolated and degenerating Islet cells on 15th day of the experiment. H&E X 200

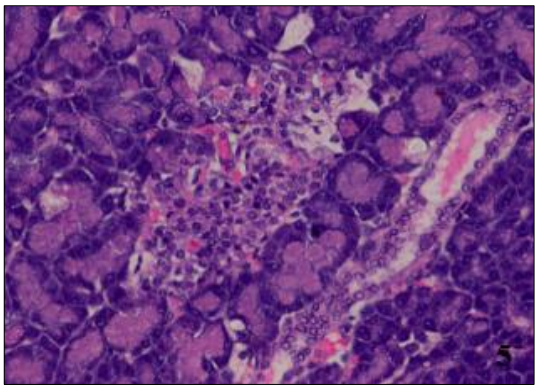

Plate 5: Pancreas of diabetic control animal showing complete loss of normal architecture with shrunken and vacuolated Islet cells on Day 30 with increase in alpha cells. H&E X 200

Group-III (Glibenclamide treatment group): In glibenclamide treatment group, on 15th day the microscopic changes were similar to those observed in diabetic control group. There was affection of both exocrine and endocrine portion. The exocrine portion revealed loss of normal architecture of pancreatic lobules with necrosis, severe vacuolations of acinar cells and wide separation of lobular tissue by edema and haemorrhage. The islets were reduced in number per lobules, irregular and smaller in size and revealed typical STZ induced lesions characterized by presence of swollen and highly vacuolated and necrotic beta cells.

By 30th day post treatment, there was an appreciable improvement in the shape, number and size of the islets which were well demarcated from exocrine portion. Small sized newly formed islets were observed in the midst of exocrine portion adjacent to intra lobular pancreatic ducts. The islets were compact and showed hyper cellularity with alpha cells however several round cells with cytoplasmic granularity resembling beta cells were also present. Occasional highly swollen and vacuolated cells indicating STZ effect were also observed in a few islets.

By 45th day of treatment the microscopic picture was similar to that of 30th day which also showed ductular hyperplasia extending into the exocrine portion as individual or small clusters of cells and presence of newly formed beta cells in small number adjacent. Some of the β-cells in the islets revealed well formed cytoplasmic granularity. However, there was persistence of STZ effect in a few cells.

On 90th day of treatment in addition to the changes observed at 45th day, there was more progressive ductular epithelial hyperplasia comprising endocrine cells either within or in adjacent area. The exocrine portion was compact and the islets revealed normal architectural details with cord like arrangement of the cells (Plate 6).

Guiot *et al.* (1994) and Wang *et al.* (2008) [10] attributed the improvement to increased proliferation as well as recruitment of subpopulation of β cells and thereby increase in the β cell mass upon treatment with glibenclamide. This could be possibly due to insulin induced regeneration of endocrine pancreas, resulting in improved histological appearance, size and number of islets (Adewole and Ojewole, 2007) [2] In addition, as indicated by Paris *et al.* (2004) [20], the improved β-cell mass could be on an account of β-cell neogenesis from ductal epithelial cells. This was well substantiated by ductal hyperplasia and presence of newly formed small clusters of β-cells or individual cells adjacent to ductal hyperplastic tissue with insulin synthesis observed microscopically and histochemically in the present study.

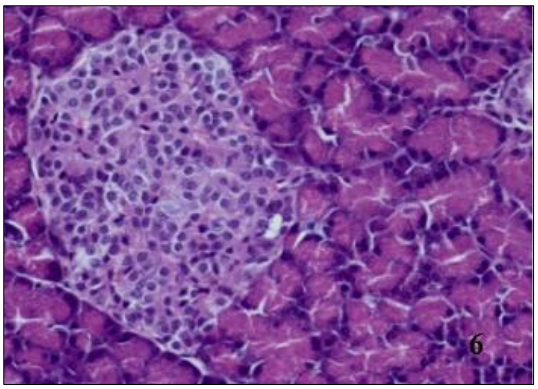

Plate 6: Section of pancreas from glibenclamide treated animal showing compact arrangement of islet with increase in cellularity on Day 90 of the study. H&E X 200

Group IV– diabetic rats treated with *Trigonella foenum graecum*: In the present study in Groups IV treated with *Trigonella foenum graecum* showed microscopically a progressive and significant improvement in morphology of pancreatic islets from 15th to 90th day of the experiment (Plate 7).

The exocrine portion revealed vacuolar and necrotic changes similar to that of diabetic control group in the acinar cells on 15th day of treatment and the architecture improved and was well maintained on 30th, 45th and 90th day of the study.

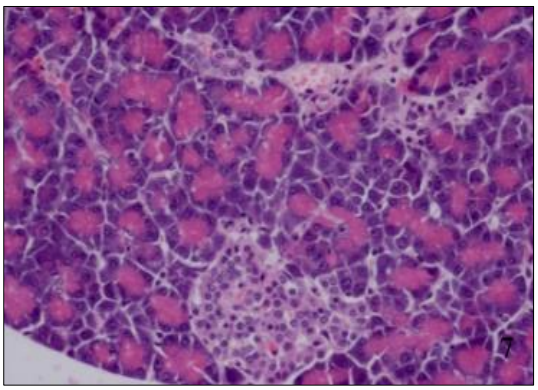

Plate 7: Section of muscle from a diabetic rat treated with *Trigonella foenum* graecum rat appearing normal on 90th day. H&E X 200

Group V– diabetic rats treated with *Trigonella foenum graecum* along with Glibenclamide half dose

Microscopically Group V rats treated with *Trigonella foenum graecum* along with Glibenclamide half dose showed a progressive and significant improvement in morphology of pancreatic islets from 15th to 90th day of the experiment (plate 8).

The exocrine component of pancreas appeared normal throughout the study. On Day 15, the islets were more in number and were hyper cellular with more number of alpha cells and comprised occasional highly vacuolated and swollen cells. The exocrine component was compact.

On Day 30, 45 and 90 the islets were more in number and comparatively larger than those of the 15th day. The islet morphology was normal with normal distribution of alpha cells at the periphery and beta cells at the centre of the islet. There was a progressive increase in the number of beta cells from 30th day to 45th day and remained comparable to 45th day on 90th day. There was also hyperplastic change involving duct epithelium on 30th, 45th and 90th day.

Histopathologically, in pancreas there was a progressive reconstruction of normal architecture of islets from Day 15 to Day 90 post treatment in *T.foenum graecum* alone as well as in combination with glibenclamide half dose in the present study. In comparison, the improvement in the pancreatic architecture was better in combination group suggesting a synergistic effect between Trigonella and glibenclamide in modifying the tissue architecture.

The increase in both alpha and beta cells from 30th day was confirmed by special staining technique as well as by immunohistochemistry in the present study. There was also ductal epithelial hyperplasia which was progressive in nature with formation of new small islets within the duct or in the adjacent area which were positive for insulin immunohistochemically. New formation of insulin positive islets cells indicated beta cell neogenesis and possible role of biological component of Trigonella seeds in islet cell regeneration during treatment of diabetes. The results of the present study were in accordance with those of Zafar *et al.* (2009) and Eman and Elaziz (2011) [31, 8] who also observed restoration of histological architecture of islets of Langerhans on treatment with *T.foenum graecum* extract in diabetic rats. They attributed the restoration of islets to regeneration and repair of damaged beta cells by the stimulating effect of 4-hydroxyisoleucine of *T.foenum graecum* extract.

In the present study, hyperplastic change involving the exocrine portion was also observed with formation of acini lined by cells with hyperchromatic nuclei and basophilic cytoplasm in which the zymogen granule formation was sparse or nil which could also be attributed to the biological effect of 4 HI of *Trigonella foenum graecum*.

A slightly better improvement in the morphology of the pancreas with respect to endocrine and exocrine component in treatment group with Trigonella and glibenclamide half dose indicate a synergistic effect in improving the architecture with the mechanisms specific for them.

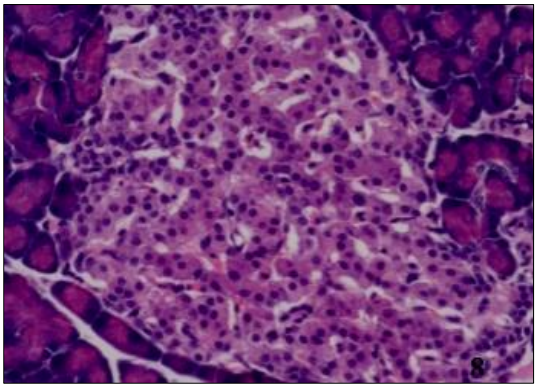

Plate 8: Pancreas from a diabetic rat treated with combination of *Trigonella foenum* graecum and glibenclamide showing large and well formed islet on Day 90 of the treatment. However note a few apoptotic cells. H&E X 200

Immnohistochemical evaluation of Insulin secretion by beta-cells

In the present study, immunohistochemical demonstration of insulin was carried out to evaluate insulin secretory function of β-cells in various treatment groups using polyclonal anti-insulin antibody. Appearance of dark brown granular staining of cytoplasm of β-cells was considered as positive reaction and based on the level of expression and percentage of cells showing positivity, the functional status was evaluated (Plate 9).

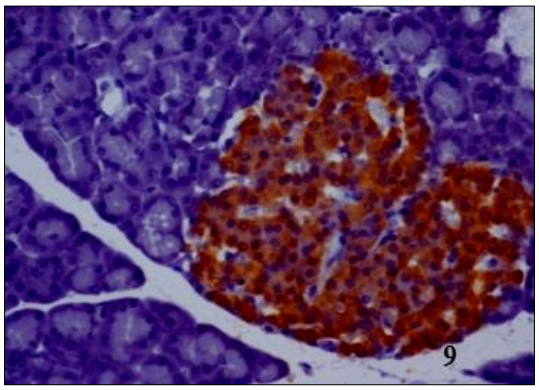

Plate 9: Islet of langerhans from a normal control animal showing intensely stained insulin positive beta cells in large number. IHC X 200

Group-I (Normal control group): In the normal animals, all the islets revealed intensely positive cells in large number which showed granular brown staining restricted to cytoplasm. The granules were compactly arranged, limited by a regular membrane. The nucleus was unstained and appeared lightly basophilic. The α-cells and exocrine component were negative for immunostaining. The mean percentage value of insulin secretory cells was found to be 79.50± 2.05 on 15th, 30th, 45th and 90th day of the present study respectively.

In the present study, immunohistochemical staining of pancreas for insulin revealed polyhedral shaped beta cells with dark brown coloured granules in the cytoplasm with unstained round to oval shaped nucleus. The functional beta cells revealed a large number of granules densely occupying

the cytoplasm which varied in their size. Occasional beta cells with sparsely scattered granules were also observed. In control rats the mean percentage value of beta cells with insulin was 78.74 ±0.92. In a normal individual the beta cells are the most abundant cells found in the islets constituting approximately 80 per cent of the endocrine cells with 15 per cent of alpha cells, 4 per cent of delta cells and 1 per cent of pancreatic polypeptide cells.

Group-II (Diabetic control group): The islets of Langerhans in pancreas of diabetic animals revealed a drastic reduction in the number of insulin positive cells. The insulin positive cells appeared irregular with light coloured scattered of granular material in the cytoplasm. The intensity of colouration of the granules was light (Plate 10). Some of islets with complete absence of insulin immune reaction were also observed.

The mean percentage positivity of insulin secretory cells at 15th, 30th, 45th and 90th day of the experiment were 3.50 ± 0.50, 2.50 ± 0.50, 2.83 ± 0.31 and 3.00 ± 0.00 respectively which were significantly lesser (P≤0.001) in comparison with that of normal control group on all the days of observation (Plate 10 and 11).

In the present study, there was a drastic decline in the number of insulin positive cells in the islets of diabetic rats due to the specific destruction of beta cells by STZ. However, a small number of cells with mean percentage value of 3.50 ±0.33 showing immunopositivity were observed which indicated survival of a few cells with retained insulin secretory function even after STZ treatment (Bolkent *et al.,* 2005; Adewole and Ojewole, 2007 and Adeghate *et al*., 2010) [6, 2, 1,]. This was proved by Adeghate *et al.* (2010) [1] ultrastructurally, who demonstrated intact cytoplasmic organelles such as RER with little or no secretory granules in a few β-cells which indicated that not all cells after STZ treatment become necrotic. In addition the immature β-cells which do not express GLUT2, escape STZ cytotoxicity which is the route of entry of STZ into the cells (Schnedl *et al*., 1994 and Thulesen *et al*., 1997) [24, 29].

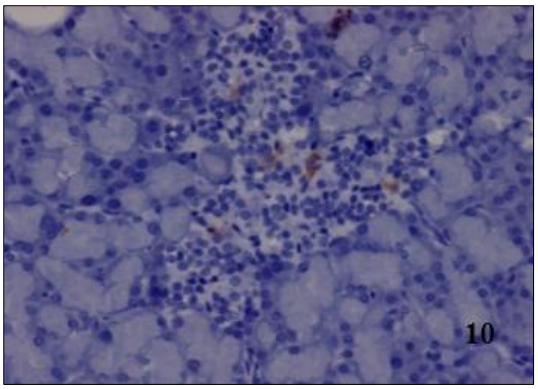

Plate 10: Islet of Langerhans from the diabetic control animal showing few insulin positive cells on Day 30 of the study. IHC X 200

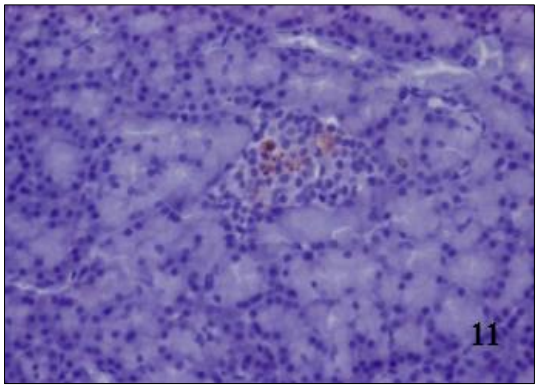

Plate 11: Islet from diabetic control animal showing mild immunoreactivity for insulin at Day 15 of the experiment. IHC X 200

Group-III (Diabetic rats treated with glibenclamide): In the glibenclamide treated diabetic rats there was a progressive increase in the number of insulin positive cells from 15th to 90th day of treatment. On 15th day the number of insulin positive cells was less and was comparable to that of diabetic control rats. On 30th day of the experiment, a drastic increase in the number of insulin positive cells per islet was observed which were comparatively larger, compact and darkly stained. However a few cells revealed dispersion of granules which were lightly stained. On 45th and 90th day a further increase in the number of beta cells was observed. However, the values were significantly lesser compared to normal control rats. In some beta cells the insulin granules appeared to be marginated to the cell membrane (Plate 12). From 30th day onwards small clusters or individual insulin positive cells were found within the ducts, adjacent to ducts as well as within exocrine portion of pancreas. The mean percentage of insulin positive cells was 7.50±0.50, 27.00±1.00, 42.33±1.68and 46.50±1.50 on 15th, 30th, 45th and 90th day of experiment respectively which were significantly higher (P≤0.001) compared to diabetic rats but significantly lesser (P≤0.001) in comparison with that of normal control rats (Plate 12).

In the glibenclamide treatment group, there was a progressive increase in the number of immune positive cells The findings indicated that glibenclamide improved the number of beta cells in STZ diabetic rats on treatment by 30th day itself and reached maximum by 90th day. However the increase in the number of insulin positive beta cells did not reach the number of normal control rats. The possible reason for the increase in the number could be sulphonylurea induced insulin release from surviving cells which in turn stimulate and enhance beta cell proliferation, maturation and functional activity of cells, as experimentally proved by Ling *et al*. (2006) and Adewole and Ojewole (2007) [2].

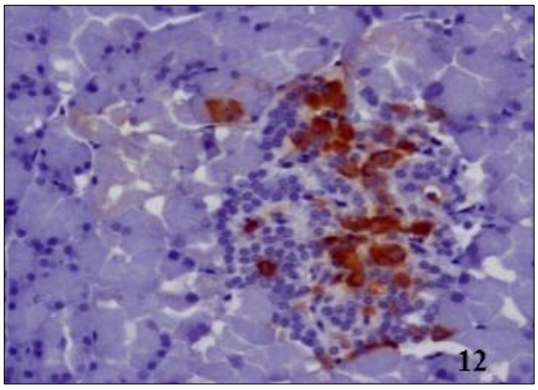

Plate 12: Islet from the animal treated with glibenclamide showing improvement in immunoreactive cells on Day 30. IHC X 200

Groups IV – diabetic rats treated with *Trigonella foenum graecum*,

The immunohistochemical examination in diabetic rats treated with *Trigonella foenum graecum*, revealed progressive improvement in the number of insulin positive cells from 15th day to 90th day (Plate 13).

The insulin immune positive cells on 15th day were lightly stained and significantly more ($P < 0.001$) than that of diabetic group. The number of insulin positive beta cells increased on 30th, 45th and 90th day. The beta cells were large, polyhedral and consisted darkly stained granular cytoplasm resembling normal beta cells. The cells were concentrated more at the centre of the islet. However, a few cells were also observed at the periphery. Insulin immune positive cells were also observed in the exocrine portion as well as within and adjacent to the ductal structures.

The immunohistochemical examination in diabetic rats treated with *Trigonella foenum graecum* revealed progressive improvement in the number of insulin positive cells from 15th day to 90th day. The improvement in immunostaining of β-cells coincided with an improvement in the serum insulin level.

The mean percentage number of insulin positive cells was significantly higher ($P \leq 0.001$) in comparison with that of diabetic control group and the percentage values were comparable to those of groups III, and IV but lesser than the combined groups V. The increase in the number of beta cells could be attributed to the beta cell regeneration or repair induced by 4-hydroxyisoleucine and antioxidant effect of *T.foenum graecum* extract (Shah *et al*., 2009; Zafar *et al.*, 2009 and Eman and Elaziz *et al*, 2011) [26,31, 8].

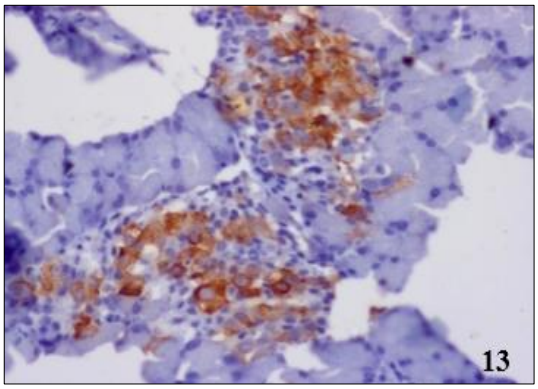

Plate 13: Islet from the animal treated with Trigonella foenum graecum showing improvement in the number of immunoreactive cells on Day 90. IHC X 1000

V– diabetic rats treated with *Trigonella foenum graecum*, and their combination with Glibenclamide half dose

The diabetic rats treated with *Trigonella foenum graecum*, individually and in combination with half dose of glibenclamide showed a progressive increase in the number of insulin positive cells from 15th to 90th day of the experiment. Insulin immune positive cells were also observed in the exocrine portion as well as within and adjacent to the ductal structures which were intensely stained (Plate 14).

The percentage of insulin positive cells was found to be 17.50±1.50, 52.00±1.00, 73.33±4.43 and 76.00±1.00 in Group V on 15th, 30th, 45th and 90th day respectively.

In comparison with diabetic control the percentage of insulin positive cells was significantly ($P \leq 0.001$) higher on all the days of observation and the diabetic rats of Group V treated with *Trigonella foenum graecum* in combination with glibenclamide at half dose showed a significant increase in the number of insulin positive cells from 15th to 90th day of the experiment. The number of insulin positive beta cells drastically increased from 30th to 90th day. The beta cells were large, polyhedral and consisted of varyingly stained granular cytoplasm in intensity with a morphology and distribution similar to that of normal beta cells. The number of insulin positive cells was comparable to that of normal control. The cells were concentrated more at the centre of the islet. Insulin immune positive cells were also observed in the exocrine portion as well as within and adjacent to the ductal structures which were intensely stained.

The increase in the percentage of beta cells coincided with the increase in the serum insulin level in the present study. Insulin immune positive cells were also observed in the exocrine portion as well as within and adjacent to the ductal structures which were intensely stained. This clearly indicated that there was beta cell neogenesis from ductal epithelial cells or acinar cells either in response to hyperglycaemic situation or due to the stimulatory effect of the bioactive substance 4-hydroxyisoleucine of the plant (Shah *et al.*, 2009; Zafar *et al.*, 2009a and Eman and Elaziz, 2011) [26,31, 8].

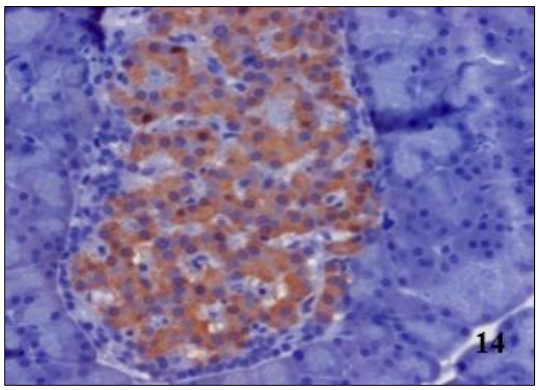

Plate 14: Pancreas from a diabetic rat treated with *Trigonella foenum graecum* and glibenclamide showing increase in the number of insulin positive cells with cord like arrangement similar to that of normal islet on Day 90 of the treatment. IHC X 200

Summary and Conclusion

The present study was focussed on evaluation of antidiabetic effect of *Trigonella foenum graecum* individually in induced diabetes in rats. The efficacy of these herbal extracts was also compared with that of glibenclamide, a novel cardinal antidiabetic drug individually and in combination.

Microscopically, in diabetic rats, both exocrine and endocrine pancreas was affected. Exocrine pancreas revealed loss of

achitecture with vacuolated, degenerated and necrotic cells in the acini. In endocrine pancreas there was a decrease in the number of islets, vacuolar degenerations, and loss of granularity, apoptosis and necrosis of cells in a progressively increasing manner.

There was a progressive reconstruction of normal architecture of acini and islets in pancreas in all the treatment groups in the present study. The exocrine portion of acini revealed improvement which was comparable to glibenclamide treatment. In endocrine pancreas compared to glibenclamide, Groups V treatment groups revealed better improvement with islets of bigger size and more compact arrangement. Tendency to form new islets near the blood vessels were constant feature seen in many of the lobules in all treatment groups.

Immunohistochemical demonstration of insulin showed drastic reduction in the number of insulin positive cells in the diabetic group. There was a improvement in number of insulin positive cells in all treatment groups. However, Groups V showed better regeneration of β-cell population which was comparable to that of control group (I) indicating the synergistic action of plant extracts and half dose of glibenclamide.

Lastly the present study high lights that the indigenous medicinal plants can be used successfully as an alternative treatment in the management of diabetes with or without antidiabetic drugs.

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