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Orignal Article

PATHOMORPHOLOGICAL AND IMMUNOHISTOCHEMICAL EVALUATION OF *PTEROCARPUS MARSUPIUM* AND *SWERTIA CHIRAYITA* HERBAL EXTRACT FOR THEIR ANTIDIABETIC EFFECT IN DIABETES INDUCED WISTER RATS

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Abstract: ***The present study was undertaken to evaluate the efficacy of Pterocarpus marsupium and Swertia chirayita individualy and in combination along with glibenclamide in streptozotocin induced diabetic rats for a period of 45 days. There was significant variation in different groups in term of Pathomorphological and Immunohistochemical findings of diabetic rats when compared to normal control rats. The alleviation of the diabetes and its complications induced by streptozotocin was observed in all the treatment groups with variable degrees of improvement. Pterocarpus marsupium and Swertia chirayita extracts were effective in alleviating streptozotocin induced diabetes and were comparable with glibenclamide. Combination of Pterocarpus marsupium with glibenclamide and Swertia chirayita with glibenclamide showed better improvement compared to individual extracts alone but statistically no significant synergetic action was seen. Synergistic effect was observed in combination of Pterocarpus marsupium and Swertia chirayita and in combination of Pterocarpus marsupium and Swertia chirayita along with glibenclamide.***

Keywords: *Pterocarpus marsupium, Swertia chirayita*, Diabetes, Glibenclamide, Streptozotocin

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Dedication: This work is dedicated to Dr. P.D. Gupta on his 85th birthday.

INTRODUCTION

Diabetes mellitus is a metabolic disorder associated with high blood glucose levels, either due to less production of insulin by the pancreas or due to inability of body cells to respond to the insulin produced. As per WHO March 2013 fact sheet 347 million people worldwide have diabetes and more than 80 per cent of diabetes deaths occur in low and middle income countries. It is also projected that the deaths due to this will be double between 2005 and 2030 (WHO 2013 [1].

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 [2]. 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.

Herbs for treatment of diabetes is not new. Many studies have confirmed the benefits of medicinal plants with hypoglycemic effects in management of diabetes. The effects of these plants may delay the development of diabetic complications and correct the metabolic abnormalities. Moreover, during the past few years some of the new bioactive drugs isolated from hypoglycemic plants showed antidiabetic activity with more efficacy than oral hypoglycemic agents [3].

With this background the present study was taken up with the following objectives: 1. To evaluate the antidiabetic effect of *Pterocarpus marsupium* and *Swertia chirayita* individually and in combination in induced diabetes in rats. 2. To study pathomorphological, and Immunohistochemical changes in induced and treated diabetic rats.

MATERIALS AND METHODS

The present study was carried out at the Department of Veterinary Pathology, Veterinary College, Hebbal, KVAFSU, Bangalore, to evaluate the antidiabetic efficacy of *Pterocarpus marsupium* and *Swertia chirayita* individually as well as in combination in rats. Also, the hypoglycemic effects of these plants were compared with an oral hypoglycemic agent glibenclamide.

Experimental animals: Genetically normal adult female *Wistar albino* rats weighing 170-180 g were procured from RRL Instruments and Animals supplier, Bangalore for the study. They were maintained under standard laboratory conditions and offered *ad libitum* of standard commercial rat feed (Amruth Feeds, Bangalore) and clean drinking water. The experiment was carried out for a period of 45 days after obtaining permission from Institutional Animal Ethics Committee.

Sources of plant extracts:

***Pterocarpus marsupium* :** The alcoholic heart wood extract of *Pterocarpus marsupium* used in the present study was obtained from Himalaya Drug Company, Bangalore. The powdered extract was weighed according to body weight and dissolved in water to make the final concentration and administered to the experimental animals.

***Swertia chirayita*:** The alcoholic plant extract of *Swertia chirayita* used in the present study was obtained from Katyani Exports, Pitampura, New Delhi, India.

Glibenclamide solution : Glibenclamide (Daonil®, 5 mg) an oral hypoglycaemic drug purchased from local chemist shop was dissolved in distilled water (82.33 ml) to make a concentration of 60 µg/ml solution.

Administration of plant extracts and glibenclamide: Throughout the period of experiment, the plant extracts and glibenclamide were administered orally to their respective groups by using clean gavaging/rat feeding needle attached to an appropriate disposable syringe during morning hours daily for a period of 45 days.

Experimental design: After procurement, the rats were maintained under standard laboratory conditions for a period of 15 days for acclimatization in the experimental room. Then the rats were divided into nine different groups with twelve animals in each based on the body weight. Care was taken to maintain the intra group weight variation to be less than 20 g and inter-group weight variation by 30 g (Table 1).

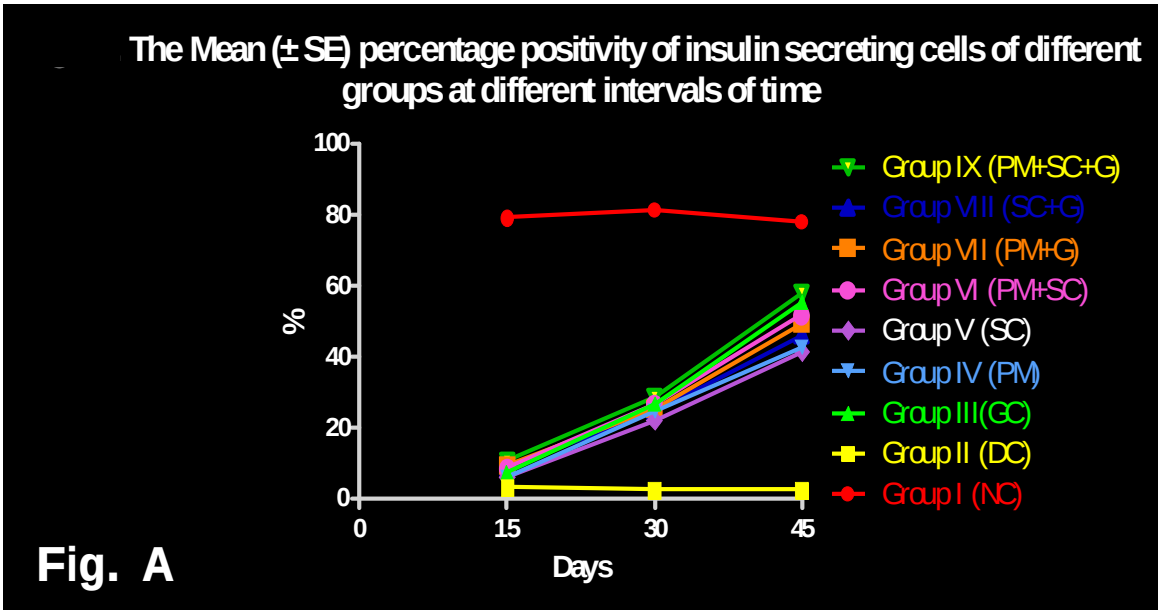

Table 1: The animal groups and their treatments. The rats of Group I and II were gavaged only with saline and the rats of all other groups with their respective treatments daily for 45 days.

Group I	Normal control: Used for studying baseline values of the parameters
Group II	Diabetic control: Streptozotocin induced diabetic rats
Group III	Diabetic rats supplemented with glibenclamide at a dose of 600 µg/kg
Group IV	Diabetic rats supplemented with extract of *Pterocarpus marsupium* at the dose rate of 500 mg/kg body weight
Group V	Diabetic rats supplemented with extract of *Swertia chirayita* at the dose rate of 500 mg/kg body weight
Group VI	Diabetic rats supplemented with combined extract of *Pterocarpus marsupium* and *Swertia chirayita* at the dose rate of 250 mg/kg body weight each
Group VII	Diabetic rats supplemented with combined extract of *Pterocarpus marsupium* and glibenclamide at the dose rate of 250 mg/kg and 300 µg/kg body weight, respectively
Group VIII	Diabetic rats supplemented with combined extract of *Swertia chirayita* and glibenclamide at the dose rate of 250 mg/kg and 300 µg/kg body weight, respectively
Group IX	Diabetic rats supplemented with combined extract of *Pterocarpus marsupium*, *Swertia chirayita* at the dose rate of 250 mg/kg each and glibenclamide at the dose of 300 µg/kg body weight, respectively

Table 2: The Mean (± SE) percentage positivity of insulin secreting cells of different groups at different intervals of time. Mean values with different superscript differ significantly [a]comparison with Group I, [b]comparison with Group II, [c]comparison with Group III. Values are statistically significant at ≤ 0.05

Groups	Days Post Treatment		
	15	30	45
Group I (NC)	79.50±2.50	81.50±2.50	78.16±1.01
Group II (DC)	3.50±0.50[a]	2.50±0.50[ac]	2.66±0.21[ac]
Group III (GC)	7.50±0.50[a]	27.00±1.00[ab]	55.16±0.65[ab]
Group IV (PM)	6.00±1.00[a]	24.50±0.50[ab]	42.83±0.90[abc]
Group V (SC)	6.00±1.00[a]	22.00±1.00[ab]	41.33±1.22[abc]
Group VI (PM+SC)	8.50±0.50[a]	26.50±1.50[ab]	52.16±1.01[ab]
Group VII (PM+G)	9.50±0.50[a]	25.50±0.50[ab]	49.16±1.30[abc]
Group VIII (SC+G)	8.50±0.50[a]	24.50±0.50[ab]	46.33±1.76[abc]
Group IX (PM+SC+G)	10.50±0.50[ab]	29.00±1.00[abc]	58.00±2.20[ab]

Pathology: Two animals from each group were sacrificed humanely on day 15, 30 and the rest at the end of the study on 45th day. The representative tissue samples of 3-5 mm thickness were collected in 10 per cent NBF for Immunohistochemistry examination.

Immunohistochemical detection of insulin: 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.

Materials immunochemicals: Primary antibody: Ready to use Flex Polyclonal Guinea Pig Anti-Insulin shown to react with insulin antigen was procured from Dako Cytomation, Denmark. It was stored at 2 to 8 ^{o}C until use.

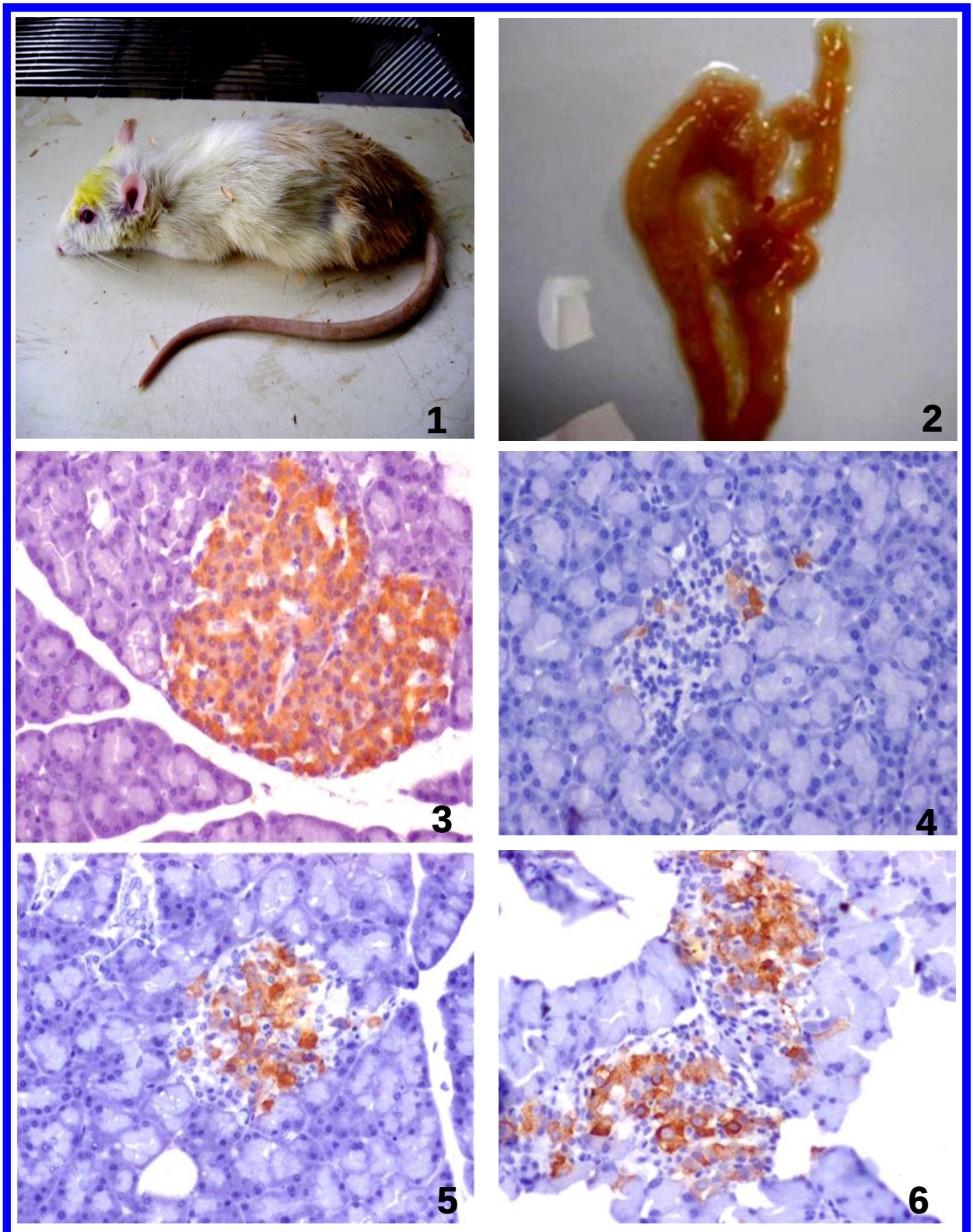

Fig. 1: Diabetic animal showing poor body condition, dehydration and ruffled soiled hair coat on 15th day of the experiment. **Fig. 2:** Pancreas and other visceral organs of normal control animal showing normal architecture on 45th day of the experiment. **Fig. 3:** Islet of langerhans from a normal control animal showing intensely stained insulin positive beta cells in large number. IHC X 200. **Fig. 4:** Islet of Langerhans from the diabetic control animal showing lightly stained insulin positive cells indicating degenerative cells on Day 45 of the study. IHC X 200. **Fig. 5:** Islet from a glibenclamide treated animal showing increase in number of beta cells in the islet on Day 45 of the study. IHC X 200. **Fig. 6:** Islet from diabetic animal treated with *Pterocarpus marsupium* showing an improvement in the number of insulin positive cells on Day 30. IHC X 200

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 2 to 8 ^{o}C until end.

Section adhesive 3-aminopropyltriethoxy-silane (APES): Procured from Sigma chemicals, USA, Hydrogen peroxide (H_2O_2) in methanol (3 %) and DAB plus substrate

Method : Tissue sections were mounted on 3-aminopropyltriethoxy-silane (APES) coated slides and dried at 37 ^{o}C for three hours. Later stored at 4 ^{o}C for further processing later. The paraffin tissue sections were deparaffinized using xylene and rehydrated using descending grades of ethanol. Endogenous peroxidase was blocked by covering the whole section with 3 per cent of H_2O_2 in methanol (100 µl). This was incubated at room temperature for fifteen minutes and later washed with 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 and later washed with 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 Immunoglobulin conjugated with HRP was added to section 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 sections. This was 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 with distilled water, dehydrated with ascending grades of ethanol and cleared with xylene and cover slipped with DPX mounting media.

RESULT AND DISCUSSION

Immunohistochemistry : In the present study immunohistochemical demonstration of insulin was carried out to identify and enumerate β-cells in various treatment groups using polyclonal 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 of islets was evaluated.

Normal control group (Group I): most of the islets revealed intensly stained positive cells in large number. The α-cells and acini of exocrine pancreas were negative and the IHC positive β-cells revealed densely stained cytoplasmic granules that were compactly arranged and limited to the regular membrane (Fig A). The mean percentage of insulin secretory cells were 79.50 ± 2.50, 81.50 ± 2.50 and 78.16 ± 1.01 on 15th, 30th and 45th day, respectively (Table 1 and Fig. 1). 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 (Fig. 2 and 3).

Diabetic control group (Group II): The islets of revealed a drastic reduction in the number of insulin positive cells. The mean percentage of insulin secretory cells were 3.50 ± 0.50, 2.50 ± 0.50 and 2.66 ± 0.21 on 15th, 30th and 45th day, respectively (Table 1 and Fig. 1). Most of the IHC positive cells appeared swollen and irregular with scattering of granular material in the cytoplasm indicating degenerating cells (Fig. 4).

The progressive decrease in the size of pancreas in the present study is due to the cytotoxic effect of streptozotocin on β-cells of islets of Langerhans [4].

In the present study, the damage to the exocrine acinar cells could be probably due to secondary response to excessive free radical liberation and antioxidant depletion in STZ cytotoxicity [5]. The decrease in the number of islets and cellularity in the islets could be attributed to the cytotoxic effect of streptozotocin which is specific for β-cells of islets as indicated by earlier workers [6]. The change in

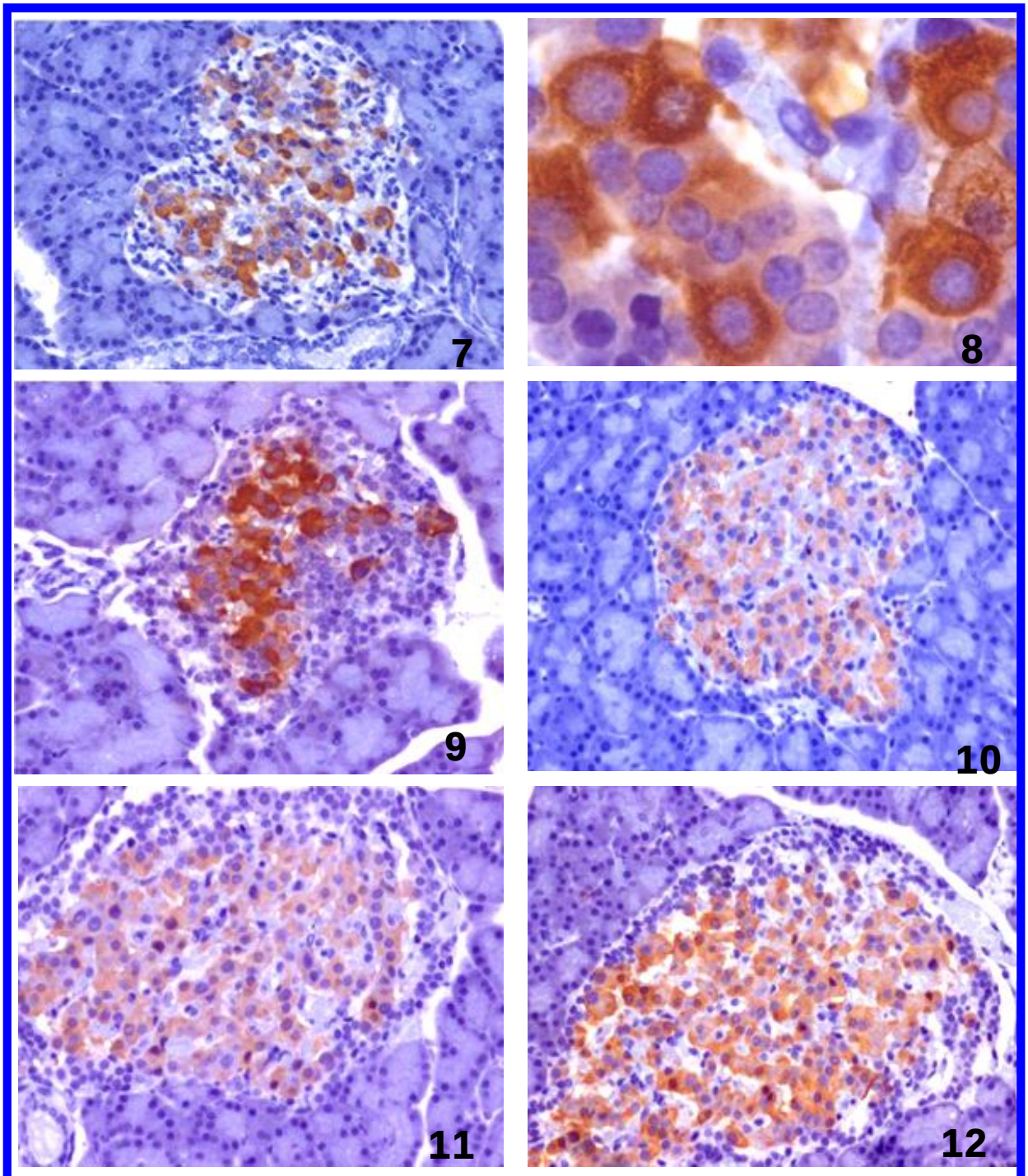

Fig. 7: Pancreas from diabetic animal treated with *Swertia chirayita* showing more number of insulin positive cells with compact arrangement of granules in the cytoplasm on Day 45 of the treatment. IHC X 200. **Fig. 8:** Section of pancreas from a diabetic rat treated with combination of *Pterocarpus marsupium* and *Swertia chirayita* showing a few insulin immunopositive beta cells at 15th day of treatment. IHC X 1000. **Fig. 9:** Pancreas from a diabetic rat treated with *Pterocarpus marsupium* and glibenclamide showing increase in the number of insulin positive cells with compact arrangement of granules in the cytoplasm on Day 45 of the treatment. IHC X 200. **Fig. 10:** Section of pancreas from a diabetic rat treated with *Swertia chirayita* and glibenclamide showing a few insulin positive cells on Day 15 of the treatment. IHC X 200. **Fig. 11:** Pancreas from adiabetic rat treated with combination *Pterocarpus marsupium, Swertia chirayita* and glibenclamide showing insulin positive cells in the midst of ductal epithelium cells on 90th day of treatment. IHC X 200. **Fig.12:** Pancreas from a diabetic rat treated with *Pterocarpus marsupium, Swertia chirayita* and glibenclamide showing increase in the number of insulin positive cells with morphology comparable to that of normal islet on Day 45 of the treatment. IHC X 200

the shape of the β-cells could be due to partial injury to the cells by STZ [7]. The mild fibrotic change in the affected islets late in the present study could be due to replacement of necrosed β-cells by substitution with fibrous connective tissue. Similar type of findings were observed by previous workers in STZ induced diabetic studies [8-10].

Glibenclamide treatment group (Group III): there was a progressive increase in number of insulin positive cells. The mean (± SE) percentage of positive cells showed an increase in values from 7.50 ± 0.50 on 15th day to 55.16 ± 0.65 on 45th day (Table 1 and Fig. 1). The number of degenerating cells were less compared to the diabetic control group and β-cells with more compact insulin positive granular material in the cytoplasm increased as the treatment progressed. The improvement was significantly higher (≤0.001) compared to diabetic control group at 30th and 45th days of observation and were not comparable with normal control with significantly lesser values (Fig. 5).

Immunohistopathological observations of Pancreas studies have shown that the drug has a positive action on glycogen deposition with direct action on the synthesis of GLUT2 rather than GLUT4 proteins and at the glycogen phosphorylase level. The effect of glibenclamide on the insulin levels, beta cells and on the altered metabolism of various macromolecules may improve the liver's microscopic architecture [11].

Combined treatment group (Group VI, VII, VIII and IX): Immunohistologically, in the pancreas there was a progressive reconstruction of normal architecture of acini and islets from day 15 to day 45 post treatment in *Pterocarpus marsupium* treated rats in the present study. The islets revealed hypercellularity with advancement of time on treatment. Initially more number of α-cells and few β-cells were seen and with the advancement of time the percentage of β-cells increased from 6.00 ± 1.00 to 42.83 ± 0.90 on day 15 to day 45 respectively which was well demonstrated by immunohistochemistry (Fig. 6, Table 1 and Fig. A).

Similar to the glibenclamide treated rats all the treatment groups (Group IV to IX) also showed progressive improvement in the insulin positive cells at regular intervals post treatment (Plate 61-70). The mean (± SE) percentage positive cells showed increase in values from 6.00 ± 1.00, 6.00 ± 1.00, 8.50 ± 0.50, 9.50 ± 0.50, 8.50 ± 0.50 and 10.50 ± 0.50 on 15th day to 42.83 ± 0.90, 41.33 ± 1.22, 52.16 ± 1.01, 49.16 ± 1.30, 46.33 ± 1.76 and 58.00 ± 2.20 on 45th day of Groups IV, V, VI, VII, VIII and IX, respectively (Fig. 1 and Fig A). The values were significantly higher (≤ 001) compared to diabetic control group on 30th and 45th day. However, in comparison to normal control animals the values were significantly less and with glibenclamide group, the values were comparable on 15th and 30th day without significant variation, but on 45th day only Group VI and Group IX were comparable and the other groups showed significantly lesser values (Figs.7-12).

The immunohistochemistry of combined treatment groups revealed that Groups VI and IX showed better regeneration of β-cell population which were comparable to the glibenclamide control indicating the synergistic action of these groups compared to individual treatment groups of *Pterocarpus marsupium* and *Swertia chirayita* groups. Special staining for β-cells also revealed better regeneration of β-cell population.

Based on the above, it may be concluded that β-cell regeneration capacity of combined treatment groups is better than individual treatment groups of *Pterocarpus marsupium* and *Swertia chirayita*. Among the combined treatment groups, Group VI and IX showed better improvement which were comparable with glibenclamide control.

SUMMARY AND CONCLUSION

Grossly, in the diabetic control rats, pancreas was slightly congested, reduced in size and appeared as thin gelatinous strip. Similar lesions were observed grossly in treatment groups also but the extent and severity of the lesions were progressively reduced as the traetment advanced. Immunohistochemical demonstration of insulin showed drastic reduction in the number of insulin positive cells in the diabetic group. There was improvement in the number of insulin positive cells in various treatment groups. However, Groups VI and IX showed better regeneration of β-cell population which were comparable to the glibenclamide control indicating the synergistic action of these groups compared to individual treatment groups of *Pterocarpus marsupium* and *Swertia chirayita*.Combination of

Pterocarpus marsupium and *Swertia chirayita* extracts alone (Group VI) and with glibenclamide (Group IX) showed significant synergistic effect in alleviating the STZ induced diabetes and its complications.This indicated that combining *Pterocarpus marsupium* and *Swertia chirayita* with glibenclamide could provide an opportunity to reduce the dose of glibenclamide, which may help in minimizing the adverse effect of glibenclamide as well as achieve enhanced therapeutic effect.

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