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Anti-hyperglycaemic activity of ethanol extract and chloroform extract of *Indigofera tinctoria* leaves in streptozotocin induced diabetic mice (Family-Papilionaceae)

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ABSTRACT

The objective of the present study was to investigate anti-diabetic and nephroprotective activity of *Indigofera tinctoria* leaves, using STZ-induced diabetic rats as model for clinical type-1, type-2 diabetes. At a regular interval of an experimental protocol blood glucose, urinary creatinine, total proteins and organs to body weight ratio were studied. The histo-pathological study was carried out by STZ-induced diabetic and anti-diabetic rat's pancreas. Statistical analysis of the results shown that in STZ-induced diabetic rats chloroform and alcohol extracts of *Indigofera tinctoria* leaves at 40, 80, 160 and 200 mg/kg doses. Significant effect of alcoholic extract from 4^{th} day to 16^{th} day of the study. *Indigofera tinctoria* leaves extract improved renal creatinine clearance and reduce renal total protein loss demonstrating nephroprotective properties. The organ to body weight ratio studies carried out on last day, shown pancreas and liver specific effects of *Indigofera tinctoria* leaves. These results were also supported by histo-pathological studies. We conclude from the present study that *Indigofera tinctoria* leaves alcoholic extract long-term treatment may be beneficial in the management of type-1, type-2 diabetes.

Keywords: Diabetes, Anti-diabetes, *Indigofera tinctoria,* nephropathy, nephroprotection, streptozotocin, and Anti-hyperglycemic.

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INTRODUCTION

Diabetes is a group of metabolic disorders of fat, carbohydrate, and protein, metabolism that result from defects in insulin secretion, insulin action (sensitivity) or both [1]. Diabetes is associated with sustained high glucose in blood behind certain level leading to long term damage, dysfunction and failure of various organs including eyes, kidneys, nerves, heart and blood vessels [2]. Hence, this disease is referred as the 'silent killer'. It is called as silent killer because it is often diagnosed too late on the damage may already have been done. It impacts not only people with the disease but also their families and costing societies heavily in treating many serious complication that arise in undiagnosed or poorly rated diabetes. The cost of non treating diabetes is much greater than the proper treatment [3]. Diabetes is world-wide distribution and the incidence of both type-1 and type-2 diabetics are rising. It is estimated that, in the year 2000, 150million people world had diabetes, and this is expected to double by 2010 [4]. According to international diabetes federation (IDF) diabetes is turning out to be bigger monster than aids. As for the recent statistics released by international diabetic federation (IDF), every ten seconds a person dies from diabetes related causes across the world. Every year 3.8 million people die of this disease, more than 246 million people ranging from 20 to 79 years, live with diabetes. About 50% are undiagnosed, 308 million's have impaired glucose tolerance and every year 7 million young people are adding up to these population. If it is allows to develop unchecked staggering 380 million relieve with diabetes in 2024 [5]. World Health Organization (WHO) estimates that by 2025 as many as 200 to 300 million people worldwide will develop diabetes.

The current modern pharmacotherapy of diabetes mellitus using pharmaceuticals and biological become ineffective due to their severe side effects. Therefore management of diabetes without any side effects is still a challenge for the medical system and the scientists working in this area [6]. Alternative strategies are urgently needed to control all the pathological aspects of the disorder and its pandemics [7]. Indian system of medicine, ayurveda has cited more than 150 plants for their possible applications in the treatment of carried out in different animal models have shown hypoglycemic activity [8].

Indigofera tinctoria leaves are most commonly known as neeli it is a small erect shrub is cultivated extensively. Juice of the leaves and indigo in powder are using anti-diabetic [9], epilepsy and liver spleen, enlargement. Juice is also given in asthma, whooping cough; palpitation of the heart, formulation containing *Indigofera tinctoria* leaves has been reported to have anti diabetic activity. Therefore the present study was under taken to evaluate anti diabetic activity of *Indigofera tinctoria* leaves using STZ induced diabetes [10]. The outcome of the study will prove and validates; an effort was also being made to find out its applicability in diabetes induced nephropathy.

MATERIALS AND METHODS

Plant material collection and authentication:

In the present study, the root part of *Indigofera tinctoria leaves* was collected from Bagalkot region (Karnataka India) and shade dried, in the month of July. The plant was authenticated by taxonomist and consultant Dr V.V Shidlingappanavar; Basaveshwar Herbal

Folk Research centres Bagalkot Karnataka India. The leaves were dried under room temperature, until free from the moisture. Finally, the dried leaves were subjected to get coarse powder and then passed through sieve no. 44 to get uniform powder. The sieved powder was stored in air tight, high-density polyethylene containers before extraction.

Acute toxicity study by using OECD 420 guidelines:

A slighting study is included for guideline 420 in order to choose an appropriate starting dose and to minimize the number of animals used. Pre specified fixed doses of 5, 50, 300 or 2000mg/kg are used both in the sighing study and the main study. There is an option to use an additional dose level of 5000mg/kg, but only when justified by a specific regulatory need. Group of animals are dosed in a step wise procedure, with the animal dose being selected as the expected to produce some sings of toxicity, until the objective is achieved; that is, the classification of the test substance based on the identification of doses causing evident toxicity, except when there are no effect at the highest fixed dose [11].

Drug and chemicals:

Streptozotocin (Cisco Research Laboratories Pvt.Ltd, Mumbai, India.), Glibenclamide was obtained as gift sample from Gujarat; Mumbai.), All chemicals used in this study were of AR grade.

Plant preparation:

The powdered root was subjected to hot continuous, successive extraction (soxhlet) 24 hours cycle with petroleum ether, chloroform and methanol (50-55ºC), then the solvent was distilled off, and excess solvent was completely removed by using a rotary flash evaporator to get chocolate coloured semisolid extract. The obtained semisolid mass completely dried in mini lyophilizer. Its percentage yield was calculated in terms of air-dried weight of plant material. The crude drug was defatted with petroleum ether. The obtained extracts were subjected to evaluate for its anti-diabetic activity. The percentage yields of chloroform and methanol extracts were (0.75% and 2.11%) respectively.

Animal selection

Healthy adult male *Wistar* albino (250-300g) rats 8-10 weeks old were used in this study. The rats were housed in polypropylene cages and maintained under suitable nutritional and environmental (12-hr light: 12-h dark cycle; 25±3ºC; 35-60% humidity) conditions throughout the experiment. All the pharmacological experimental protocols were approved by the Institutional animals' ethics committee, (HSKCP/IAEC. Clear /2004-05. Dated: 27-12-2004) H.S.K. College of pharmacy, Bagalkot- Karnataka.

Instruction of experimental diabetics

Diabetes was induced in overnight fasted rats by Intra-peritoneal injection of streptozotocin (50 mg/kg, i.p.) dissolved in 0.9% ice-cold saline immediately before use. Seventy-two (72) hours after streptozotocin administration blood samples were withdrawn by retro-orbital puncture and glucose levels determined to confirm diabetes. The diabetic

rats were exhibiting blood glucose levels in the range of 180 and 280 mg/100ml were selected for the studies.

These diabetic rats were sub-divided in to 11 groups of 6 rats each, were used for the study. The study was carried out for 16 days, and extract was given daily for 13 days. Blood was collected by retro-orbital puncture under light ether anesthesia on the 1st, 4th, 7th, 10th 13th and 16th day. Urine was collected on the last day of study for 24 hrs by using metabolic cage for further biochemical estimation.

Diagnostic kits

Glucose Reagent Kit (Aspen Laboratories Pvt.Ltd, Delhi.), Creatinine Reagent Kit (Aspen Laboratories Pvt.Ltd, Delhi), Protein- CSF kit (Biolab- diagnostics (I) PVT.LTD, Tarapur Boisar, Maharastra.

Instruments

Research Centrifuge (REMI-24), Borosil Soxhlet Extractor, Auto-Analyzer (Star 21 plus), Research Microscope (Metzer), Afcoset Digital Balance (E-R-180 A) and Mini Lyotrap (LTE Scientific LTD, Great Britain).

Estimation of serum and urine bio-chemical parameters:

Serum glucose

Blood samples (2 ml) were collected from the rats by retro-orbital puncture under mild ether anesthesia on the 1st, 4th, 7th, 10th, 13th, and 21st day of the study. The serum was immediately separated by centrifugation and the glucose level was measured by GOD/POD method using glucose reagent kit and by auto-analyzer.

Urine creatinine

Creatinine level in urine was estimated by alkaline picrate method using creatinine kit by auto- analyzer**.**

Urine total protein

Concentration of total proteins in urine was estimated auto & manual method using Protein-CSF kit by auto-analyzer**.**

Organ to body weight ratio

At the end of the study, animals were sacrificed and adrenal glands, kidneys, liver, heart and pancreas were isolated and weighed in wet condition to measure organ to body weight ratio.

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Statistical Analysis

Results were expressed as mean blood glucose levels ± S.E.M. (standard error of the mean). Data were analyzed by using student's t-test. P values less than 0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

Table 1. Effect of *Indigofera tinctoria* leaves extracts on urine creatinine and total proteins in streptozotocin induced diabetic rats.

Treatment and dose	Urine	
	Creatinine	Total proteins
Normal	0.8295 ± 0.030	0.6435 ±0.02
Control	1.682 ±0.020***	0.8358 ±0.03***
Glibenclamide (5mg/kg)	0.6657 ± .117***	0.6622 ± 0.032***
Chloroform extract (40 mg/kg)	0.9735 ± 0.079*	0.7133 ± 0.172*
Chloroform extract (80 mg/kg)	0.9640 ± 0.075*	0.6643 ±0.025*
Chloroform extract (160 mg/kg)	0.8952 ± 0.054*	0.5747 ± 0.021**
Chloroform extract (200 mg/kg)	0.8196± 0.080**	0.5932± 0.022**
Alcohol extract (40 mg/kg)	1.260± 0.159*	0.5850 ±0.015**
Alcohol extract (80 mg/kg)	0.978± 0.118***	0.5612 ±0.020***
Alcohol extract (160 mg/kg)	0.9657 ±0.050***	0.5612± 0.012***
Alcohol extract (200 mg/kg)	0.8325 ±0.024***	0.5612 ±0.014***

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Table 2. Effect of *Indigofera tinctoria* leaves extracts on serum glucose (mg/dl) in streptozotocin induced diabetic rats.

Treatment and dose	Serum glucose					
	Day's					
	1	4	7	10	13	16
Normal	119.7±9.8	113.5±9.5	122.4±8.9	91.3± 3.1	87.5± 6.6	84± 3.0
Control	218.6±17.9	219.9±17.6	219.7±16.8	215.3±15.1	189.2± 7.3	179.2± 6.2
Glibenclamide (5 mg/kg)	158.3±8.6*	154.1±8.8**	143.0±8.6**	144.3±6.5**	130.1±6.5***	120.2±8.6***
Chloroform extract (40 mg/kg)	205.7± 8.6	219.9± 17.6	199.4± 9.4	189.9± 6.8	182.9± 7.5	173.3± 5.6
Chloroform extract (80 mg/kg)	212.8± 6.0	209.1± 6.3	200.9± 4.3	193.4± 5.8	185.2± 6.0	175.0± 5.3
Chloroform extract (160 mg/kg)	164.3± 8.1	167.3± 7.8	164.6± 4.5	163.8± 4.0	150.3± 3.0	147.3± 4.1
Chloroform extract (200 mg/kg)	208.5± 7.6	203.0± 7.5	193.6± 3.8	190.6± 3.6	187.4± 3.3	181.9± 3.5
Alcohol extract (40 mg/kg)	236.9±14.0	230.0±14.2	226.2± 21.3	214.4± 15.3	196.6± 10.0	168.9± 11.0*
Alcohol extract (80 mg/kg)	207.5± 8.5	200.8± 8.1	193.2± 8.1	188.3± 4.8*	180.1± 6.2**	174.3± 6.5**
Alcohol extract (160 mg/kg)	187.0±3.9**	176.7±3.9**	133.4±5.2***	131.2±2.1***	125.3± 1.8***	119.5± 1.5***
Alcohol extract (200 mg/kg)	184.6±2.9**	178.0 2.0***	149.1±4.7***	129.8±5.0***	117.4±10.4***	101.3± 4.0 ***

Effect of normal (saline), tween 80 (2%), glibenclamide (5 mg/kg), chloroform extract 40, 80,160 and 200 mg/kg and alcoholic extract 40, 80, 160 and 200 mg/kg on urine creatinine & total proteins in STZ-induced diabetic rats. Urine was collected on 16th day; creatinine and total proteins were estimated by using creatinine kit and protein-CSF kit respectively by auto analyzer. The results were analyzed by student't' test and by comparing mean of treatment group with control and control group with normal for coming to conclusion and presented as mean± SEM. The 'p' value <0.05 was considered as significant. *p<0.05, **p<0.01, ***p<0.001

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Table 3. Effects of *Indigofera tinctoria* leaves extracts on organs to body weight ratio in (mg/gm) streptozotocin induced diabetic rats.

Treatment	Adrenal gland	Kidney	Liver	Pancreas	Heart
Normal	0.1267±0.0033	0.2350 ± 0.010	9.205 ± 0.049	0.6367 ± 0.031	0.8883 ± 0.025
Control	0.1650±0.004	0.2883±0.003	9.918 ± 0.178	0.7600± 0.028	0.8617 ± 0.034
Glibenclamide (5 mg/kg)	0.1133 ±0.0021	0.1654±0.0181	8.747± 0.243	0.5817±0.003	0.7483±0.039
Chloroform extract (40 mg/kg)	0.1633 ± 0.004	0.2550 ± 0.021	9.435 ± 0.336	0.8150 ± 0.031	0.9500 ± 0.019
Chloroform extract (80 mg/kg)	0.1650 ± 0.006	0.2600 ± 0.022	9.462 ± 0.334	0.8150 ± 0.031	0.9783 ± 0.003
Chloroform extract (160 mg/kg)	0.1650 ± 0.006	0.2600 ± 0.022	9.277 ± 0.218	0.7717 ± 0.030	0.9683 ± 0.008
Chloroform extract (200 mg/kg)	0.1517 ± 0.005	0.2450 ± 0.023	9.255 ± 0.217	0.7233 ± 0.020	0.9383 ± 0.011
Alcohol extract (40 mg/kg)	0.1567 ± 0.004	0.2800 ± 0.019	9.785 ± 0.079	0.7617 ± 0.016	0.9467 ± 0.013
Alcohol extract (80 mg/kg)	0.1317 ± 0.005	0.2467 ± 0.009	9.167 ± 0.186	0.6517± 0.018	0.9133 ± 0.017
Alcohol extract (160 mg/kg)	0.1283 ± 0.004	0.2233 ± 0.006	8.440 ± 0.053	0.5967 ± 0.017	0.8767 ± 0.003
Alcohol extract (200 mg/kg)	0.1083 ± 0.0030	0.1933 ± 0.017	8.022 ± 0.186	0.5633 ± 0.023	0.8300 ± 0.004

Effect of normal (saline), tween 80 (2%), glibenclamide (5 mg/kg), chloroform extract 40, 80,160 and 200 mg/kg and alcoholic extract 40, 80, 160 and 200 mg/kg on organs to body weight ratio in (mg/gm) STZ-induced diabetic rats. On the final day adrenal glands, kidney, liver, heart and pancreas were isolated and weighed in wet condition to measure organ to body weight ratio. The results were analyzed by student't' test and by comparing mean of treatment group with control and control group with normal for coming to conclusion and presented as Mean± S.E.M. The 'p' value <0.05 was considered as significant. *p<0.05, **p<0.01, ***p<0.001.

Effect of *Indigofera tinctoria leaves* extracts on serum and urine biochemical parameters:

Single dose intra-peritoneal (i.p) treatment of rats with streptozotocin (50 mg/kg) significantly ($p < 0.001$) raised blood sugar level from 3rd day to 16th day of the study. As shown in table 1. Glibenclamide (5 mg/kg), orally has reduced streptozotocin increased blood sugar level significantly ($p < 0.001$) except on first day of treatment. The *Indigofera tinctoria leaves* chloroform and methanolic extract 40, 80, 160 and 200 mg/kg, oral route significantly ($p<0.001$) attenuated streptozotocin induced increased blood sugar level from 4th to 16th day of study. However, except chloroform extract 40 and 80 mg/kg all other doses of chloroform and methanolic extracts failed to produce any acute reversal effect in streptozotocin diabetic animals. The reversal effect of methanolic extract 80, 160, 200 mg/kg and chloroform extract 160 and 200 mg/kg dose was almost reverted to the level of normal. The effect of streptozotocin alone treatment gradually and significantly increased blood sugar level from 85 to 219 mg/dl on 16th day of study.

As shown in Table 2 creatinine clearance was significantly ($p<0.05$) reduced from 0.8325 0.024***. Total proteins loss was increased 0.5612 ±0.014*** non- significantly ($p>0.01$) in streptozotocin treated normal and control group. Higher doses of chloroform extract (160 and 200 mg/kg) significantly increased ($p<0.05$ and 0.01). Renal creatinine clearance when compared to streptozotocin diabetic rats, all doses of chloroform extract also significantly ($p<0.01$) reduced by 80, 160 and 200 mg/kg dose of methanolic extract. However, this reversal effect was not observed at 40 mg/kg methanolic and chloroform extract. Reduced clearance of creatinine by streptozotocin was significantly ($p<0.05$ and $p<0.01$) reversed at all doses of *Indigofera tinctoria leaves* alcoholic extract. The effect of methanolic extract 80, 160 and 200 mg/kg on serum glucose and urine biochemical profile was much higher than the effect of standard drug glibenclamide throughout the study.

Effect of *Indigofera tinctoria leaves* extracts on organs to body weight ratio:

Sixteen days treatment of diabetic rats with 160 and 200 mg/kg dose of chloroform extract and 160 and 200mg/kg alcoholic extracts significantly increase the weight of kidney ($p<0.01$) when compared to non STZ treated normal group. In control STZ diabetic rats the liver and heart to body weight ratio was significantly reduced from 8.022 ± 0.186 to24±0.98 ($p<0.001$) and 0.8300 ± 0.004 ($p<0.05$) respectively. These effects were attenuated significantly by the treatment with chloroform and alcoholic extract. Mass of pancreatic gland was reduced but non-significantly from 0.5633 ± 0.023 to 1.9±0.21. However, the pancreatic mass was significantly increased in a dose dependent manner when compared with control STZ diabetic rats.

Histopathological evaluation of pancreas:

Diabetic rats revealed degeneration and lytic changes in islets of langerhans of pancreas similar to earlier study [8]. It was also observed the islets were shrinken, inflammatory cellular

infiltration with fibrosis. Treatment of these diabetic rats with glibenclamide inhibited streptozotocin induced shrinking of islets of langerhans of the pancreas, inflammatory cellular infiltration and enlarged pancreatic cells. *Indigofera tinctoria leaves* chloroform extract treatment reversed all the effects of streptozotocin linear and dose dependently. However, more prominent effect of methanolic extract was observed at moderate doses. The chloroform extract increased the size of islets of langerhans inhibited lymphocytic infiltration and vascular changes. The effect of methanolic extract was much higher than the chloroform extract and shows inhibitory effect only on lysis and shrinking.

DISCUSSION

Diabetes mellitus is a serious risk factor for the development of multiple organ damage as a result of multiple and complex mechanism [12]. The present study shows that prolonged administration of *Indigofera tinctoria* leaves methanolic extract protects against chronic streptozotocin induced hyperglycemia and kidney damage in rats. The results of this study obtained from streptozotocin induced diabetes significantly reduced and beneficial in type-I and type-II. In streptozotocin induced creatinine clearance was significantly lower and urinary albumin excretion was significantly higher relative to the normal control group indicating severe effect due to diabetes, similar to earlier reports [11]. Development of micro-albuminuria as a symptom of nephropathy has been widely considered and accepted as a prevalence of cardio vascular disease in human diabetes [13]. In the present study urinary profile was also considered to evaluate nephro-protective effect of *Indigofera tinctoria leaves* along with anti-hyperglycemic effect. *Indigofera tinctoria leaves* alcoholic extract treatment significantly improve renal creatinine clearance whereas glibenclamide produce significant effect on STZ induced renal dysfunction. Streptozotocin induced increased renal protein loss was significantly reversed by the treatment with glibenclamide and *Indigofera tinctoria,* methanolic extracts.

Intra-peritoneal administration of STZ selectively destroys the pancreatic insulin secreting ß- cells living less number of active cells and resulting in type-1 diabetic state [7]. Similar results were obtained in the present study by both histopathological examinations and by serum glucose estimation. The results of the present study have shown that of *Indigofera tinctoria* methanolic extract have potent hyperglycemic effect after chronic treatment but does not have acute effects in STZ diabetic rats. In these IDDM model the effect of glibenclamide and all other groups demonstrated significant hypoglycemic effect similar to the earlier effect of glibenclamide [7]. Glibenclamide is known to produce its effects by the stimulation of insulin release [5]. It is also known that glibenclamide is effective in moderate diabetic rats not in severe diabetic animals [7]. The similar activity of the chloroform and methanolic extract of *Indigofera tinctoria* may indicate that these extracts also act by stimulation of the islet cells of pancreas and insulin like effects on peripheral tissues. However, the effect of the extracts was much higher and potent then compares to glibenclamide even in crude form[7]. IDDM is characterized by the infiltration of lymphocytes in to the pancreatic islets followed by the destruction of ß- cells which leads to overt diabetes [14]. It has also been reported that rat pancreas with STZ induced diabetes, islet cells are shrunken with lytic and vascular changes

[13]. Histopathological examination in the present in study are demonstrated all these histological changes in STZ diabetic control group of *Indigofera tinctoria* methanolic extract treatment prominently inhibited effect of STZ on ß- cells of islets of langerhans in rat pancreas and was observed to be enlarged and free from lymphocytic infiltration. This result support and substantiates anti-hyperglycemic effects as evidenced in bio-chemical profile. These cytoprotective and nephroprotective activities could be related to its anti- oxidant property as reported by earlier study.

The results in STZ diabetic rats provide strong supportive evidence about its possible utility in type-I diabetes [15].These results supports earlier study carried out in antioxidant activity of *Indigofera tinctoria* in streptozotocin nicotinamide induced type-2 diabetic rats [12].

As per U.S. FDA guidelines we selected three gradient doses and one safety dose (5 times of minimum dose). The organs to body weight ratio was considered to evaluate organs specific effect of the treatments and also study chronic toxicity if any by microscopic observation. STZ treatment significantly reduced kidney, liver, heart and pancreas to body weight ratio showing direct effects on all these organs [16]. STZ lower pancreas to body weight ratio was significantly inhibited by all treatment groups due to enlargement of pancreatic ß-cells and hyperplasia as evidenced in histopathological study. Effect of *Indigofera tinctoria* chloroform and methanolic extracts on STZ modulated organs to body weight ratio was highly variable may be due to unknown reasons.

The Compliment an effort of the scientists working on its micro propagation and cultivation. The qualitative phytochemical analysis of the alcoholic extract indicates that it contains sterols, triterpenoids, flavonoids, alkaloids and their glycosides [9]. The methanolic extract shown the presence of glycosides, Saponines and tannins. These constituents may be responsible for observed anti-diabetic and nephro-protective activity.

As per the mechanism of action concerned, we can propose that the anti-hyperglycemic activity of extracts may be due to potentiating the pancreatic secretions by pancreatic ß-cells enlargement, inflammatory cell infiltration, anti-oxidant, cytoprotection and by increasing the glucose uptake. Thus the folk use of this plant has been validated by the study. However more studies are required to identify the active constituents and to explore molecular mechanism of action.

CONCLUSION

Our results on streptozotocin induced diabetic rats, *Indigofera tinctoria* leaves extract possess significant anti-hyperglycemic activity on chronic treatment, indicating its possible applications in type-I, type-II diabetics. More prominent and significant results were obtained with methanolic extract then compared to chloroform extract. However, both these extracts have failed to produce acute anti-hyperglycemic effects in all these models shows that, it has long-term anti-diabetic activity without hypoglycemic side effects. The anti-hyperglycemic

effects observed with alcoholic extract were also prominent than glibenclamide demonstrating it as potent and safe anti-diabetic component then compared to glibenclamide even in crude form. The histopathological study has shown anti-inflammatory, cellular infiltration, cytoprotective, pancreatic enlargement and anti-lipedmic properties indicating not only its efficacy in overcoming from diabetes but also autoimmune disorders. The organ to body weight ratio study indicated pancreatic and liver specific actions, thereby probably stimulating insulin secretion and other liver mediated hypoglycemic effects. *Indigofera tinctoria* alcoholic extract possess anti-diabetic, nephroprotective and cytoprotective activities probably due to the presence of anti-oxidant active constituents present in high numbers than compared to chloroform extract.

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