



Pharmacological Evaluation of *Indigofera argentea* for Its Antidiabetic Activity and Ameliorative Potential on End Organ Damage in Streptozotocin-Induced Diabetic Rats

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Abstract

Research in drug discovery from medicinal plants involves a multidimensional approach including botanical, phytochemical, biological and molecular techniques. The objective of the study was to evaluate the antidiabetic effects of 70% methanolic extract of *Indigofera argentea* (Ia.Cr), a plant from Cholistan desert of Pakistan, and its ameliorative potential on end organ damage in streptozotocin-induced diabetic rats. Male Wistar albino rats were divided into different groups each comprising of six animals. Diabetes was induced in 12 h fasted animals by injecting streptozotocin (STZ, 60 mg/kg; i.p). Blood glucose levels were checked after 72 h and then two weeks after administration of STZ, the animals having levels >250 mg/dl were considered diabetic and included in further studies. The normal control and diabetic control groups were administered distilled water (4 ml/kg/day), standard control group was administered metformin (500 mg/kg/day), and the treatment groups were given Ia.Cr at the doses of 30, 100, 300 mg/kg/day; p.o. for 28 days. Blood of animals was drawn by cardiac puncture, sera separated and analyzed. Histological examination of liver, kidney and heart of one representative animal from each group was performed. STZ was found to induce diabetes mellitus in rats and also caused end organ damage that was demonstrated by hepatic markers (increased levels of ALT, AST, ALP & decreased levels of albumin), elevated renal markers (urea, creatinine and uric acid), lipid profile (increased levels of cholesterol, triglycerides, LDL, and decreased levels of HDL) and histological findings. Ia.Cr was found to produce dose-dependent antidiabetic effects and highly significant (P<0.001) results at the dose of 300 mg/kg, as compared to the diabetic control group. The study concludes that *Indigofera argentea* possesses the antidiabetic effects and prevents the end organ damage caused by STZ.

Keywords: *Indigofera argentea*; Anti diabetic activity; Streptozotocin; Hyperlipidemia

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Introduction

Diabetes mellitus is a group of metabolic disorders which is characterized by hyperglycemia due to defects in insulin secretion, action or both. Chronic hyperglycemia is associated with long-term damage including dysfunction and failure of organs, especially eyes, kidneys, nerves, heart, and blood vessels. Diabetes has reached epidemic proportion and needs strategies for early diagnosis and effective management [1]. Global prevalence of diabetes has increased dramatically as the number of people with diabetes has risen from 108 million in 1980 to 422 million in 2014 [2].

Diabetes can be classified into four clinical categories; Type 1 diabetes which is due to autoimmune destruction of the beta (β) cells and leads to absolute deficiency of insulin, Type 2 diabetes that is characterized by hyperglycemia due to decreased secretion of insulin by β cells of pancreas and insulin resistance, Gestational Diabetes Mellitus (GDM) which is diagnosed during pregnancy and is not clearly overt diabetes, and other specific types of diabetes due to other causes; e.g. genetic defects in β cell function or insulin action, drug- or chemical-induced diabetes, and, any disease of the exocrine pancreas that diffusely injures the pancreas, can cause diabetes [3].

Diabetes leads to severe complications if remains untreated. Generally, these complications are categorized as microvascular and macrovascular. Microvascular complications include diabetic retinopathy, diabetic nephropathy and diabetic neuropathy. Macrovascular complications comprise of the coronary artery disease, peripheral artery disease and stroke [4]. The mechanisms

of diabetic complications involve increased flux of Polyol pathway, increased Hexosamine pathway activity, protein kinase C activation and increase levels of Alpha-Glucosidase Enzyme (AGE). All these mechanisms ultimately lead to increased production of Reactive Oxygen Species (ROS) that causes cell damage [5].

Mostly diabetic patients use oral hypoglycemics and insulin for the management of high blood glucose levels. However, all these treatments have limited efficacy and are associated with undesirable side effects. For example, metformin causes Metformin Associated Lactic Acidosis (MALA), although not very common, but the morbidity and mortality rate by MALA is very high. Gastrointestinal and Central Nervous System (CNS) disturbances are also reported by the patients. Chronic use of thiazolidinedione and acarbose was reported to cause liver injury [6]. Sulphonylureas and meglitinides can cause severe hypoglycemia. Major side effects of oral hypoglycemics are dizziness, headache, neurological deficit, idiosyncratic liver cell injury, digestive problems, and even death due to severe hypoglycemia [7].

Due to high risk of adverse effects by almost all the pharmacological agents used in the management of diabetes, the interests are shifted to develop new drugs having less risk of side effects. Now the traditional medicines, particularly the plants, have extensively been studied and used as alternative. Medicinal plants remain a powerful source for new drugs contributing about 90% of the newly discovered pharmaceuticals [8].

A large proportion of world's population use herbal drugs for their beneficial effects. Scientific studies on traditional herbal drugs may provide a substantial alternative for their use in the management of diabetes and prevention of its complications that lead to end organ damage and even the organ failure. Moreover, the high cost and poor accessibility of currently available allopathic medicines in the rural areas of developing countries like Pakistan make it more difficult to manage the disease. There are many plants which are being used for medicinal purposes, many of them have been used for decades to prevent and control the progression of various diseases. Natural products have always been a good alternative therapy for the management of diabetes.

Indigofera argentea, a medicinal plant, indigenous to Cholistan, is commonly known as Jantar, and is used by folk healers in the management of diabetes, and its seeds mixed with flour are used in the treatment of skin diseases. It is used to increase the fertility of soil and as green manure. It is also used as good food for livestock. *In vivo* and *ex vivo* studies showed that *I. argentea* has significant analgesic, anti-inflammatory and antipyretic activities [9]. It has shown antibacterial potential against *Bacillus subtilis*, *Klebsiella pneumoniae*, *Proteus vulgaris* and *Pseudomonas aeruginosa* as compared to ciprofloxacin [10].

Different animal models have been established to study different compounds having proposed antidiabetic potential for the induction of diabetes but Streptozotocin (STZ) is proved to be a better diabetogenic agent than other chemical agents because of its greater reproducibility and more stability in solution than alloxan. STZ is not only diabetogenic but also nephrotoxic, hepatotoxic [11], and can cause hyperlipidemia [12]. STZ model imitates many of the acute and chronic complications of human diabetes and so assumed the established similarities of some of the structural, functional and biochemical abnormalities to human disease.

The aim of the study is to investigate the antidiabetic effects of the crude extract of *Indigofera argentea*, and to assess its protective potential against streptozotocin-induced complications like hepatotoxicity, nephrotoxicity and hyperlipidemia.

Material and Methods

Chemicals and equipment

Streptozotocin was purchased from Bioworld (USA), citric acid monohydrate and trisodium citrate dehydrates from Sigma-Aldrich, USA. Digital electronic balance (Shimadzu, Japan), grinder (National, Japan), vortex mixer (Serulin bioscience, Korea), incubator, centrifuge machine, rotary evaporator (Heidolph, Germany), glucometer (Bayer, Germany), automatic chemistry analyzer (Beckman coulter, Japan) and microscope were used.

Preparation of the crude extract of *Indigofera argentea* (Ia. Cr) and biochemical analysis

The 70% methanolic aqueous extract of *Indigofera argentea* was prepared according to the protocol with some minor modifications [13]. One kg of *I. argentea* was purchased from local market and identified from the botanist. A specimen of the plant was deposited at the herbarium of Pharmacology research lab, the Islamia University of Bahawalpur (IUB), and voucher no. 1A-WP-09-14-76 was issued for future reference. After cleaning, dried plant material was ground into coarse powder and soaked in aqueous methanol (30:70) for 3 days with occasional stirring at room temperature. Plant material was filtered thrice. The filtrate was concentrated to thick, semi-solid mass; i.e. crude extract (Ia.Cr), by using rotary evaporator. Percent yield of the extract was calculated; the extract was labeled and stored in refrigerator for further use. The prepared extract was screened for the analysis of secondary metabolites.

Pharmacological section

Experimental animals: Adult male Wistar albino rats, 250 g to 300 g, were housed in animal house of pharmacology research laboratory, IUB, in polycarbonate cages with temperature ($22 \pm 2^\circ\text{C}$) at 12 h light dark cycle and relative air humidity $45 \pm 5\%$. Each cage contained six rats and all the animals were provided food and drinking water ad libitum. The study was conducted as per guidelines of the Institutional Research Ethical Committee (PREC).

Induction of diabetes: Diabetes was induced in 12 h fasted animals by administering single intraperitoneal injection of streptozotocin (60 mg/kg) in 0.1 M citrate buffer (pH 4.5, 2 ml/kg; i.p.). After administration of STZ, animals were given 10% dextrose water for next 48 h, as damage of pancreatic islets causes more release of insulin causing severe hypoglycemia and even death of the animals. Blood glucose levels were checked after 72 h and two weeks after administration of STZ with glucometer by taking blood from the tail vein [14] and the animals having blood glucose levels more than 250 mg/dl were considered diabetic and included in the study [15].

After induction of diabetes, normal control and intoxicated (diabetic control) groups received normal saline (4 ml/kg; p.o.) and the standard group received metformin at the dose of 500 mg/kg; p.o. Three treatment groups were given Ia.Cr at different doses; i.e. 30, 100 and 300 mg/kg; p.o. for twenty eight days as single daily dose. Blood glucose levels of animals were checked on day 0, 7, 14, 21 and 28.

Serum analysis: At the end of the study, the animals were anesthetized with ketamine/xylazine in combination of 10:1 (2 ml/kg; i.p.) and blood samples were taken by cardiac puncture. The blood

samples were centrifuged (4000 rpm for 15 min); sera were separated and analyzed. Renal functions were evaluated by analyzing urea, creatinine and uric acid levels; while, liver performance by checking the levels of alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase and albumin, and lipid profile was evaluated by determining the levels of total cholesterol, triglycerides, low density lipoproteins and high-density lipoproteins.

Histopathology: Heart, liver and kidney from one representative animal of each group were dissected out under anesthesia, washed with normal saline, preserved in formalin (10%) for 24 h and embedded in paraffin wax [16]. With the help of microtome, samples were sectioned out in 5 μ m slices and then stained with eosin-hematoxylin dye. These sections were examined under microscope for histopathological changes in liver, kidney and heart. Photomicrographs were also taken and observed.

Acute toxicity study: Acute toxicity studies were performed according to the guidelines of Organization for Economic Co-operation and Development (OECD) [17]. Swiss albino mice weighing 18 g to 30 g were grouped into 5 groups each comprising of five animals. The normal behavioral parameters; i.e. alertness, grooming, convulsions, hyperactivity, lacrimation, salivation, urination, touch response, pain response, writhing reflex, corneal reflex, gripping strength and righting reflex, were observed. Animals were kept on fasting and received only water ad libitum. One group received vehicle and other four groups received different doses of Ia.Cr in increasing order; i.e. 300, 1000, 3000 and 10,000 mg/kg. Behavioral changes, response parameters and mortality were observed and recorded at 0.5, 1, 2, 4, 6, 12, 24, 48 and 72 h, and then on 7th and 14th day.

Statistical analysis

The results were analyzed statistically and expressed as mean \pm S.E.M. One Way Analysis of Variance (ANOVA) was performed on the collected data to compare the significance level between different groups. Results were considered as non-significant if $p > 0.05$, significant (*) if $p < 0.05$, more significant (**) if $p < 0.01$ and highly Significant (***) if $p < 0.001$.

Results and Discussion

Phytochemical analysis of Ia.Cr

Presence of alkaloids, amino acids, carbohydrates, flavonoids, glycosides, proteins, phenols, quinones, saponins, tannins and terpenes was confirmed by different tests used for phytochemical analysis.

Pharmacological evaluation of Ia.Cr on STZ-induced diabetes

Blood glucose levels were checked before induction of diabetes, after induction of diabetes, and on 7th, 14th and 28th days of the treatment. The treatment of rats with different doses of Ia.Cr (30, 100 and 300 mg/kg) showed significant decrease in the blood glucose levels (Table 1).

Mechanisms of hypoglycemic action in the medicinal plants have been reported to be the inhibition of renal glucose reabsorption, increased secretion of insulin from β -cells of the pancreas, increased tissue uptake of glucose by enhancement of insulin sensitivity, regeneration or repair of the β -cells, stimulation of glycogenesis and hepatic glycolysis, protective effect on the destruction of the β -cells and/or prevention of oxidative stress that is possibly involved in pancreatic β -cells destruction [18,19]. The fiber of plants may

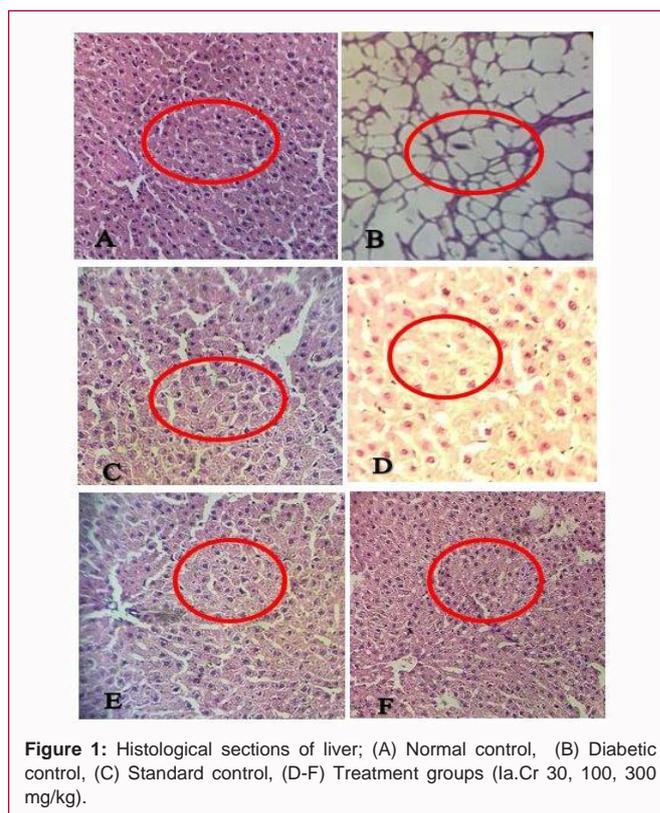


Figure 1: Histological sections of liver; (A) Normal control, (B) Diabetic control, (C) Standard control, (D-F) Treatment groups (Ia.Cr 30, 100, 300 mg/kg).

also interfere with carbohydrate absorption and contribute to decrease blood glucose levels [20]. The antidiabetic potential of *Indigofera argentea* may be due to the presence of phytochemical constituents such as flavonoids, saponins, alkaloids, glycosides and tannins. Flavonoids and alkaloids have been reported to possess the α -glucosidase inhibitory activity [21]. AGIs delay the absorption of carbohydrates from small intestine which decreases the glucose level in blood. Saponins have the ability to induce the release of insulin from the pancreas [22] while tannins have been reported to possess the antioxidant activity and enhance glucose uptake and inhibit adipogenesis [23].

Pharmacological evaluation of Ia.Cr on STZ-induced hepatotoxicity

The hepatic markers; such as, Alanine Transaminase (ALT), Aspartate Transaminase (AST), alkaline phosphatase and serum albumin levels in diabetic group showed significant results when compared with the normal control group and standard control group treated with metformin. The treatment of rats with different doses of Ia.Cr (30,100 and 300 mg/kg) showed significant decrease in the enzyme levels in serum as shown in Table 2.

STZ induces hepatocellular damage causing excessive release of the enzymes into the serum due to tissue injury. When the integrity of the hepatocellular membrane is compromised, there is release of the marker enzymes (ALT, AST and ALP) into the blood. Ia.Cr. and metformin treated groups showed a significant reduction in the levels of the enzymes when compared to the diabetic control group, indicating the protective effects of Ia.Cr in STZ-induced liver injury. The biochemical contents of Ia.Cr possess antidiabetic activity that further limits the liver injury. Flavonoids, alkaloids and phenols have been reported to possess antidiabetic, anti-inflammatory, antioxidant, antihypertensive, antihyperlipidemic and antihepatotoxic effects

Table 1: The effects of Ia.Cr on blood glucose levels.

Treatment Groups	0 Day	7 th Day	14 th Day	21 st Day	28 th Day
Normal control	93.50 ± 4.22	95.33 ± 2.09	100.3 ± 2.60	102.3 ± 2.09	99.83 ± 2.50
Treatment groups	1 st day	7 th day	14 th day	456.4 ± 8.22 ^{###}	439.8 ± 25.01 ^{###}
Normal control	93.50 ± 4.23	95.33 ± 2.10	100.3 ± 2.61	130.9 ± 6.25 ^{***}	117.2 ± 5.42 ^{***}
Treatment groups	2 nd day	7 th day	14 th day	368.5 ± 6.54	267.2 ± 5.69 [·]
Normal control	93.50 ± 4.24	95.33 ± 2.11	100.3 ± 2.62	162.5 ± 6.25 [™]	157.3 ± 2.71 [™]
Treatment groups	3 rd day	7 th day	14 th day	130.9 ± 2.78 [™]	127.5 ± 2.83 [™]

Mean ± SEM (n=6); P<0.05(*), P<0.01(**), P<0.001(***) vs. Diabetic control group; P<0.001(###) vs. Normal control group

Table 2: The effects of Ia.Cr on liver markers.

Treatment	ALT Levels (IU/L)	AST Levels (IU/L)	ALP Levels (IU/L)	Serum Albumin Levels (mg/dl)
Normal control	39.3 ± 1.33	73.83 ± 2.78	94.0 ± 1.77	3.78 ± 0.04
Diabetic control	160.7 ± 3.63 ^{###}	196.3 ± 3.88 ^{###}	406.0 ± 6.22 ^{###}	2.31 ± 0.03 ^{###}
Standard control (Metformin)	41.50 ± 1.50 [™]	77 ± 1.86 [™]	108.5 ± 4.37 [™]	3.73 ± 0.03 [™]
Treatment group I Ia.Cr (30 mg/kg)	139.3 ± 2.30 [·]	179.8 ± 1.44 [·]	282.2 ± 4.62 [·]	2.56 ± 0.02 [·]
Treatment group II Ia.Cr (100 mg/kg)	93.67 ± 2.07 [™]	151.0 ± 4.24 [™]	163.3 ± 3.20 [™]	3.00 ± 0.05 [™]
Treatment group III Ia.Cr (300 mg/kg)	46.67 ± 0.84 [™]	84.33 ± 2.36 [™]	123.2 ± 2.21 [™]	3.57 ± 0.04 [™]

Mean ± SEM (n=6); P<0.05(*), P<0.01(**), P<0.001(***) vs. Diabetic control group; P<0.001(###) vs. Normal control group

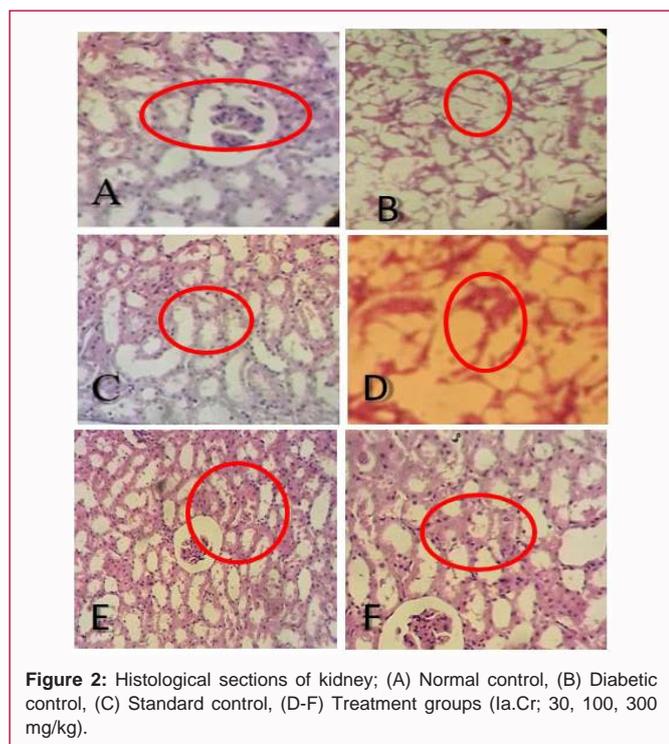


Figure 2: Histological sections of kidney; (A) Normal control, (B) Diabetic control, (C) Standard control, (D-F) Treatment groups (Ia.Cr; 30, 100, 300 mg/kg).

[24,25]. Saponins and terpenoids have also showed antioxidant effects that may protect the hepatocellular injury [26]. Low levels of serum albumin observed in the diabetic control group may be due to the oxidative stress caused by streptozotocin [27] and the groups treated with different doses of Ia.Cr showed increased levels of serum albumin which could be due to the antioxidant potential of chemical constituents present in *Indigofera argentea*.

Effects of Ia.Cr on histopathological parameters of liver

Histological analysis showed that the treatment of Ia.Cr caused marked changes in preventing the STZ-induced diabetic injury; however, at the dose of 300 mg/kg of Ia.Cr, highly significant changes were found to restore the normal hepatic architecture as shown

Figure 1.

Pharmacological Evaluation of Ia.Cr on STZ-induced nephrotoxicity

Nephroprotective potential of Ia.Cr, in STZ-induced nephrotoxicity, was evaluated by measuring the levels of serum urea, creatinine and uric acid in normal control and diabetic control groups. The treatment groups that were given Ia.Cr (30, 100 and 300 mg/kg) showed significant decrease in the levels as compared to diabetic control group, especially at the dose of 300 mg/kg as shown in Table 3.

The results revealed that Ia.Cr showed protective effect against STZ-induced injury which may due to its chemical constituents having anti-inflammatory potential such as flavonoids, alkaloids, saponins and tannins.

Histopathological analysis of kidney

The observations indicated that streptozotocin intoxication caused the renal damage as evidenced by the tubular necrosis and fibrosis observed in diabetic control group (Figure 2B) as compared to normal control group (Figure 2A). Treatment with Ia.Cr showed marked changes in preventing the STZ-induced kidney damage; however, at the dose of 300 mg/kg of Ia.Cr, highly significant changes were found to restore the normal kidney architecture, which was comparable with metformin treated group.

Pharmacological evaluation of Ia.Cr on lipid profile

The values of serum cholesterol, triglycerides, LDL and HDL of metformin treated group and Ia.Cr (30, 100 and 300 mg/kg) were determined. The values in Ia.Cr treated groups, at the doses of 100 and 300 mg/kg, were found more significant when compared with the diabetic group, as shown in Table 4.

Streptozotocin significantly increased the levels of cholesterol, triglycerides and Low-Density Lipoproteins (LDL) that was confirmed when normal control group was compared with diabetic control group. When the diabetic rats were treated with different doses of Ia.Cr, the dose- dependent decrease in levels was observed that was comparable with the standard control group, treated with metformin.

Table 3: The effects of Ia.Cr on renal markers.

Treatment	Serum Urea Levels (mg/dl)	Serum Creatinine Levels (mg/dl)	Uric Acid Levels (mg/dl)
Normal control	20.17 ± 1.30	0.56 ± 0.03	1.40 ± 0.02
Diabetic control	39.67 ± 1.05###	1.90 ± 0.04###	4.69 ± 0.07###
Standard control (Metformin)	24.00 ± 1.06***	0.68 ± 0.06***	1.85 ± 0.01***
Treatment group I Ia.Cr (30 mg/kg)	34.50 ± 0.61 [†]	1.60 ± 0.04 [†]	4.04 ± 0.05 [†]
Treatment group II Ia.Cr (100 mg/kg)	31.00 ± 0.57 ^{††}	1.40 ± 0.03 ^{††}	3.72 ± 0.02 ^{††}
Treatment group III Ia.Cr (300 mg/kg)	25.83 ± 1.57***	0.70 ± 0.05***	2.92 ± 0.03 ^{†††}

Mean ± SEM (n=6); P<0.05(*), P<0.01(**), P<0.001(***) vs. Diabetic control group; P<0.001(###) vs. Normal control group

Table 4: The effects of Ia.Cr on lipid profile.

Treatment	Serum Cholesterol Levels (mg/dl)	Serum Triglycerides Levels (mg/dl)	Serum LDL Levels (mg/dl)	Serum HDL Levels (mg/dl)
Normal control	95.00 ± 2.72	74.83 ± 2.04	16.33 ± 0.55	44.83 ± 0.87
Diabetic control	255.3 ± 7.67###	216.7 ± 5.03###	42.00 ± 1.75###	15.50 ± 1.11###
Standard control (Metformin)	98.00 ± 4.38***	77.67 ± 2.56***	20.00 ± 0.25***	30.83 ± 1.30***
Treatment group I Ia.Cr (30 mg/kg)	225.8 ± 5.46 [†]	160.3 ± 2.01 [†]	35.00 ± 1.46 [†]	19.00 ± 0.36 [†]
Treatment group II Ia.Cr (100 mg/kg)	177.5 ± 4.54 ^{††}	100.3 ± 2.231 ^{††}	27.00 ± 0.68 ^{††}	23.50 ± 0.84 ^{††}
Treatment group III Ia.Cr (300 mg/kg)	111.8 ± 3.11***	84.17 ± 1.66***	22.67 ± 0.61***	31.83 ± 0.87***

Mean ± SEM (n=6); P<0.05(*), P<0.01(**), P<0.001(***) vs. Diabetic control group; P<0.001(###) vs. Normal control group

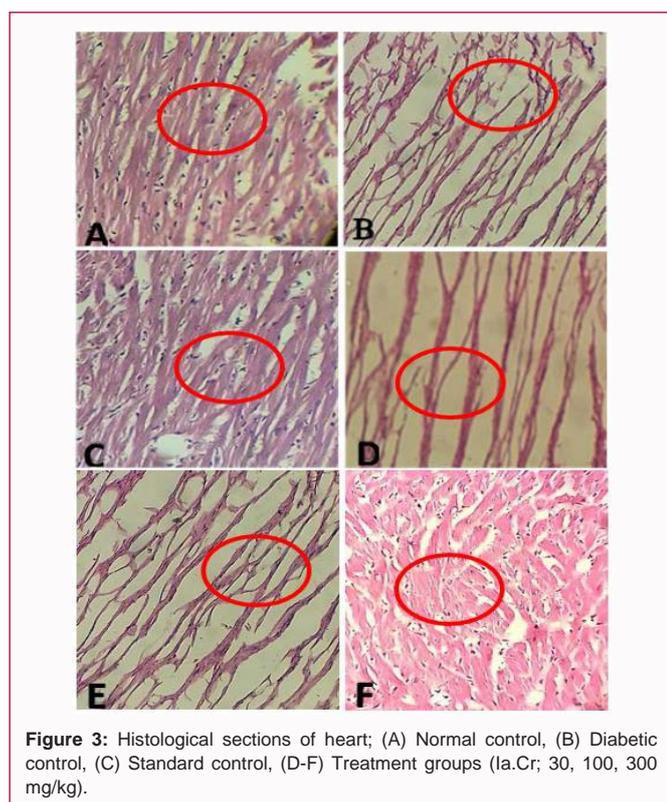


Figure 3: Histological sections of heart; (A) Normal control, (B) Diabetic control, (C) Standard control, (D-F) Treatment groups (Ia.Cr; 30, 100, 300 mg/kg).

These results suggested that Ia.Cr decreased hyperglycemia and hyperlipidemia, which could be due to inhibiting the progression of oxidative stress in STZ-induced diabetic rats.

Histopathological analysis of heart

Normal control group showed single, oval and centrally located nuclei of cardiomyocytes and cardiac myofibers were regularly arranged (Figure 3A). The same pattern was found in control group, treated with metformin (Figure 3C). However, nuclei of the cardiomyocytes in diabetic control group showed deformation in sizes and shape, and the cardiac myofibers were seen in disarrayed

pattern as compared to the normal control group, which could be due to the degeneration of the structural proteins in diabetes mellitus (Figure 3B). Ia.Cr treated groups showed the comparable histological findings as that of control group especially at the dose of 300 mg/kg, which showed regularly arranged cardiac myocytes and centrally located nuclei.

Results of acute toxicity studies

Ia.Cr was found safe up to the dose of 10 g/kg in acute toxicity studies. No signs of toxicity were observed during initial 24 h and further no lethality was recorded after 48 h till 14 days.

Conclusion

The results indicated that Ia.Cr reduced the levels of hepatic and renal marker enzymes and reduced hyperlipidemia significantly. It is, therefore, concluded that *Indigofera argentea* has high therapeutic potential against STZ-induced diabetes and end organ damage which could be due to the presence of flavonoids and other biochemical constituents like alkaloids, saponins and phenolic compounds. However, further studies are encouraged to explore the mechanistic basis of Ia.Cr for its protective effects against diabetes and organ damage.

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