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HISTOPATHOLOGICAL ALTERATIONS IN ALLOXAN INDUCED DIABETIC MICE LIVER AND KIDNEY AFTER *CARISSA SPINARUM* METHANOLIC LEAF EXTRACT TREATMENT

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ABSTRACT: *Carissa spinarum* is an evergreen, thorny shrub found in the Himalayan areas of the Indo-Pakistan subcontinent. The plant has a number of ethnomedicinal applications. Pharmacologically this plant is used for the treatment of asthma and pulmonary diseases, anticancer, diarrhea, hepatoprotective, cardioprotective and reproductive dysfunction. The present study is aimed to study effects of *Carissa spinarum* methanolic leaf extract in alloxan treated mice liver and kidney tissues. First group mice served as control and were given distilled water only. Second group mice were given *Carissa* leaf extract orally to a dose of 800 mg/kg body weight. Mice were injected with intraperitoneal injection of alloxan monohydrate at a dose of 120 mg/kg body weight and were further divided into three groups. Third group mice served as diabetic control and were given distilled water. Fourth and fifth group diabetic control mice were given *Carissa* leaf extract orally to a dose of 600 and 800 mg/kg bodyweight for 28 days. Sixth group mice were given glimepiride at a dose of 2mg/kg body weight. Fasting blood sugar levels were determined after regular intervals and prior to dissection. A significant decrease in blood glucose levels with extract administered groups from initial value 267.96 ± 1.602 mg/dl to final value 168.03 ± 1.598 mg/dl was observed as compared to diabetic mice during the period of experiment up to 28 days. The histopathological studies of liver and kidney of diabetic mice revealed degeneration of normal tissue architecture and various other complications, reparative changes were observed after treatment with *Carissa spinarum* leaf extract.

INTRODUCTION: Diabetes is a complex multisystemic disorder characterized by a relative or absolute insufficiency of insulin secretion and disturbance in carbohydrate, protein, and lipid metabolism[1]. It is a major degenerative disease in the world today, which affects a minimum of 15 million people with complications like hypertension, atherosclerosis, and microcirculatory disorders [2].

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Diabetes is broadly classified into insulin-dependent, in which the pancreas is not able to synthesize and secrete insulin and insulin-independent in which the body cells do not possess normal receptors for insulin also called Type-1 and Type-2 diabetes respectively. Both types are characterized by high blood glucose levels in the blood resulting in polyuria, polydipsia and polyphagia. The therapeutic remedies of diabetes cover a vast region of the healthcare market. Though there are so many hypoglycemic medicines available in the market but the demand for natural remedies still persists. This is due to the avoidance of side effects posed by these synthetic agents and the price related issues. Several plants are used for treatment of various diseases.

These are natural and do not have any synthetic compounds that may harm the health of the consumer.

Carissa spinarum also is known as ‘‘conkerberry’’ or ‘‘Bush Plum’’ is a large shrub, which belongs to the family of Apocynaceae. It is an erect thorny shrub, with forked branches. It generally grows up to a height of 2-3 meters. Leaves are ovate, leathery with reticulate venation. Leaves exude white latex when plucked from the stem. Flowers are short-stalked, sweetly scented, bisexual and complete. Roots and leaves are rich in tannins, carissone, palmitic acid, benzyl benzoate, farnesene, stigmasterol, ursolic acid, lupeol, campesterol,17-hydroxy-11-oxo-nor-β-amyrone and urs-12-ene-3β, 22β-diol-17-carboxylic acid [3]. The plant has many medicinal properties *viz*. roots are bitter, stomachic, anthelmintic and are used to treat malaria, wound, inflammation, stomach ache, bleeding after delivery, muscle cramps, dysentery, ulcer, diabetes, male and female weakness, skin disorders and antidote for snake bite. Leaves are used to treat remittent fever, jaundice, hepatitis, and chest pain [4]. Keeping in view the medicinal properties of *Carissa spinarum* and presence of various alkaloids in leaves this plant was selected to study its effects in alloxan-induced diabetes in mice liver and kidney.

MATERIALS AND METHODS:

Collection of Plant Material: Fresh leaves of *Carissa spinarum* were collected from different areas of district Mandi, Himachal Pradesh and were identified and authenticated by taxonomists of HPU.

Preparation of Plant Extract: The collected plant leaves were cleaned and dried under shade for fifteen days and powder of dried leaves was formed. 1 kg powdered plant sample was extracted thrice in a ratio of methanol: water- 80:20 at 25 °C for 24 h each. For filtration Whatman no. 1 filter paper was used and then the filtrate was concentrated on a rotary evaporator under reduced pressure at 50°C, and dry extract was stored at 4 °C for further investigation.

Acute Toxicity Study: The study was carried out to determine the therapeutic dose of the methanolic leaf extract. For acute toxicity study evaluation of *Carissa spinarum* methanolic extract was suspended in distilled water and administered orally at a dose of 200, 500, 1000 and 2000 mg/kg body weight. The general activity of mice was monitored for 1 h after dosing periodically during first 24 h and then daily for 14 days. Changes in normal behavior of mice and their weights were monitored and time at which sign of toxicity or death appeared.

Grouping of Mice: Group 1- Normal mice designated as control received only distilled water, Group 2- Mice that receive methanol leaf extract of *Carissa spinarum* at a dose of 800 mg/kg body weight, Group 3- Mice injected with alloxan intraperitoneally at a dose of 120 mg/kg body weight, Group 4- Diabetic mice that receive extract at a dose of 600 mg/kg body weight, Group 5- Diabetic mice that receive extract at a dose of 800 mg/kg body weight, Group 6- Diabetic mice receive glimepiride at a dose of 2 mg/kg body weight.

Administration of Alloxan Monohydrate: Alloxan monohydrate (Sigma chemicals) was used to induce diabetes in mice. A freshly prepared solution of alloxan monohydrate in distilled water was injected to overnight fasted animals intraperitoneally at a dose of 150 mg/kg body weight but mortality was observed. So, dose was recalculated and mice were injected with alloxan at a dose of 120 mg/kg body weight. Then animals with blood sugar greater than 180 mg/dl were selected for further study and divided into groups. The experimental procedures were carried out in strict compliance with the Institutional Animals Ethics Committee (IAEC/BIO/1-2013).

Body Weight: Body weights of normal mice, diabetic mice, and diabetic mice treated with extract were taken after 7, 14, 21, 28 days of investigation.

Determination of Blood Glucose Levels: Blood samples were collected by cutting tips of tails of mice after 72 h of alloxan injection and before dissection using glucometer strips and expressed as mg/dl. Blood glucose levels were measured using glucometer every time on 7, 14, 21, 28 days of the experiment.

Histological Study: Tissues were fixed in Bouin's fixative for 24 h. After thorough washing in running tap water excess of fixative was removed from the tissues. Tissues were dehydrated finally in different grades of alcohol (30%, 50%, 90%, 100%) and embedded in paraffin wax (58-60 ºC). Sections were cut and employed for hematoxylin-eosin staining.

Haematoxylin-Eosin Staining: Paraffin cut sections of tissue (4-6µm) were stretched on albuminised slides in warm water. Sections were subjected to overnight dewaxing in xylene at 37 ºC followed by hydration in descending grades of alcohol (100%, 90%, 70%, 30%). These were kept in distilled water and subjected to hematoxylin staining for 10-15 min. Sections were passed through acid water (1-2 drops of HCl in distilled water) and again washed in distilled water and dehydrated in ascending grades of alcohol (30-90%). They will be kept in eosin stain for 2-3 minutes and again in absolute alcohol. These were cleared in xylene and mounted in DPX. Slides were dried in the oven, examined, and photographed for further studies.

RESULTS:

Acute Toxicity Study: There was no mortality amongst the assigned dose groups of animals and did not show any toxicity or behavioral changes at a dose level of 2000mg/kg. This finding suggested that the methanolic extract is safe or non-toxic to mice, and hence doses of 600, 800 mg/kg were selected for the study.

Body Weight: In the present study, alloxan-induced diabetic mice showed significant (P<0.01) reduction in body weight. Administration of methanolic leaf extract of *Carissa spinarum* (600 and 800 mg/kg) and glimpiride (2g/kg) significantly (P<0.01) increased the bodyweight within 28 days **Fig. 1**.

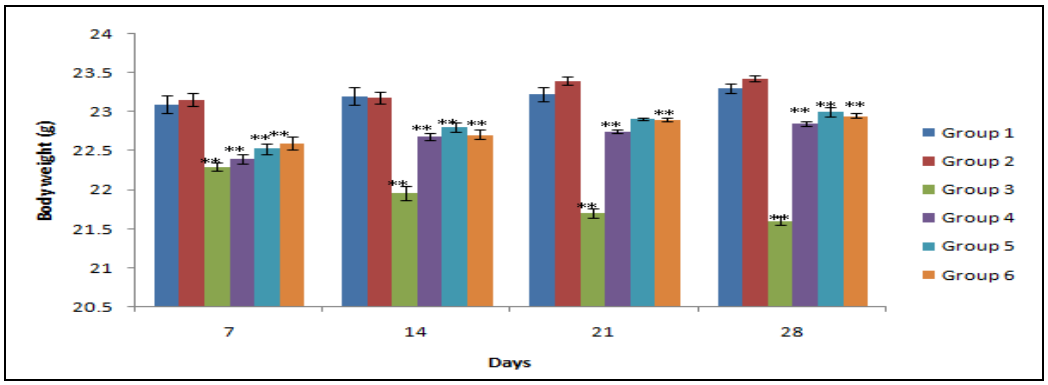

FIG. 1: CHANGES IN BODY WEIGHT (g) OF NORMAL (GROUP 1), *CARISSA SPINARUM LEAF* EXTRACT 800 mg/kg (GROUP 2), ALLOXAN INJECTED (GROUP 3), DIABETIC MICE TREATED WITH *CARISSA SPINARUM* LEAF EXTRACT 600 mg/kg (GROUP 4), DIABETIC MICE TREATED WITH *CARISSA SPINARUM* LEAF EXTRACT 800 mg/kg (GROUP 5), DIABETIC MICE TREATED WITH GLIMPIRIDE 2 mg/kg BODY WEIGHT (GROUP 6) DURING 7-28 DAYS PERIOD. Values are mean ± SEM; n = 3 (p**< 0.01).

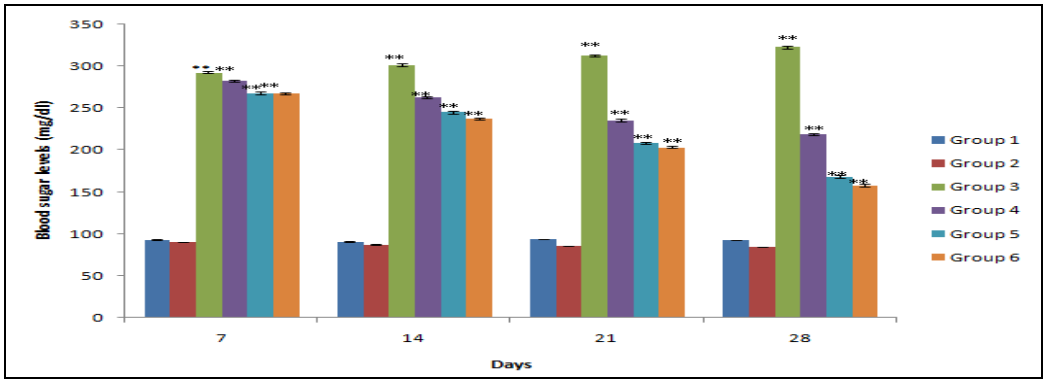

FIG. 2: CHANGES IN BLOOD GLUCOSE LEVELS (mg/dL) OF NORMAL (GROUP 1), *CARISSA SPINARUMLEAF* EXTRACT 800 mg/kg (GROUP 2), ALLOXAN INJECTED (GROUP 3), DIABETIC MICE TREATED WITH *CARISSA SPINARUM* LEAF EXTRACT 600 mg/kg (GROUP 4), DIABETIC MICE TREATED WITH *CARISSA SPINARUM* LEAF EXTRACT 800 mg/kg (GROUP 5), DIABETIC MICE TREATED WITH GLIMPIRIDE 2 mg/kg BODY WEIGHT (GROUP 6) DURING 7-28 DAYS PERIOD. Values are mean ± SEM; n=3 (p**< 0.01).

Blood Glucose Levels: The levels of glucose in blood, of alloxan-induced diabetic mice, were significantly ($p<0.01$) elevated as compared with control mice. Oral administration of leaf extract of *Carissa spinarum* to diabetic mice for 28 days caused significant reduction in blood glucose levels from an initial level of 282.63 ± 1.53 mg/dl to 218.66 ± 1.047 mg/dl and 267.96 ± 1.602 mg/dl to 168.03 ± 1.598 mg/dl for 600 mg/kg and 800 mg/kg doses respectively **Fig. 2**.

Histopathology of Kidney: Normal kidney section **Fig. 3A** demonstrated the blood filtration unit, nephrons or uriniferous tubules in the kidney and renal corpuscle or malpighian corpuscle consisting of normal glomerulus surrounded by the urinary space in the cortical region. In alloxan-induced kidney tissue glomeruli were congested and hence widen the urinary space of renal corpuscle. Areas of hemorrhage, vacuolar degeneration in tubular epithelial cells were also observed. Degeneration in form of karyolysis, damaged tubules, and congested medullary rays was seen in interstitium. Kidneys revealed vascular congestion and nephrotic changes both in cortical and medullary regions. Glomeruli were congested and frequently revealed hypercellularity and reduced peri-glomerular space. Some glomeruli revealed hypocellularity, hypersegmentation or shrinkage with the widening of peri-glomerular space. Convoluted tubules in the cortical region revealed degenerative changes characterized by swollen epithelium with indistinct cell boundaries and increased eosinophilia. The kidney of the alloxanated diabetic mice showed vacuolar degeneration in some tubular epithelial cells and cell debris scattered in tubular lumina. Increase in thickness of tubular epithelial cells with narrowing of lumen, massive cellular infiltration and areas of hemorrhage in interstitial tissue were seen **Fig. 3B-C**. *Carissa spinarum* leaf extract-treated mice kidney sections showed near to normal mice kidney architecture with minute cellular infiltration. Convoluted tubules were intact and regular. An expanded glomerulus with well defined urinary space and reformation of renal tubular epithelium was also observed. The extract treated mice kidney showed features of healing *i.e.* regenerating renal corpuscles, absence of inflammatory cells and normal basement membrane and capillaries. A decrease in the mucopolysaccharide and hyaline deposits and respective diminution of cellular infiltration was also observed **Fig. 3D-F**.

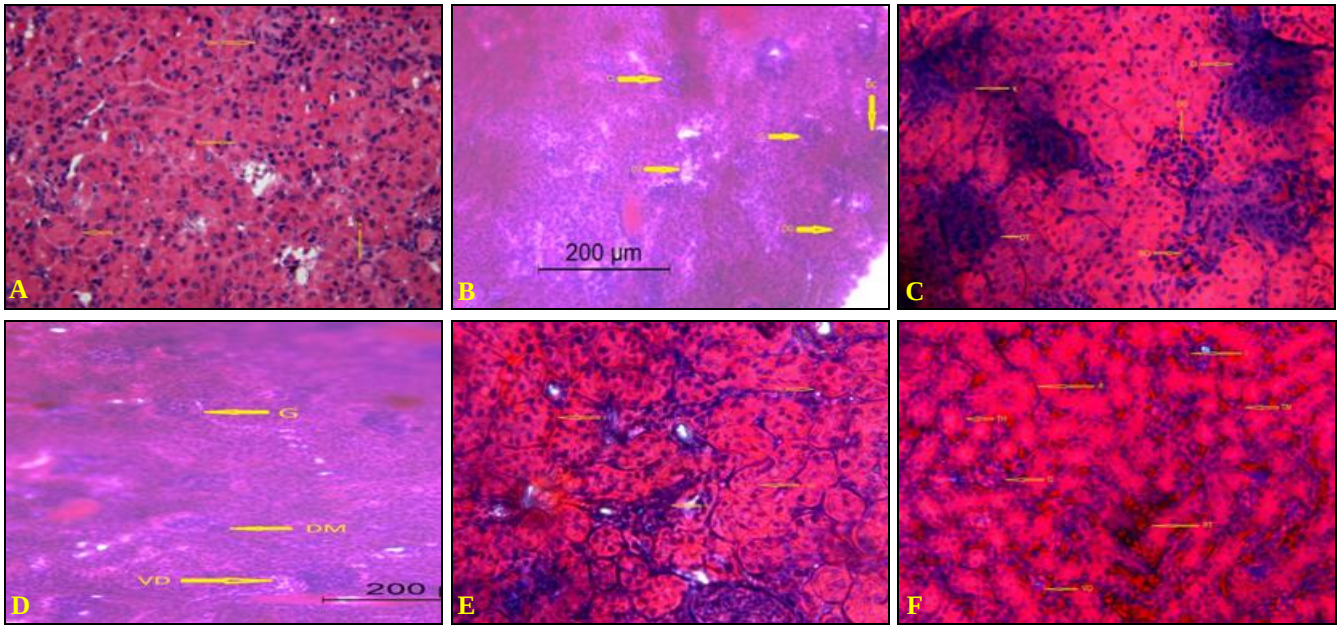

FIG. 3: A: T. S. OF NORMAL KIDNEY SHOWING GLOMERULUS (GM), WELL DEFINED RENAL TUBULE (RT), THICK AND THIN LOOPS OF HENLE (TH, TN) AND WELL DEFINED RENAL ARCHITECTURE. B-C: T. S. OF ALLOXAN INDUCED DIABETIC MICE KIDNEY TISSUE SHOWING DEGENERATING GLOMERULUS WITH WIDENED URINARY SPACE (DG), DEGENERATING RENAL TUBULES (DT), CELLULAR INFILTRATION (CI), KARYOLYSIS (K), BLOOD CELL INFILTRATION (BCI) AND DEFORMED RENAL ARCHITECTURE. D-F: T. S. OF *CARISSA SPINARUM*LEAF EXTRACT TREATED KIDNEY TISSUE SHOWING REPAIRATIVE CHANGES WITH REDUCTION IN THICKENING OF EPITHELIAL WALLS OF RENAL TUBULES (R), REDUCTION IN VACUOLAR DEGENERATION AND DIMINUTION IF CELL INFILTRATION (DM)

Liver: Liver of normal mice showed polygonal hepatocytes. Nuclei were large and rounded under normal conditions. Hepatocytes deliver endocrine secretions into bile ducts. Liver cells border a vascular space called sinusoid and fibrous connective tissue forming supporting framework of liver shown in **Fig. 4G**. Alloxan induced mice liver showed changes in nuclear size from congested nuclei to a large enucleated area because of distortion in hexagonal shape of hepatocytes and formation of pyknotic nuclei. Degeneration of fibrous connective tissue resulted in formation of vacuolated hepatocytes with condensed nuclei and infiltration of blood cells was also observed. Widened blood vessels were also seen due to hemolysis of blood cells. The clumping of nuclei at various places was also seen **Fig. 4H-I**. *Carissa spinarum* leaf extract treated diabetic mice liver tissue showed reparative changes **Fig. 4J-L** in size of nuclei and cellular infiltration was also decreased. At 28 days stage mice liver showed similar hepatocytes arrangement like the normal liver mice. The reformation of fibrous connective tissue was also seen. Very less or little infiltration of nuclei was also seen. Blood vessels also become compact and revealed very less hemolysis.

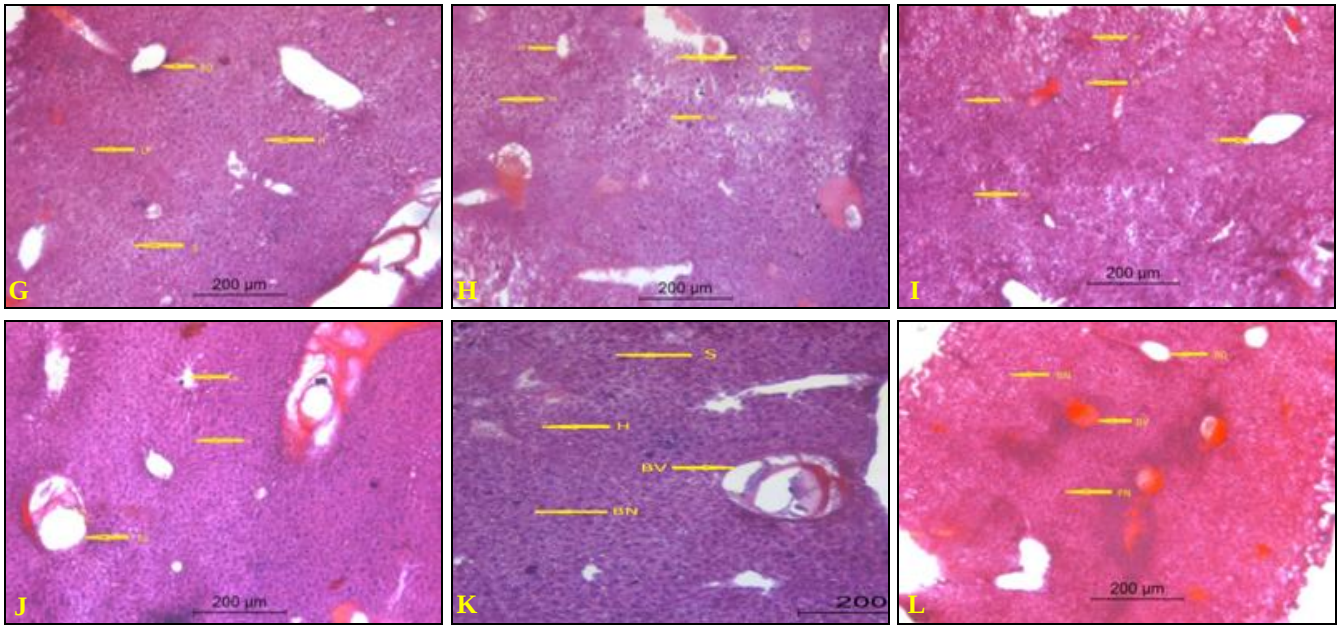

FIG. 4: G: T.S. OF NORMAL LIVER TISSUE SHOWING POLYGONAL HEPATOCYTES (H), LIVER PLATES (LP), SINUSOIDS (S), BILE DUCT (BD). H-I: T. S. OF ALLOXAN INDUCED MICE LIVER TISSUE DEPICTING DEGENERATION OF FIBROUS CONNECTIVE TISSUE (CTF), LARGE ENUCLEATED AREA (ENA), NUCLEAR DEGENERATION (ND), SOME BINUCLEATE NUCLEI (BN), DEGENERATING LIVER CONNECTIVE TISSUE AND INFILTRATION OF BLOOD CELLS (INF). J-L: T. S. OF *CARISSA SPINARUM* LEAF EXTRACT TREATED LIVER TISSUE SHOWING REFORMATION OF DEGENERATED LIVER TISSUE ARCHITECTURE WITH NORMAL POLYGONAL HEPATOCYTES (H) REPAIRED NUCLEI AND MILD CELLULAR INFILTRATION

DISCUSSION: Diabetes is manifested by multiple disturbances in the metabolic processes of the body, which are directly attributed to an insufficient supply of insulin. This investigation aimed to study histopathological changes in liver and kidney of alloxan-induced diabetic mice after treatment of *Carissa spinarum* methanolic leaf extract. Liver sections of alloxan-induced diabetic mice showed marked structural alterations in the liver as a result of absence of insulin. The major alteration was periportal fatty infiltration and necrosis of hepatocytes. Liver lesions in Alloxan-induced hypoglycemia included congestion, hepatosis, and necrosis. Alloxan-STZ induced hypoglycemia additionally revealed hepatitis and Kupffer cell hyperplasia. Acute hepatopathy, like nephropathy, may be ascribed to direct drug-induced toxicity and hypoglycemia or hyperinsulinemia.

The present study evaluation of methanolic leaf extract of *Carissa spinarum* showed that the oral administration of leaf extract to alloxan-induced diabetic mice reduced fasting blood glucose in dose-independent manner which suggested their inherent hypoglycemic effect. The alloxan-induced

diabetic mice showed two to three fold increase in blood glucose levels as compared to the normal control mice. Administration of diabetogenic drug, alloxan monohydrate, caused selective massive destruction of the β-cells of islets of Langerhans resulting in hyperglycemia [5]. The lowering effect of blood sugar levels by *Carissa spinarum* extract, in the same manner, might suggest that the extract may have been absorbed in the cell system through active transport where a particular concentration saturation of the extract occurred resulting in the rest of extract being excreted. Similar results have been reported by other workers [6]. The plant's antihyperglycemic action maybe by stimulation of pancreatic secretion of insulin. In this context a number of other plants have also been reported to have antihyperglycemic and insulin-releasing stimulatory effect [7].

The study on the effect of the aqueous extract of *C. carandas* on alloxan-induced and normoglycemic Wister rats found that the doses of 500 and 1000 mg/kg of the extract significantly decreased the blood glucose levels of alloxan diabetic Wister rats at 4, 8 and 24 h. They concluded that the plant extract doses had both significant hypoglycemic as well as anti-hyperglycemic effects [8].

The decrease in body weight was observed in alloxan-induced mice as a result of abnormal carbohydrate mechanisms. In an earlier study it was reported that a great reduction in protein synthesis was observed in almost all the tissues due to insufficient ATP and insulin [9]. An increase in body weight of alloxan-induced mice after treatment with leaf extract was observed. Similar results were observed with the plant extract of *E. jambolana* in diabetic albino rats [10].

Histopathological changes in kidney of alloxan-induced mice showed renal glomerular congestion with widened urinary space. Vacuolar degeneration in tubular epithelium, karyolysis, and damaged tubules was also observed. Cellular infiltration and renal hypertrophy were also visualized. The kidney histopathology of alloxan-induced diabetic mice showed marked tubular damage, hemorrhage in the Bowman's space due to glomerular damage. The results indicated a primary and a secondary effect of the diabetic state on the kidney of the mice. The primary effect, the diabetes factor was associated with hyperglycemia and was responsible for dilatation of proximal and distal tubules in the cortex. The secondary effect named the individual response factor was associated with inflammatory processes [11].

Diuresis is a common feature associated with diabetes which may be the reason for structural changes observed with glomerulus [12]. The excellent recovery of renal function expected with the treatment of *Carissa spinarum* can be explained by the regenerative capability of the renal tubules. Similar results have been observed with the treatment of alloxan-induced diabetic mice with *Trigonella foenum graecum* seed powder [13].

The liver of alloxan-induced diabetic mice showed marked structural alterations as a result of absence of insulin. The major alterations were, degeneration of fibrous connective tissue, infiltration of blood cells, clumping of nuclei at various places and necrosis of hepatocytes. Similar results were shown on diabetic mice which showed acute morphological alterations in the liver involving hepatocyte hypertrophy, bile duct hyperplasia and an increased number of intracytoplasmic acidophilus pellets [14]. This damage is partially reversed by the *Carissa spinarum* leaf extract treatment and is similar to that observed by *Vinca rosea* extract in alloxan-induced diabetic rats [15].

Various researchers concluded that complications caused by alloxan in the liver can be overcome by restoring the liver functions back to normal through effective control over hyperglycemic conditions [16, 17]. The present study showed that *Carissa spinarum* leaf extract treatment displayed noticeable capacities to reverse renal and hepatic tissue degeneration and disarrangement.

CONCLUSION: The results of the present study revealed that *Carissa spinarum* methanolic leaf extract is able to reverse and protect renal and hepatic tissues against diabetes mellitus induced organ injuries.

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CONFLICTS OF INTEREST: The authors declare that they have no conflict of interest.

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