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CARISSA SPINARUM: A POTENT RENOPROTECTIVE AGENT AGAINST ALLOXAN INDUCED DIABETES

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ABSTRACT

Carissa spinarum also known as ‘‘conkerberry’’ or ‘‘Bush Plum’’ is a large shrub, which belongs to the family of Apocyanaceae. This species is widely distributed in tropical region and in India. A chemical study of this plant led to the isolation of 12 compounds, including a coumarin, two cardiac glycosides and nine lignans. These isolated compounds have been evaluated for several biological activities, including antiherpetic, cytotoxic, antioxidant and antibacterial effects. Present study was aimed to study effects of *Carissa spinarum* methanolic leaf extract in alloxan treated mice pancreas. Mice were injected with alloxan monohydrate (150 mg/kg BW) intraperitoneally and selected diabetic mice were divided into four groups with six mice in each group. Diabetic mice were given *Carissa* leaf extract orally to a dose of 600 and 800 mg/kg body weight and glimepiride (2mg/kg body weight) for 28 days daily. The histopathological studies of kidney of diabetic mice revealed degeneration of renal architecture, but after supplementation of *Carissa spinarum* leaf extract reformative changes were observed. Significant changes in serum urea, creatinine, enzyme activities of SOD, CAT, GPx, glutathione levels and lipid peroxidation levels were recorded after extract administration to alloxan induced oxidative stress in diabetic mice.

KEYWORDS: *Carissa spinarum*, diabetes, kidney, oxidative stress, histology.

1. INTRODUCTION

Phytotherapy has been a source of medicinal products and there have been many attempts to use herbal medicines for the treatment of diabetes over the years (Kooti *et al*., 2016; Ota and Ulrih, 2017). It is evident from various plant based studies that plant medicines show a potential hypoglycemic activity in diabetes-induced animals. Novel compounds with antihyperglycemic potential isolated from the plant sources were administered to control diabetes mellitus. Studies reveal the role of crude extracts of plants with potential antidiabetic activity in alloxan and streptozotocin-induced diabetic animals. Diabetes mellitus (DM) is a metabolic disorder characterized by the presence of high levels of glucose in blood that occurs either due to deficiency or malfunction of insulin (Liu *et al*., 2019). It is mainly a disease of adulthood but affects both old and young people with females and patients aged 61-65 years mostly affected (Debrah *et al*., 2020). The major factors responsible for type 2 diabetes include obesity, sedentary lifestyle, increased consumption of high energy diets, sugar-sweetened beverages.

Carissa spinarum is a widely distributed *Apocyanaceae* plant in tropical regions and is well known for its medicinal properties. *C. spinarum* is known to possess an extensive range of phytochemicals. Pharmacological importance of the plant fruits has been evaluated by several researchers through in vitro and in vivo advances. The presence of ursolic and betulinic acid in the ethanolic extract of the leaves of *C. spinarum* adds one positive attribute to the plant as these compounds were reported to possess a wide spectrum of activity including antibacterial, anti-fungal, anti-cancer, anti-platelet aggregation and anti-mycotic (Feyissa and Melaku, 2016). These activities of *C. spinarum* have been reported from the crude extract and their different fractions and isolates from fruit, leaves and roots. *Carissa spinarum* contains alkaloids, tannins, carbohydrates, glycosides, saponins, terpenoids, flavonoids and steroids as reported by Liu *et al*., (2021) reported that the phenolic compounds extracted from *Carissa spinarum* showed antioxidant, anti-inflammatory and hepatoprotective activities. Considering the various beneficial effects of *Carissa spinarum* the present study was designed to study the effects of *Carissa spinarum* methanolic leaf extract on alloxan induced experimental diabetic mice for 28 days.

2. MATERIALS AND METHODS

2.1 Preparation of extract

Fresh leaves were collected from Joginder Nagar, District Mandi, Himachal Pradesh, India. The leaves were removed from the stems, rinsed in clean water and then dried in shadow for weeks. After drying, the leaves were ground to powder with the aid of a blender to obtain approximately 1kg. 1 kg powdered plant sample was extracted thrice in a ratio of methanol: water- 80:20 at 25°C for 24 hours each. Whatman No. 1filter paper was used for filtration, then filtrate was concentrated on rotary evaporator under reduced pressure at 50°C and dry extract was stored at 4°C for further investigation.

2.2 Animal usage

Healthy pathogen free Swiss albino mice weighing 25-30 g were procured from Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, India, and protocol of present investigation was approved by Institutional Animals Ethics Committee (IAEC/BIO/1-2013). Mice were kept in polypropylene cages on soft chip bedding and maintained in the animal house of Department of Biosciences under suitable hygienic conditions with suitable light and temperature. Mice were provided with pellet feed (Hindustan Lever Limited, New Delhi, India) and water *ad libitum.*

2.3 Administration of alloxan monohydrate

Alloxan monohydrate (Sigma chemicals) was used to induce diabetes in mice. A freshly prepared solution of alloxan monohydrate in normal saline solution was injected to overnight fasted animals intraperitonealy at a dose of 150 mg/kg body weight.

2.4 Induction of Diabetes

The animals were fasted overnight and diabetes was induced by a single intraperitoneal injection of a freshly prepared alloxan dissolved in normal saline solution. After injection mice had free access to food and water, and given a 15% glucose solution to drink overnight to counter hypoglycemic shock. The mice were considered as diabetic if their blood glucose values reached above 180 mg/dl on day 3 after alloxan injection. The blood glucose levels were measured by using one touch glucometer (Gluco one). After diabetes confirmation, mice were allowed for 7 days to acclimatize to the diabetic condition, and mice with hyperglycemia (blood glucose above 180 mg/dl) were chosen for further study. Treatment with extract started on day 8 after alloxan injection which was also considered as the first day of treatment and continued further until end of the study period.

2.5 Grouping of Animals

Mice of the same age group (3 months old) were divided into six groups. Six mice in each group.

- Group I: Control animals, received only distilled water.
- Group II: Received methanolic leaf extract of *Carissa spinarum* (800 mg/kg BW) daily for 28 days.
- Group III: Injected with alloxan intraperitonealy at a dose of 150 mg/kg BW.
- Group IV: Diabetic mice received *Carissa spinarum* methanolic leaf extract (600 mg/kg BW) daily for 28 days.
- Group V: Diabetic mice received *Carissa spinarum* methanolic leaf extract (800 mg/kg BW) daily for 28 days.
- Group VI: Diabetic mice received glimepiride (2mg/kg BW) daily for 28 days acted as standard. This group was maintained for better comparison of the protective effect of *Carissa spinarum* methanolic leaf extract against diabetes induced complications.

All the chemicals used in the study were of analytical grade and obtained from SD fine chemicals (Mumbai, India) and HIMEDIA (Mumbai, India).

2.6 Tissue harvesting

Animals were sacrificed at 7, 14, 21, 28 days of experiment by cervical dislocation. Pancreas was immediately dissected out, cleaned in normal saline quickly blotted and weighed.

2.7 Histopathological studies

The tissues after excision were fixed in Bouin's fixative for 24 hours. 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^{0}c). Thin 5-6 µm sections were cut on a Spencer type rotary microtome and employed for haematoxylin eosin staining.

2.8 Biochemical studies

Spectrophotometric assays were performed on Hitachi VSU-double beam spectrophotometer and Bausch and Lomb spectronic-20. SOD levels in tissue were estimated by method of Kakkar *et al.*, (1998), units/min/mg of protein. Estimation of enzyme activity of catalase was done by method of Sinha, (1972)**.** A portion of tissue is used for the estimation of glutathione (GSH) content by method of Moron *et al.*, (1979). Glutathione peroxidase was assayed by the procedure of Wendel (1980). Levels of malondialdehyde, index of lipid peroxidation was estimated according to method of Dhindsa *et al.* (1981) using thiobarbituric acid (TBA). Creatinine level in serum was estimated by method of Owen *et al.*, (1954). Level of urea in serum was determined by method of Netelson, (1957).

Statistical analysis

The results were obtained as mean ± SEM. Statistical significance was determined by one way Anova with post-hoc Tukey HSD to find out mean differences between groups. The differences were considered significant at $^{**}p<0.01$.

3. RESULTS

3.1 Histopathological observations

Histological architecture of mice kidney shows normal epithelium of convoluted tubules, thick and thin loop of Henle. Glomerulus was surrounded by well defined urinary space. Proximal and distal convoluted tubules of nephrons were uniformly distributed along with blood vessels in the cortex. Renal medulla consisted of tubules and collecting ducts (**Fig. 1).** Kidney sections of alloxan-diabetic mice showed glomerular alterations. There was a reduction in Bowman's space to the glomerulus. Furthermore, degeneration of renal tubules including intracytoplasmic vacuoles in the epithelial cells of these tubules was also witnessed. Deposition of eosinophilic materials in the intermediate substantial of medulla in the kidney of diabetic mice was also noticed (**Fig. 2-3)**. Treatment of diabetic mice with CS leaf extract for 28 days led to restoration of changes caused by alloxan induction. Mild cellular hypertrophy with mild cellular haemorrhage was noticed. Renal tubules showed reduction in thickening of tubular membrane. Glomeruli also regained their original shape and size with normal epithelial lining. Atrophy of glomerular capillaries causing Bowman's space dilatation with expansion of mesangial tissue was observed **(Fig. 4-6).**

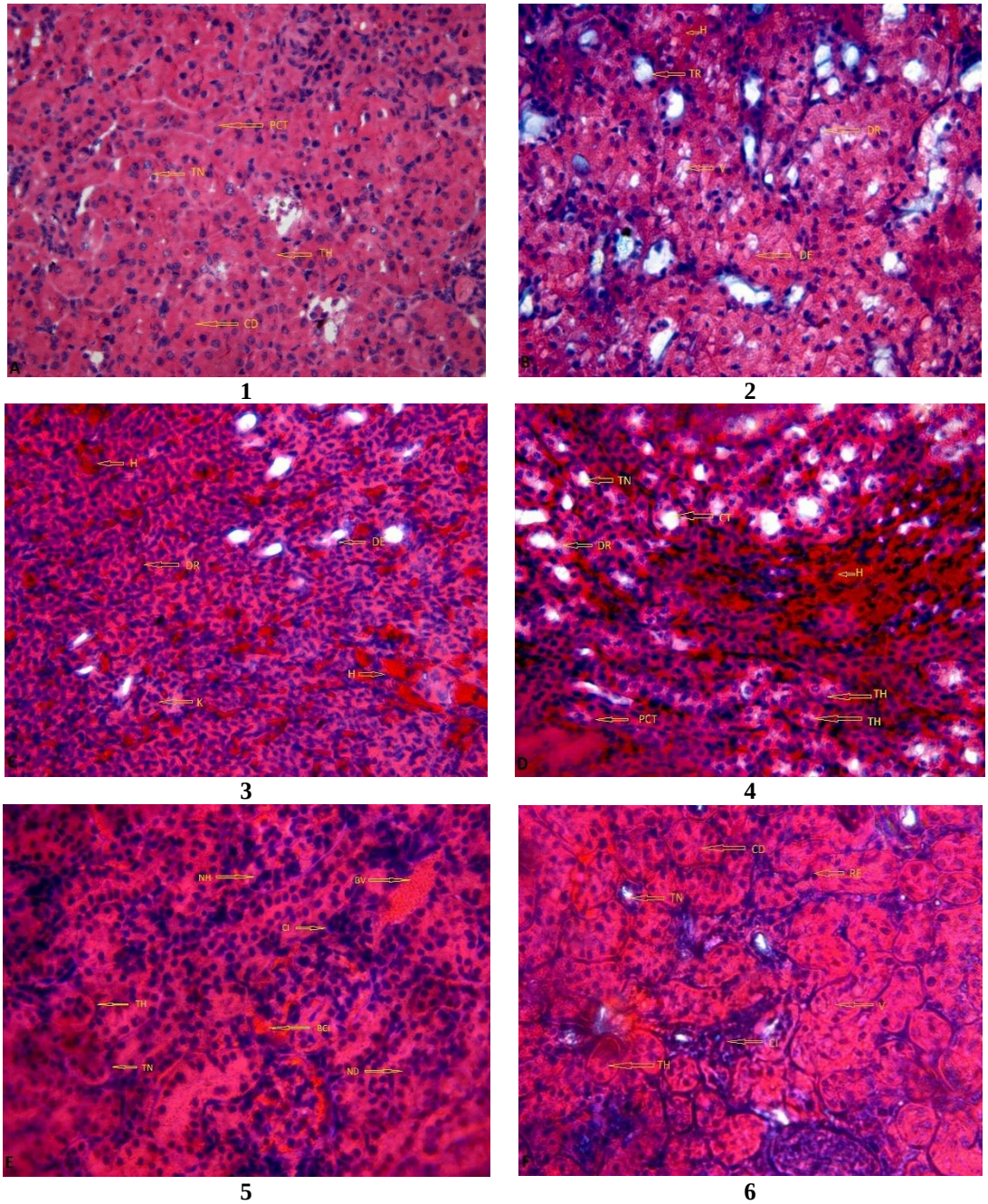

Fig. 1: T.S. of normal kidney showing thick (TH) and thin (TN) loops of Henle, proximal convoluted tubule (PCT), collecting ducts (CD). Fig. 2-3: T.S. of alloxan treated mice kidney with degenerating renal tubules (RT), areas of haemorhage (H), vacuolar degeneration (V), thickening in renal tubular epithelium (TR), degenerating

renal tubular epithelium (DE) and karyolysis (K). Fig. 4-6: T.S. of CS leaf extract treated mice kidney for 28 days showing thick (TH) and thin (TN) loops of Henle, nuclear degeneration (ND), mild cellular infiltration and regenerating tubular epithelium (RE).

3.2 Biochemical parameters

Effects of *C. spinarum* leaf extract on Oxidative Stress Markers and Renal function indicators

The SOD activity was found to be reduced in the kidney of animals treated with alloxan ($p < 0.01$). SOD values in mice treated with alloxan along with CS leaf extract was significantly higher ($p < 0.01$) on the 28th day (Table 1). CAT values in alloxan induced diabetic mice decreased in alloxan induced diabetic mice as compared to control mice whereas an increase in CAT values was observed after supplementation of CS extract (600 and 800 mg/kg bw) and glimepiride (2 mg/kg bw) to diabetic mice on 28th day (Table 1). GSH and GPx values also showed decrease in alloxan induced diabetic mice ($p<0.05$, $p<0.01$) on 28th day. However significant increase was noticed in GSH and GPx values after supplementation of CS leaf extract (600 and 800 mg/kg BW) and glimepiride (2mg/kg BW) to diabetic mice (Table 1). Lipid peroxidation level was elevated significantly in diabetic mice compared to control mice. Administration of CS leaf extract (600, 800 mg/kg BW) and glimepiride (2mg/kg BW) to diabetic mice reduced the LPO level significantly on the 28th day (Table 1). Urea and creatinine are renal function indicators. Alloxan administration to mice caused increase in serum urea and creatinine levels as compared to control mice indicating abnormal kidney functioning. Whereas extract treatment to diabetic mice for 28 days restored the urea and creatinine levels near to normal up to certain extent (Table 2).

Table 1: The effect of *Carissa spinarum* on antioxidative markers of alloxan induced mice kidney after 28 days of extract treatment.

Groups	SOD	CAT	GSH	GPx	LPO
Units	U/mg protein/min		nM/mg protein	µMGSH utilized/min/mg protein	nmoles/mg tissue
Control	14.45±0.30	8.57±0.12	5.52±0.11	13.34±0.04	8.42±0.20
CSE	15.14±0.23**	9.13±0.22**	6.25±0.18**	14.50±0.36*	6.75±0.26
ALX	8.97±0.09**	5.22±0.12	1.88±0.17*	7.05±0.16*	13.58±0.22**
ALX+E1	12.73±0.23	7.44±0.23*	4.13±0.22	10.47±0.38	9.92±0.31*
ALX+E2	13.26±0.22**	7.75±0.11**	4.50±0.30**	11.16±0.26*	9.36±0.13
ALX+G	12.42±0.28	7.09±0.21**	3.91±0.19**	10.08±0.37	9.67±0.30**

Each value is mean±S.D. for six mice in each group. The data for various biochemical parameters were analysed using one way Anova post hoc Tukey's test. Values were considered statistically significant at $p^{*}<0.05$, $p^{**}<0.01$. SOD: Superoxide dismutase, CAT: Catalase, GSH: Glutathione, GPx: Glutathione peroxidase, LPO: Lipid peroxidation. CSE: Carissa spinarum extract, ALX: Alloxan, E1: CSE (600 mg/kg BW), E2: CSE (800 mg/kg BW), G: Glimepiride (2mg/kg BW).

Table 2: The effect of *Carissa spinarum* on renal function markers of alloxan induced mice after 28 days of extract treatment.

Groups	Urea (mg/dl)	Creatinine(mg/dl)
Control	34.70± 0.46	0.77±0.03
CSE	29.23±0.65*	0.41±0.02
ALX	65.64±0.62	2.11±0.07**
ALX+E1	49.59±0.52	0.91±0.08
ALX+E2	42.80±1.54	0.83±0.09**
ALX+G	45.46±2.39**	0.93±0.13

Each value is mean±S.D. for six mice in each group. The data for various biochemical parameters were analysed using one way Anova post hoc Tukey's test. Values were considered statistically significant at $p^{*}<0.05$, $p^{**}<0.01$. CSE: Carissa spinarum extract, ALX: Alloxan, E1: CSE (600 mg/kg BW), E2: CSE (800 mg/kg BW), G: Glimepiride (2mg/kg BW).

DISCUSSION

As mentioned by Liu *et al*., (2021) the phenolic compounds extracted from *Carissa spinarum* showed antioxidant, anti-inflammatory and hepatoprotective activities. The impaired glucose metabolism in diabetes leads to aggravated free radical formation and enhanced triglyceride and lipoprotein levels. The free radicals initiate lipid peroxidation stimulating glycation of proteins, inactivation of antioxidant enzymes thus leading to secondary diabetic complications (Aquacheri *et al*., 2015). Histologically, the kidney sections of alloxan induced diabetic mice in the present study showed marked increase in the glomerular size, mesangial matrix expansion, necrosis and vacuolation of renal tubules. The glomerular enlargement is not due to the increase in the size of the mesangium or in the width of the basement membrane, but due to enlargement of the intercapillary volume. Same results associated with study were reported by (Kim *et al*., 2008 and Muhammad *et al*., 2009) who showed tubular epithelial changes and enlargement of lining of cells of tubules.

CS leaf extract administration to diabetic mice for 28 days led to restoration of changes caused by alloxan induction. Mild cellular hypertrophy with mild cellular

hemorrhage was noticed. Glomeruli also regained their original shape and size with normal epithelial lining. Similar pathological changes were observed after supplementation of root extract of *Musa balbisiana* which decreased the pathological changes induced in diabetic rats. The glomeruli appear to be normal with mild hemorrhages, which may be due to the protective effect of extract. Root extract may decrease the GFR, which in turn may be responsible for narrowing of the bowman's space of glomerulus (Kalita *et al*., 2016). As it is predictable from the current study that activities of free radical scavenging enzymes were diminished by administration of alloxan (ALX). As the activities of important antioxidant enzymes were degraded, the concentration of superoxide anion and hydrogen peroxide (H_2O_2) radicals enhanced, eliciting the production of ROS and dissipation of lipid peroxidation. Similar results were reported by Alezandro *et al*. (2013) which stated that the levels of SOD, CAT, GSH, GSH-Px were diminished in all the tissues of diabetic individuals. Supplementation of CS leaf extract to alloxan induced diabetic mice led to restoration of the activities of GSH, GSH-Px, CAT and SOD to an extent. Al-Attar and Alsalmi, (2019), reported that the values of GSH, SOD, and CAT were declined, and the value of MDA was significantly enhanced in diabetic animals. However, the possible mechanism of the studied extracts attributed to their antioxidant role which was studied by GSH, SOD, MDA, and CAT levels.Lipid peroxidation inhibitory activity of methanolic extract of *C. spinarum* leaves was indicated in the current study. The methanolic extract indicated fairly good lipid peroxidation inhibitory activity and antioxidant activity. Supplementation of *Carissa spinarum* leaf extract in dose dependent manner improves the renal function parameters. Similar findings were reported by a study conducted on *S. alata* showing the tendency to prevent the liver and renal tissues from the damage caused by the oxidative stress during diabetes in STZ induced experimental rats (Sugumar *et al*., 2016).

CONCLUSION

The results and observations concluded the protective role of methanolic leaf extract of *Carissa spinarum* against alloxan induced oxidative stress thus it can be used as a supplement phytotherapy for prevention and management of diabetes and aggravated oxidative stress. Conclusively this research outcome showed that CS possesses antioxidative and renoprotective potential and is capable of restoring tissue damage to an appreciable extent in alloxan induced diabetic mice.

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Conflict of interest

None of the authors has any conflict of interest.

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