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ATTENUATION OF OXIDATIVE STRESS AND HYPOLIPIDEMIC PROPERTIES OF CARISSA SPINARUM LEAF EXTRACT IN ALLOXAN INDUCED DIABETIC MICE

Biological Science

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ABSTRACT

Carissa spinarum L. is an evergreen, thorny shrub found in the Himalayan areas of Indo-Pakistan subcontinent. Number of ethno-medicinal applications of this plant has been reported. Pharmacologically this plant is used for the treatment of asthma and pulmonary diseases, anticancer, diarrhea, hepatoprotective, cardioprotective and reproductive dysfunction. Present study is aimed to study effects of *Carissa spinarum* methanolic leaf extract in alloxan treated mice liver tissues. Mice were given intraperitoneal injection of alloxan monohydrate to a dose of 150 mg/kg body weight and selected diabetic mice were divided into four groups with three mice in each group. First group served as control and were given distilled water. Second group mice were given *Carissa* leaf extract orally to a dose of 800 mg/kg body weight. 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 liver of diabetic mice revealed degeneration of renal architecture, but repairative changes were observed after treatment with administration of *Carissa spinarum* leaf extract. Significant changes in serum lipid profile and 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, liver, oxidative stress, lipid profile.

1. INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder characterized by the presence of high levels of blood glucose that occurs either due to insulin's deficiency or malfunction [1,2]. Generally, people suffering with diabetes encounter uncountable misery and devastating complications that may lead to morbidity and mortality. According to a survey by International Diabetes Federation, 5 million deaths were recorded due to diabetes [3]. Earlier, it has also been reported that more than 2.5% of the world's total population suffer from DM [4]. With its increased prevalence rate, along with other complications, warrants an urgent need to search for effective treatment strategies. Medicinal plants have been documented as having beneficial properties used for the management of various ailments because of presence of certain bioactive compounds called phytochemicals and secondary metabolites that protect humans against diseases. The availability of some synthetic drugs used in the treatment of a specific disease is common but because of the high cost and side effects associated with their use, attention is currently been focused on the use of medicinal plant products in the management or prevention of various diseases.

Carissa spinarum L. also known as Conkerberry is an evergreen shrub belonging to family Apocyanaceae with spines, bearing white tubular flowers and edible berries [5]. Traditional medicinal uses of this plant in Indian subcontinent are for the treatment of worms in animals and humans, wound healing, malaria, dysentery, sexual weakness and for snake bites [5-8]. Uses of this plant such as anticonvulsant, hepatoprotective, antiarthritic, antibacterial, antioxidant has also been reported [6-9]. Its roots, leaves, stem and fruit all are used for treatment of various ailments. Leaves are used to treat cancer, jaundice, hepatitis, pain inhibitors[5,6].

Oxidative stress may be defined as the imbalance between the over production of reactive oxygen species (ROS) or oxidants and the antioxidant response in cells or tissues [10]. Excessive accumulation of ROS results in oxidative damage to cells, tissues and organs, which alters many biological functions and involve different pathological processes [11-14]. It has been reported by various studies that oxidative stress leads to development of several chronic liver diseases, such as nonalcoholic steatohepatitis (NASH), hepatocellular carcinoma (HCC), liver fibrosis and cirrhosis [15]. Therefore, antioxidative therapy may prove beneficial for the management of these liver diseases. Based on previous literature studies, plant-derived antioxidants were extensively utilized in disease prevention [16,17].

Therefore, considering the traditional use of the *Carissa spinarum* as an alternative medicine and its inherent phytoconstituents, we aim to evaluate the hepatoprotective, antihyperlipidemic and antioxidant effects of leaf extract of *Carrissa spinarum* in alloxan induced diabetes model.

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: The mice of first group designated as normal (control) animals, received only distilled water.
- Group II: The second group of mice that received methanolic leaf extract of *Carissa spinarum* at a dose of 800 mg/kg body weight daily for 28 days.
- Group III: The third group of mice were injected with alloxan intraperitonealy at a dose of 120 mg/kg body weight.
- Group IV: Diabetic mice that received *Carissa spinarum* methanolic leaf extract at a dose of 600 mg/kg body weight daily for 28 days.
- Group V: Diabetic mice that received *Carissa spinarum* methanolic leaf extract at a dose of 800 mg/kg body weight daily for 28 days.
- Group VI: Diabetic mice that received glimepiride at a dose of 2mg/kg body weight 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. Liver was immediately dissected out, cleaned in normal saline quickly blotted and weighed.

3. 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.

4. Biochemical studies

Spectrophotometric assays were performed on Hitachi VSU-double beam spectrophotometer and Bausch and Lomb spectronic-20.

4.1 Determination of serum lipid profile

Estimation of cholesterol was carried out by the method of Zlatkis *et al* [20]. Serum triglycerides were measured by the method of Foster and Dunn [21]. Determination of serum HDL- Cholesterol was carried out by the method of Burstein *et al* [22]. By using Freidelwald formula the concentration of LDL and VLDL cholesterol in serum was calculated [23].

VLDL-Cholesterol = Triglycerides / 5

LDL-Cholesterol = Total-Cholesterol – (HDL + VLDL)

The levels of HDL, LDL and VLDL-cholesterol were expressed as mg/dl.

4.2 Markers of oxidative stress

SOD levels in tissue were estimated by method of Kakkar *et al*. [24]. The specific activity of the enzyme is expressed as unit/min/mg protein for tissues. Estimation of enzyme activity of catalase was done by method of Sinha [25]**.** The specific activity of the enzyme is expressed as unit/min/mg protein for tissues. A portion of tissue is used for the estimation of glutathione (GSH) content by method of Moron *et al*. [26].

GSH is measured by its reaction with Di-thio-bis-nitrobenzoic acid (DTNB) to give a compound that absorbs at 412 nm. The amount of reduced glutathione expressed as nM/mg of protein. Glutathione peroxidase (GPx) is an antioxidant enzyme located in cytoplasm and mitochondria. Glutathione peroxidase was assayed by the procedure of Wendel [27]. The enzyme activity is expressed in terms of µM of glutathione utilized / min /mg /protein. Levels of malondialdehyde, index of lipid peroxidation was estimated according to method of Dhindsa *et al*. [28] using thiobarbituric acid (TBA). The MDA contents were calculated in n moles/mg of fresh tissue weight.

STATISTICALANALYSIS

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 $^{*}p<0.05$ and $^{**}p<0.01$.

5. RESULTS

5.1 Effect of *C. spinarum* on liver histopathology

Light microscopic examination of paraffin embedded and haematoxylin-eosin stained control liver sections of mice showed the normal architecture of hepatic lobules with hepatocytes radiating from the central vein to the periphery of lobules that contain the portal areas (Fig. A).

At 28 days of alloxan administration in mice there was aggregation of kupffer cells across the sinusoidal spaces. Vacuolations appeared in the cytoplasm of some hepatocytes. Hepatocytes close to portal areas in some lobules appeared with empty cytoplasm and darkly stained nuclei and lost most of their cytoplasmic masses and turned into empty spaces with peripheral cytoplasmic layer. An extensive parenchymal necrosis and degeneration was noticed at certain regions. Degenerating nuclei were surrounded by a clump of macrophages. Some of nuclei were enlarged than normal size. Multiple necrotic gaps due to severe necrosis was seen due to extensive fibrosis as a result of degeneration (Fig. B, C). Fewer effects of alloxan administration could be seen after 28 days of extract treatment. However after *Carissa spinarum* extract treatment the hepatocytes showed various regenerative changes from regaining their normal outline to decrease in size of the enlarged nuclei. Few pycnotic nuclei were noticed. Sinusoidal gaps were also reduced along with cytoplasmic vacuolation. Necrotic gaps formed due to extensive fibrosis were also lowered. Mild cellular infiltration with some enucleated hepatocytes were also seen. Mild polymorphonuclear cell infiltrations around integrated blood vessels, sinusoidal epithelium and rounded nuclei with nucleoli were depicted in the section (Fig. D, E). Glimepiride treated diabetic mice liver showed pycnotic nuclei at many places with well developed connective tissue at day 28. Nuclear and cell degeneration was spotted at some areas. Cytoplasmic vacuolization and sinusoidal dilation was also noticed at certain areas. Some enucleated areas were also seen in the section. Nuclear hypertrophy and cell necrosis was also observed at some areas (Fig. F).

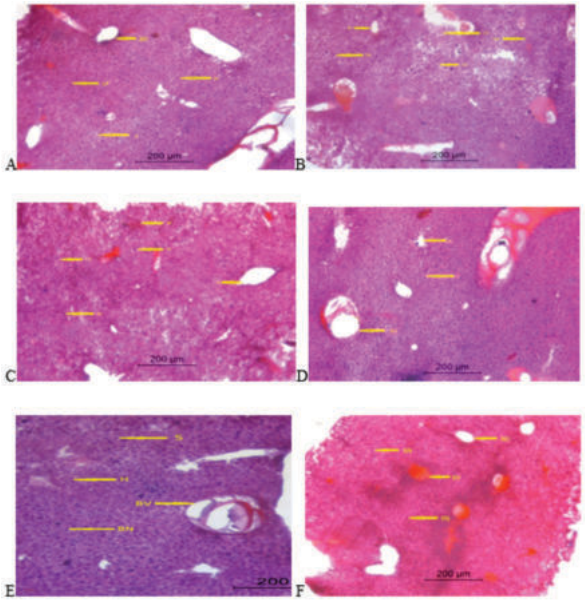

Fig **A:** T.S. of normal liver tissue showing polygonal hepatocytes (H), liver plates (LP), sinusoids (S), bile duct (BD). Figs. **B, C:** T.S. of alloxan induced mice liver tissue depicting degeneration of fibrous connective tissue (CTF), large enucleated area (ENA), nuclear degeration (ND), some binucleate nuclei (BN), degenerating liver connective tissue and infiltration of blood cells (INF). Figs. **D, E, F:** 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.

5.2 Effects of *C. spinarum* on Lipid Profiles

Diabetes is often associated with alterations in lipid metabolism and hence lipid profile was studied in the present study. Serum lipids (TC,

TG, VLDL-c, and LDL-c) were increased significantly ($p < 0.05$, $p < 0.01$) in diabetic animals in comparison to that of control group animals (Figure IV, V, VII, VIII). However, these parameters were decreased significantly ($p < 0.01$) in the group IV, V and VI. HDL-c, a beneficial lipoprotein, was decreased in alloxan induced diabetic mice group as compared to that of control mice, and the result reversed in both extract (600 and 800 mg/kg BW) as well as glimepiride ($p < 0.01$, Table VI) supplemented diabetic mice.

5.3 Effects of *C. spinarum* leaf extract on Endogenous Antioxidants and Oxidative Stress Markers

The SOD activity was found to be reduced in the liver 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 (Figure IX). 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 (Figure X). 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 (Figure XI, XII). 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 XIII).

Table I: The effect of *Carissa spinarum* on lipid profile of alloxan induced mice serum after 28 days of extract treatment

Groups	TG	TC	HDL	VLDL	LDL
Control	90.90±0.86	86.97±0.53	32.63±0.52	17.93±0.86	35.95±0.63
CSE	83.84±1.12	81.57±0.53	38.21±0.86**	15.45±0.50**	27.73±0.73**
ALX	140.26±2.63*	186.64±0.62**	24.68±0.46*	28.14±0.13*	99.47±1.91
ALX+E1	99.65±1.10	136.78±0.76	31.25±0.37	19.03±0.54*	50.93±0.84
ALX+E2	95.94±1.88**	119.29±0.69	33.09±0.42	17.75±0.04**	40.96±0.98
ALX+G	94.26±2.18	112.63±1.25**	33.44±0.77*	18.03±0.04	39.64±0.88

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 statically significant at $p^{*}<0.05$, $p^{**}<0.01$. TG: Triglycerides, TC: Total cholesterol, HDL: High density lipoprotein, VLDL: Very low density lipoprotein, LDL: Low density lipoprotein. CSE: Carissa spinarum extract, ALX: Alloxan, E1: CSE (600 mg/kg BW), E2: CSE (800 mg/kg BW), G: Glimepiride (2mg/kg BW).

Table II: The effect of Carissa spinarum on antioxidative markers of alloxan induced mice liver after 28 days of extract treatment

Groups	SOD	CAT	GSH	Gpx	LPO
Control	8.29±0.29	12.58±0.21	7.07±0.12	10.56±0.13	7.72±0.35
CSE	9.06±0.23	12.93±0.15*	8.08±0.16	11.53±0.27*	6.91±0.32*
ALX	3.53±0.26	9.66±0.18*	2.40±0.15**	5.24±0.12**	13.20±0.23*
ALX+E1	7.01±0.13**	11.79±0.16	4.78±0.14	8.65±0.10**	9.62±0.40
ALX+E2	7.13±0.25**	12.05±0.19**	5.40±0.30**	9.12±0.37*	8.97±0.26
ALX+G	6.95±0.07	10.55±0.30	4.72±0.10**	8.54±0.15*	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 statically 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).

6. DISCUSSION

Diabetes mellitus is among the most common disorder in developed and developing countries, and the disease is increasing rapidly in most parts of the world. It is a debilitating and often life-threatening disease with a group of etiologically and clinically heterogeneous disorders with common set of symptoms such as excessive thirst and hunger, muscular weakness and weight loss, excessive urination. Diabetes mellitus is also characterized by hyperglycemia resulting from insulin secretion, insulin action or both, which on exceeding the renal threshold levels, results in the excretion of glucose in the urine [29].

Carissa spinarum has been extensively used since ancient times for its various medicinal properties, which is used for the treatment of diabetes, cardiovascular disease, pain reliever, etc., in different parts of the world. For this reason, we have screened out the methanolic extract of *Carissa spinarum* to study its antioxidant and antilipidemic property.

Alloxan leads to hyperglycemia resulting in increase of blood glucose levels. Similar observations were observed in the present study. Alloxan induced diabetic animals showed increased levels of blood glucose as compared to control mice. The observation of elevated blood glucose levels in the diabetic mice might be due to the metabolism of stored glycogen and fats [33]. After supplementation of extract to diabetic mice led to decrease in blood glucose levels. Decreased blood glucose level was due to the inhibition of glucose uptake in the intestine and insulinotropic activity of the extract [34]. The possible mechanisms to explain the hypoglycemic activity exhibited by *C. spinarum* may include inhibition of intestinal absorption of glucose, facilitation of glucose-induced insulin release, enhancement of peripheral uptake of glucose, promotion of the regeneration of b-cell of islets of Langerhans and amelioration of oxidative stress attributed to the presence of a variety of phytoconstituents present in this plant [35].

Mahmoud and Al-Salahy [36] demonstrated that hyperglycemia plays an important role resulting in the liver injury by inducing an enlargement of sinusoids, dilation of cisterns and a loss of RER ribosomes in the hepatocytes of fish treated with repeated doses of glucose and fish that received alloxan only. Histopathological studies of diabetic liver section showed a degenerative structure with central vein congestion and cellular swelling. ROS, which was generated from STZ, may be responsible for all these hepatic damages [37]. After CS leaf extract administration to diabetic mice at 2 doses (600, 800 mg/kg BW) led to restoration of liver histoarchitecture to an extent. Similar results were reported in the study in which it was observed that administration of *Allium sativum* (garlic) extract to alloxan induced diabetic rats revealed preserving cellular architecture, reappearance and cellular restoration, vascular congestion, reappearance of hepatocytes with pyknotic nuclei migrating from the sinusoidal lining layer as moderate restoration and complete regeneration in the liver tissues when compared to non-diabetic and diabetic control groups [38].

The unavailability of insulin and elevation of the counter-regulatory hormone glucagon led to activation of hormone sensitive lipase which stimulate lipolysis and enhanced release of free fatty acids from adipose tissue [39]. Hypertriglyceridemia and reduced plasma HDL-C is commonly present in T2DM and has been reported by Kharroubi *et al*. [40]. Similar observations were found in our study where cholesterol, total glycerides, LDL and VLDL cholesterol levels increased but HDL levels were decreased in comparison to control mice in alloxan administered mice. There was a significant decrease (**$p<0.01$) in the total triglyceride, cholesterol, LDL and VLDL level of diabetic mice treated with methanolic leaf extract of *C. spinarum*. Whereas HDL levels of extract treated diabetic group mice showed an increase. The results of present study are in concordance with another research report where similar types of changes in lipid profile were observed in alloxan-induced diabetic rats after administration of plant extract [41,42].

Diabetic mice showed decreased activities of SOD, CAT, GSH and GPx. Similar results were reported by Alezandro *et al.* [43] which stated that the levels of SOD, CAT, GSH, GSH-Px were diminished in all the tissue of diabetic individuals. Supplementation of CS leaf extract in alloxan induced diabetic mice restored the activities of GSH, GSH-Px, CAT and SOD in liver of the diabetic mice to an extent.

Significant increase in lipid peroxidation products and decrease of some antioxidants were observed in diabetic animals. Al-Attar and Alsalmi [44], showed that the values of GSH, SOD, and CAT were declined, and the value of MDA was significantly enhanced in diabetic animals. 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.

7. CONCLUSION

Thus from above results it can be concluded that *Carissa spinarum* leaf extract has a potential to attenuate the diabetic and oxidative stress related pathological alterations developed in the liver tissue. The *Carissa spinarum* leaf extract demonstrated the protective effect by restoring the antioxidant biomarkers, serum lipid profile and improving the histoarchitecture of the tissue.

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Conflict Of Interest

None of the authors show any conflict of interest.

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