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EVALUATION OF ANTIDIABETIC EFFECTS OF CARISSA OPACA LEAVES EXTRACTS AGAINST ALLOXAN INDUCED DIABETES IN RATS

Jainedra Kumar*, Shamim Ahmad, Sudha, Shaily Mishra and Huma Khan

Assistant Professor Department of Pharmaceutical Technology; Translam Institute of Pharmaceutical Education and Research Meerut.

***Corresponding Author: Jainedra Kumar**

Assistant Professor Department of Pharmaceutical Technology; Translam Institute of Pharmaceutical Education and Research Meerut

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ABSTRACT

Diabetes mellitus is heterogeneous metabolic disorder characterized by disturbances of carbohydrate, fat, protein metabolism. It is often connected with the development of micro and macro vascular diseases which include a long-term damage dysfunction and failure of various organs especially the eye, nerves, heart, kidney, and blood vessels. There are approximately 20.8 million people in the United States or 7% of the population, who have diabetes. Of those, 14.6 million people have been diagnosed but an estimated 6.2 million people are unaware that they have the disease. In addition to those people with diabetes, there are an additional 41 million Americans who are pre-diabetic. Data from death certificates indicate that diabetes contributed to 224,092 deaths in the United States in 2002, making it the sixth leading cause of death. However, it is believed that diabetes is generally under-reported on death certificates and as such the toll of diabetes may be much higher than officially reported. Beyond the toll on human life, the total annual economic cost of diabetes in 2002 was estimated to be $132 billion or one out of every 10 health care dollars spent in the United States.

KEYWORDS: Diabetes mellitus, Hyperglycemia, Insulin.

INTRODUCTION

Diabetes Mellitus is a syndrome of glucose when cannot be metabolized by the cells then it remains in the bloodstream and disturbances of carbohydrate, fat and protein metabolism and disturbed in the insulin secretion or insulin action. Diabetes Mellitus may be suspected or recognized clinically by the presence of characteristic symptoms such as excessive thirst, fatigue, loss of energy, rubbing the eyes unexplained weight loss and difficulty seeing the board in school or one or more of the many complications associated with or attributable to the disease.[1]

The most characteristic feature in DM is a persistently high level of glucose in the blood. When glucose cannot be metabolized by the cells then it remains in the bloodstream. A person with diabetes, therefore, has constantly high blood glucose levels. As the glucose level becomes sufficiently high, some of the glucose is excreted in the urine. There are two types of diabetes: Type 1 DM and Type 2 DM. Type 1DM is thought to be caused by a combination of genetic and environmental factors that result in a lack or complete absence of insulin. However, the reasons are largely unknown as to why the body's immune system attacks itself, destroying over 90 percent of its own insulin-producing betacells in the pancreas. Much more common type 2 DM diabetes has been linked to obesity. Treatment for type 1DM continues to consist with insulin injections, although other strategies for taking insulin are currently being researched whereas type 2 DM requires medication and sometimes insulin injections. Both types require lifestyle changes like diet and exercise.[2] Untreated hyperglycemia can lead to a reduced number of glucose transporters, down/regulation in the number of insulin receptors as well as defects of tissue insulin signal transduction. Subsequent to these deteriorations there is anabsolute increase in the hepatic glucose output which excessincreases glucose utilization and fasting hyperglycemia occurs.[3] Finally, hyperglycemia itself manifests adverse effects on β-cell insulin secretion and insulin resistance.[4] The Process which leads to damage, of the function and failure of various organs suchas nerve, heart, blood vessels, kidney, eye etc. and creates a huge economic burden related to the management of diabetic complications.[5] The World Health Organization (WHO) have estimated that approximately 171 million people worldwide suffered from diabetes in 2000 and that it expects that number to more than double to 366 million people by 2030. It has been estimated that approximately 3.2 million deaths worldwide were attributable to diabetes in 2000, with that number expected to increase significantly with the increasing prevalence of diabetes over the next several decades.

MATERIALS AND METHODS

Identification, Collection and Authentication of plant and its leaves

Leaves of a Carissa opaca were collected from U.P (Utter Pradesh) Nagina, Bijnor and authentication of the plant were done by Dr. Amita Arjaria, Professor, Department of Botany, and Govt. Maharaja Mahavidyalaya Chhatarpur M.P India. The collected material was washed with running water. The leaves were dried under the sunshade. Dried leaves were coarsely powdered and used for extraction.[37]

Pharmacognostic studies

Morphology – Carissa opaca leaves were subjected to morphological examination based on colors, odors, smell, taste, and size of drugs leaves.

Macroscopic and Microscopic studies

Determination of physicochemical parameter

Moisture content-the percentage of active constituents in crude drugs is given in term of air-dried drugs. Hence the moisture content of the drug should be determined.

Total ash value

2 gm of drug powder was weighted accurately into silica Crucible and incinerated at 450^{0C} in muffle furnace until free from carbon. The Crucible was cooled to room temperature and weighted. Percentage of ash value was calculated with reference to air-dried substance.

Acid soluble ash

Total ash obtained from powder drug was boiled with 25 ml of (70 gm/ml) HCl for few minutes and filtered through an ashless filter paper. The filter paper was transferred into a tarred silica crucible and incinerated at 650^{0}C in muffle furnace until free from carbon. The crucible was cooled to room temperature and weighted. Percentage of ash value was calculated with reference to air-dried substance.

Preparation of extracts

*Carissa opaca*leaves at maturity were collected from NaginaBijnor, and Leaves were shades dried at room temperature for two weeks, chopped and ground mechanically. 1.5 kg of dried sample was extracted twice with 5 l of 70% methanol at 25 °C for 48 h. The extracts were filtrated through Whatman No. 1 filter paper and combined followed by Concentration using a rotary evaporator (Panchun Scientific Co., Kaohsiung, Taiwan) under reduced pressure at 40°C. After crude extraction, it was further fractionated with solvent extraction to various fractions. Each of the fractions obtained was dried using a rotary evaporator. The dry extract in powder form obtained with each solvent was weighed, (SumairaSahreen et al, chemistry central journal (2017).[38]

Method of extraction: Hotpercolation process.

Requirements: Shade dried coarse powder flower of *A. Carissa opaca.*

Solvent: Methanol, Ethanol, water chloroform-ethyl acetate.

Experimental animals

Wister rats weighing (150-170 g) of procured from Translam. They were kept in the departmental animal house in well cross ventilated room at 22 ± 2 °C, and relative humidity 44–56 %, light and dark cycles of 12 h, for 1 week before and during the experiments. The standard rodent pellet diet provided for animal and the food was withdrawn 19-25h before the experiment through the water was given ad libitum. All studies were performed in accordance with the guide for the care and use of laboratory animals, as adopted and promulgated by the Institutional Animal Care Committee, CPCSEA, India.[40]

Acute toxicity studies (Oecd 423)

An acute toxicity study was performed according to Organization for Economic Co-operation and Development guidelines. Acute toxicity category method is a method for assessing acute and oral toxicity that involves the identification of a dose level that causes mortality. The s bolus dose of the test substance feeds to young adult rodent by the oral route, and the observed for up to 15 days and record the body weight after dosing and the necropsy of all the animals. In this method prespecified fixed doses of the substance were used i.e., 5 mg/kg, 50mg/kg, 300mg/kg, 600mg/kg, 1000mg/kg, 2000mg/kg and the mortality due to these doses were observed. Generally, male animals were used for this study and each dose group should consist of 6 animals. The simple bolus dose.

Pharmacological studies

Experimental Set up for the animals (Wister rats weighing 150-170 g) were divided into six groups of six animals each. The overnight fasted mice were weighted and selected.

Ethanol extract was dosed in a stepwise procedure with the initial dose beingselected as the dose expected to procedure some signs of toxicity and wereobserved for a period Of two weeks. The toxic doses were selected based on Guideline 423. The result of the LD50 study was done by mice using guideline 423 methodswere shown in a table.

Evaluation of antidiabetic activity of *carissa opaca leaves extract alloxan induced* diabetes, model

Alloxan induced diabetes

Alloxan is an oxygenated pyrimidine derivative. Alloxan is isolated in 1818 by Brugmatelli and the name gives in 1838 by Wohler and Liebig. In 1838 the compound was discovered by von Liebig and Whole and the oldest named organic compounds that exist. Alloxan derived from the merging of two words, i.e., Allantoin and Oxaluric acid. The alloxan model of diabetes induction was first described in rabbits by Dunn, Sheehan, and McLetchie in 1943. Oxidation of uric acid and nitric acid

formed the Alloxan. The monohydrate is simultaneously prepared by oxidation of barbituric acid by chromium trioxide. 15 Moreover, alloxan has been regarded as a strong oxidizing agent that forms a hemiacetal with its reduced reaction product; dialuric acid, in which a carbonyl group is reduced to a hydroxyl necrotic cell death of pancreatic islets. The last and the 4th phase of the blood glucose response is the final permanent diabetic hyperglycemic phase during which complete degranulation and loss of the integrity of the beta cells within 24-48 h after administration of the alloxan takes place the non-beta cells and other endocrine and non-endocrine islet cell types along with extrapancreatic parenchyma remain intact, providing the evidence of selective toxic action of alloxan.

Alloxan injection has been noted to induce an insulin-dependent type I like diabetes syndrome and all the morphological features of beta cell destruction are characteristic for a necrotic cell death.[40-41]

Mechanism of action

Alloxan-induced diabetes model is used for insulin dependent diabetes mellitus. The mechanism of action of the alloxan studied which currently can be characterized quite well. Several experimental studies have demonstrated that alloxan evokes a sudden rise in insulin secretion in the presence or absence of glucose which appeared just after alloxan treatment. 34-33 alloxan-induced releases the insulin for short duration The alloxan action in the pancreas is preceded by its rapid uptake by pancreatic beta cells that have been proposed to be one of the important features determining alloxandiabetogenicity. 34-35 Alloxan reacts with two sulfide groups in the sugar binding and formation of the disulfide bond and inactivation of the enzyme. Dialuric acid is formed which and re-oxidized back to alloxan and establishing the redox cycle for the generation of reactive oxygen species (ROS) and superoxide radicals. 36-37 the superoxide radicals liberate ferric ions from ferritin and reduce them to ferrous and ferric ions. 38 In addition, superoxide radicals undergo dismutation to yield hydrogen peroxide (H2O2) in the presence of superoxide dismutase. As a result, highly reactive hydroxyl radicals are formed according to the Fenton reaction in the presence of ferrous and H2O2. Alloxan causes DNA damage, fragmentation of DNA takes place in the beta cells exposed which stimulates poly ADP-ribosylation process of participating in DNA repair. Antioxidants like superoxide dismutase, catalase and non-of diabetes in the laboratory animals by alloxan injection are the result of selective uptake of alloxan via GLUT2 into a pancreatic beta cell. 42-48 the effective prevention of redox cycling and generation of ROS can prevent pancreatic beta cell death and counteract the development of alloxan diabetes in vivo.12, 46, 49, and 50.[42]

Chemicals

Alloxan monohydrate is purchased from Merck, was purchased, preserved at 25^0C and used for the study. (daonil tab) Glibenclamide manufactured by Sanofi Aventis Pharma Pvt Ltd. India was purchased from the market.

Induction of experimental diabetes

Adult Wistar EPM (*Rattusnorvegicus Albinus*, Rodentia, Mammalia) weighing 200g to250 g. were made diabetic by a single intraperitoneal injection of alloxan monohydrate (50 mg/kg). Alloxan was first weighed individually for each animal according to the body weight and then solubilized with 0.2 ml saline (154 mNaCl) just prior to injection. Two days after alloxan injection, rats with plasma glucose level of <140 mg/dl were included in the study. Treatment with plant extracts was started 48 h after alloxan injection.[43]

Experimental design

Wister rats are divided into five groups and in each received the following treatment schedule.

- Group I: Normal control (saline).
- Group II: Alloxan treated control (140 mg/kg.ip).
- Group III: Alloxan (140 mg/kg.ip) + Carissa Opaca. Leaves extract (400 mg/kg p.o).
- Group IV: Alloxan (140 mg/kg.ip) + Carissa Opaca. Leaves extract (500mg/kg, p.o).
- GroupV: Alloxan (140 mg/kg.ip) + Standard drug, Glibenclamide (5 mg/kg, p.o).

Methanolicleaves extractsand with a standard drug, Glyburide (OS: USAN) glibenclamide (5 mg/kg) and saline solution were administered with the help of feeding cannula. Group, I served as a normal control, which received saline for 14 days.

From group II and Group V are diabetic control rats. Group III and Group V which are already received the alloxan now give the Methanolic extract (300 mg/kg, p.o), (500 mg/kg, p.o) and standard drug glibenclamide (5 mg/kg) for 14 consecutive days.

Evaluation of parameters

Change in body weight

Body weight of all rats was measured on starting the day (0 days) of theexperiment and 2nd, 5th, the 10th and 15th day of the experiment. All the body weights werenoted and reported in the table.

Sample collection

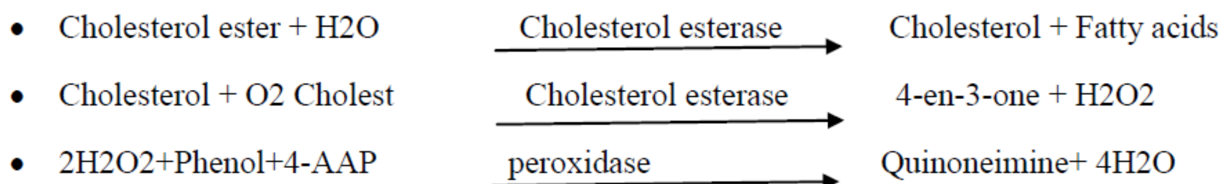
Fasting blood glucose (FBG) of all rats was determined before the start ofthe experiment. A blood sample was collected at weekly intervals from tail vein puncturetill the end of the study. In the continuous 21 days of drug treatment, a blood glucose level ofall animals was determined on 0, 7, 14 & 21 days. On day 21, blood was collected by retro-orbital under mild ether anesthesia from overnight fasted rats. Blood was collectedin EDTA tubes plasma and serums were separated by centrifugation. After centrifugationat 2000 rpm for 10 minutes, the clear supernatant was used for the analysis

of various Biochemical parameters (Cholesterol, Triglyceride, HDL, and LDL). The pancreas wasexcised and rinsed in ice-cold saline and kept in formalin solution for futurehistopathologicalexamination.

Estimation of blood glucose levels
Blood glucose level was measured using Glucometer (Acucheck-sensor).

Estimation of cholesterol
Method
Enzymatic – Colorimetric
Trinder– Endpoint

Principle
Enzymatic Colorimetric of total cholesterol according to the following
Reactions



Procedure
The reagent can be used on most analyzers, semi-automated Analyzers and Manualmethods.

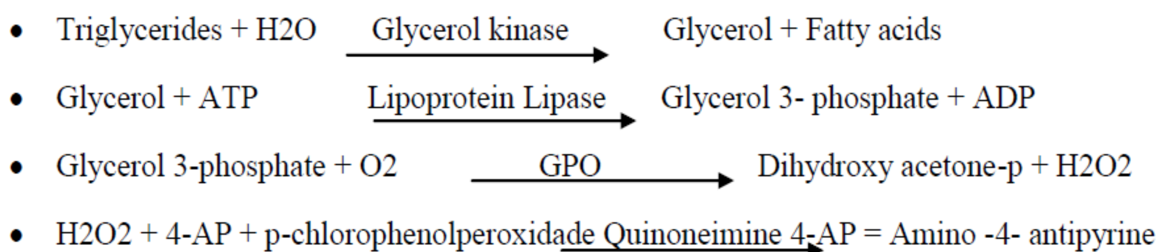
Wavelength: 505nm
Temperature: 370C

Table no. 2.8.2.1: Reading against a reagent blank.

	Blank	Standard	Sample
Reagents R	500 µL	500 µL	500 µL
Distilled water	5 µL	-	-
Standard	-	5 µL	-
Sample	-	-	5 µL

Calculation
Estimation of triglycerides
Method
Enzymatic- Colorimetric
Trinder- Endpoint

Principle
Enzymatic determination of triglycerides according to following reactions-



Procedure
The reagent can be used on most analyzers, semi-automated analyzers, and Manual methods.

Table no. Reading against reagent blank.

	Blank	Standard	Sample
Reagent r	500 µl	500 µl	500 µl
Distilled water	5 µl	-	-
Standard	-	5 µl	-
Sample	-	-	5 µl

Mix and read the absorbance (A) after 425-second incubation.

Estimation of High-Density Lipoprotein
Method
Enzymatic – colorimetric
Trinder - Endpoint

Principle

This method depends on the activity and properties of the detergent which soluble only with the HDL, HDL is released to react with enzyme cholesterol esterase. Cholesterol oxidase andchromophore or dye which provides the color. The intensity of the color formed is proportional to the HDL-c concentration in the sample.

Procedure

- Assay Conditions
- Wavelength 600 nm (600-700)
- Cuvette 1 cm light path
- Temperature 37^{0}C
- Adjust the instrument to zero with distilled water.
- Pipette into Cuvette

Table no. Reading against reagent R1.

	Blank	Standard	Sample
R 1 µl	300	300	300
Calibrator µl	-	3	-
Sample µl	-	-	3

- Mix and incubate for the 10 min at 37^{0}C
- Read the absorbance (A1) of the samples calibrated.
- Add:

Table no. Reading against reagent R2.

	Blank	Calibrator	Sample
R 2 µl	100	100	100

- Mix and incubate for 5 min at 370C.
- Read the absorbance (A2) of the samples and calibrator, against the blank.
- Calculate the increase of absorbance ΔA=A2 –A1.

Endpoint.

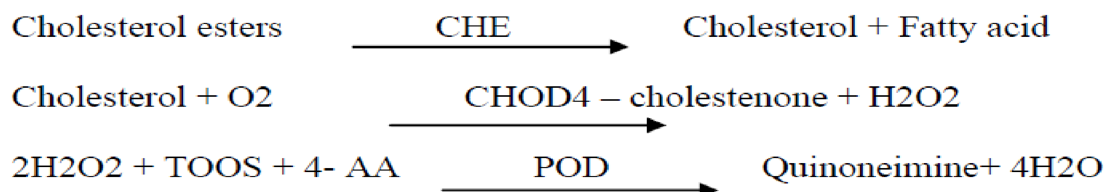
Principle:

- Elimination of lipoprotein No-LDL

Estimation of Low-Density Lipoprotein

Method

Enzymatic – colorimetric



The intensity of the color which formed is proportional to the LDL-c concentration in the sample

Procedure

- Assay Conditions.
- Wavelength 600 nm (600-700).
- Cuvette 1 cm light path.
- Temperature 370 C.
- Adjust the instrument to zero with distilled water.
- Papette in to Cuvette.

Table no. Reading against reagent R1.

	Blank	Standard	Sample
R 1 Ml	300	300	300
Calibrator µL	-	3	-
Sample µL	-	-	3

- Mix and incubate for 5 min at 370C.
- Read the absorbance (A1) of the samples calibrated.
- Add

Table no. Reading against reagent R.

	Blank	Calibrator	Sample
R 2 µl r 2 µl r 2 µl	100	100	100

- Mix and incubate for 5 min at 370C.
- Read the absorbance (A2) of the samples and calibrator, against the blank.

$$\text{LDL Cholesterol (mg/dl) in sample} = \frac{\text{A sample}}{\text{A standard}} \times \text{Standard conc.}$$

Histopathological studies
Section of the pancreas was made, stained with Haematoxylin and Eosin reagent and observed under low and high power objective for Histopathologicalchanges. The alteration and changes in the histology of pancreas and were shown in vide plate in figures.

Statistical analysis
All the values were expressed as mean ± SEM (standard error mean) for sixrats. Statistical analysis was carried out by using PRISM software package (version 5.0). Statistical significance of differences between the control and experimental groups was assessed by One way ANOVA followed by Dunnet's Multiple Comparison tests. Thevalue of probability less than 55 (P > 0.05) was considered statistically significant in tables.

Effect of *Carissa opaca*leaves extract on Oxidative Stress in Alloxan-induced diabetic rat Effect of *Carissa opaca*to extract on oxidative stress was observed by using LPO, GSH, Catalase and SOD levels and the result shown in table

Effect of *Carissa opaca leaves* extract (methanolic) on LPO, GSH, Catalase and Sod in Alloxan diabetic rat

Group	LPO (nM/mg protein)	GSH (mM/ g wet tissue)	Catalase (U/mg protein)	SOD (% inhibition of NBT
Normal Control	0.73±0.102	24.978±1.442	34.492±1.245	44.477±2.355
Diabetic Control	3.562±0.044	10.515±0.994	14.163±1.771	24.033±2.055
Standard	0.992±0.271*	21.247±1.223*	31.018±1.211*	39.002±2.854*
Extract (200 mg/kg)	2.162±0.035*	12.733±1.042*	18.128±1.09*	27.832±2.71**
Extract 400	1.866±0.14*	16.812±1.321*	26.825±1.802*	33.532±1.781*

Values are expressed as Mean±S.D. of n=5/group. *p<0.05 significant *vs*diabetic control group using one Way ANOVA (Analysis of Variance), followed by Bonferroni test.

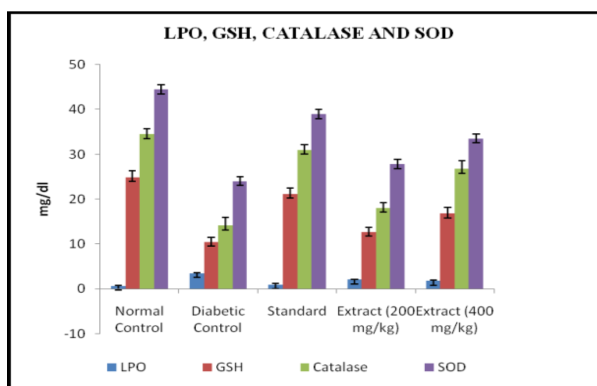


Figure no. 8: *Carissa opaca leaves* extract on LPO, GSH, Catalase and SOD.

Biochemical estimation of serum parameters
Table no. Biochemical estimation of serum cholesterol.

Group	Treatment	Dose (mg/kg)	Total Cholesterol (mg/dl)	Serum Triglyceride	Serum Protein (g/dl)	Albumin (g/dl)	Creatinine (mg/dl)
Group I	Normal Control	**-**	84.9±2.61	46.317±1.8	7.325±0.342	3.727±0.30	0.835±0.07
Group II	Diabetic Control	**150**	129.75±3.84	89.033±4.61	3.017±0.084	1.813±0.124	2.927±0.16

Values are expressed as Mean±S.D. of n=5/group. *p<0.05 significant *vs*diabetic control group using one way ANOVA (Analysis of Variance), followed by Bonferroni test.

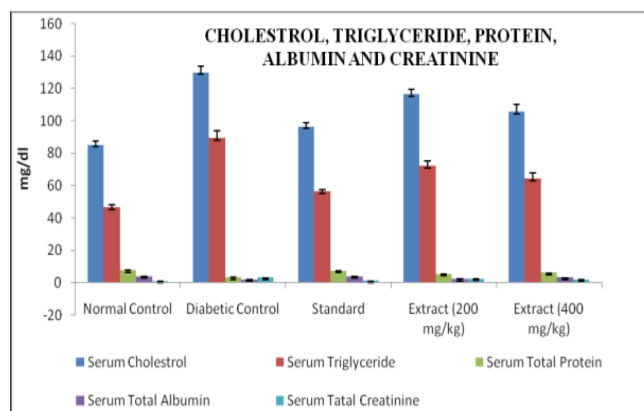


Figure no. 9: Alloxan-induced diabetes effect on Cholesterol, Triglyceride, Serum Protein, SerumAlbumin and Serum Creatinine.

The results of the cholesterol, triglyceride, serum protein, serum albumin, serum creatinine profile) showed total serum creatinine increased significantly (P < 0.05) decreased in the Group IV and Group V when compared to diabetic control group II.

DISCUSSION

Diabetes is a major problem today. Therefore in the present study, Alloxan was employed to induce diabetes. The antidiabetic activity of a Methanolic extract of leaves of *Carissa opaca*against the diabetes was studied. The extent of induction of diabetes was estimated by increasing blood glucose level, Cholesterol, triglycerides, HDL, LDL and Histopathological studies. Generally, loss of body weight is observed in diabetes. The ability extract to prevent loss of weight is due to its ability to reduce hyperglycemia. The other lipid parameters like Cholesterol, triglycerides, HDL, LDL also estimated. The level of serum lipids are usually elevated in diabetes mellitus and such as elevation represents a risk factor for coronary heart disease. This abnormally high level of lipid is mainly due to the uninhibited action of lipolytic hormones in fat depot mainly due to an action of insulin. Under normal circumstances, insulin activates lipoprotein lipase, which hydrolyzes TG. However, in diabetic state lipoprotein lipase is not activated due to insulin deficiency resulting in hypertriglyceridemia.

Acute toxicity studies were done with 200 & 400 mg/kg of methanolic extract for the experimental study. Alloxan is a broad spectrum antibiotic, which causes beta cell damage by free radical generation. Moreover, the induction of these lesions by Alloxan is followed by a cascade of intracellular events like stimulation of DNA repair (via poly-ADP-ribose synthetase), reduction of islet NAD content and subsequent inhibition of the islet function.

Treatment with Methanolic extract of *Carissa opaca* leaves brought down. Methanolic extract of leaves of *Carissa opaca* various phytochemical investigation studies showed the presence of Triterpenes, Steroids, Tannins, Flavanoids, alkaloids, and Saponin.

The recovery towards normalization of serum enzymes and histological architecture caused by a Methanolic extract of leaves of *Carissa opaca* almost similar to standard Glibenclamide.

CONCLUSION

The present study was designed with the objective to evaluate the antidiabetic effect of methanolic extract of Carissa opacaagainst Alloxan-induced diabetes. Methanolic extract (400 mg/kg) showed significant antidiabetic activity whencompared to that of extract (200 mg/kg). Standard (glibenclamide) almost reduced thediabetic condition to normal. These findings were supported by a reduction in thebiochemical parameters i.e. elevated cholesterol, triglycerides, LDL and elevated thedecrease HDL levels.

This was further supported by the histopathological finding of pancreas tissue that showed the altered architecture as well as maintenance of normal architecture. The antidiabetic activity was observed in a dose-dependent manner and the maximum antidiabetic activity was evident at 400 mg/kg dose level of *Carissa opaca*leaves, against Alloxan-induced diabetes. Methanolic extract (400 mg/kg) showed significant antidiabetic activity when compared to that of extract (200 mg/kg). Standard (glibenclamide) almost reduced the diabetic condition to normal. These findings were supported by a significant reduction in the biochemical parameters i.e. elevated.

Cholesterol, triglyceride, LDL and elevated the decreased HDL level. Thus the antidiabetic activity of methanolic extract (400 mg/kg) of *Carissa opaca*leaves and the standard almost normalized the biochemical parameters (cholesterol, triglycerides, HDL, LDL). Plant-derived natural product such as flavonoids, a Phenoliccompound has received considerable attention in

recent year due to their diversepharmacological properties including antidiabetic and antioxidant activity. There hasbeen growing interest in the analysis of certain flavonoids etc stimulated by intense researchinto their potential benefit to human health.

Antidiabetic plants an important role ininhibiting the glucose level and scavenging radical, thus providing protection to human against hyperglycemia. Realizing the fact, this research was carried out to evaluate theantidiabetic activity of leaves extract of *Carissa opaca*in Alloxan-induced diabetic rats.

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