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Assessment of Antidiabetic and Antioxidant Potential of Lyophilized Neem Leaf Powder (LNLP) Supplementation in Alloxan-Induced Diabetic Male Albino Rats

MukhtarAhmed G. Ghodesawar, *[1] Umadevi C. Jadaramkunti [2] and Ravindranath H. Aladakatti[3]

[1] *Department of Zoology, Anjuman Arts, Sciences and Commerce College, Vijayapura-586101, Karnataka,*
[2] *Department of Zoology, Government First Grade College, Hubballi-580032, Karnataka,*
[3] *Central Animal Facility, Indian Institute of Science, Bengaluru-560012, Karnataka.*

*drmukhtarahmedgg@gmail.com

Abstract

This study aimed to compare the antidiabetic and antioxidant effects of the standard drug glibenclamide and lyophilized neem leaf powder (LNLP) in alloxan monohydrate-induced diabetic male albino rats. To induce diabetes, male Wistar rats were injected with alloxane monohydrate at a dose of 100 mg/kg body weight. If the fasting blood glucose of the rats were 200 mg/dL or more after 48 hours, they were used in the study. Wistar albino rats were divided into 5 groups of 10 animals each, with Group I rats being non-diabetic (normal controls); Group II rats being diabetic control rats who did not receive any treatment; Group III rats being conventionally treated for diabetes (glibenclamide); and Groups IV and V rats being supplemented with LNLP at doses of 50 and 100 mg/kg, BW/day, respectively. Blood samples were taken to measure serum insulin and hematological parameters. Liver and skeletal muscle tissues were collected to evaluate the activity of carbohydrate metabolism enzymes, glycogen content and histopathological observation. Intestinal homogenates were prepared to study intestinal enzymatic and non-enzymatic antioxidant activity. LNLP supplementation improved haematological and serum chemistry profiles, glycogen content, and carbohydrate metabolic enzyme activities in tissues with improved microscopic architecture. A recovery in the enzymic and non-enzymic antioxidant markers levels of intestinal homogenates compared with diabetic controls and glibenclamide. Results indicate that LNLP supplementation can improve glycemic control as well as lipid profiles in diabetic rats, with improved antioxidant enzymes that have a beneficial effect on preventing diabetic complications by scavenging free radicals in diabetic rats.

Keywords: Antidiabetic, Antioxidant, LNLP, Alloxan, Glibenclamide, Biochemical indices

INTRODUCTION

Diabetes mellitus (DM), a metabolic disorder that causes hyperglycemia and insufficient production, action, or both of insulin (Zimmet *et al.,* 2016), is characterized by alterations in protein, carbohydrate, fat, and glucose metabolism. Effective treatment of DM is a major challenge for the medical community as many drugs currently on the market have a wide range of side effects. World Health Organization (WHO) on the need to develop and evaluate improved pharmacological agents to increase insulin sensitivity, prevent beta (β) cell degeneration, stimulate β-cell regeneration, and obstructing pathways leading to the various complications of diabetes is being implemented (WHO, 1994). Because of their potent phytochemical composition, medicinal plants, and many treatments using their products are highly regarded as alternative medicine sources for diabetes. Among the compounds used in these treatments are flavonoids, carotenoids, terpenoids, glycosides and alkaloids (Verma *et al.,* 2018).

One of the most adaptable medicinal plants, *Azadirachta indica* A Juss (Neem, a member of the Meliaceae family) has a wide range of biological activities. Several reviews have summarized the various therapeutic roles of neem and the significant progress made in its pharmacological potential and medical applications over the past 50 years (Islas *et al.,* 2020). Scientific studies have shown that neem leaves have a positive effect on blood glucose levels by regulating key biochemical parameters, significantly reducing hypoglycemic effect (Dholi *et al.,* 2011), hemoglobin A1c (Shailey and Basir, 2012), an increase in body weight gain and antidiabetic activity because of improved glucose metabolism (Fattah *et al.,* 2020). In rats with experimentally induced diabetes, neem leaf extract normalized altered blood glucose and serum insulin levels (Satyanarayana *et al.,* 2015), suggesting that this is important for the treatment of type 2 DM. In addition, a proprietary standardized aqueous extract of neem leaves and twigs has been shown to help control blood sugar levels, improve vascular health, and reduce systemic inflammation in people with type 2 diabetes (Pingali *et al.,* 2020).

Previous experimental studies have shown that LNLP is a good natural source of antioxidants to prevent CCl_4-induced liver damage (Aladakatti and Ghodesawar, 2018). It also appears to exert its wound-healing effects in rats through a variety of mechanisms, including free radical scavenging and immune modulation. Considering that phytochemical analysis of aqueous extracts of neem leaves revealed high levels of saponins, flavonoids, phenols, tannins, alkaloids, glycosides, proteins, triterpenoids, carbohydrates and alkaloids, these responses could be attributed to presence of various phytoconstituents (Dash *et al.,* 2017). Therefore, the current experimental study was conducted to determine the antidiabetic and antioxidant activities of LNLP supplementation in rats with alloxan-induced diabetes based on presently available information on alternative herbal treatment of diabetes.

MATERIALS AND METHODS

Preparation of lyophilized neem leaf powder (LNLP)

Neem leaves were collected locally and verified by the Department of Botany, Anjuman College of Arts, Sciences and Commerce, Vijayapura. The sample voucher number has been delivered to the botanical department's herbarium. (AASCC/2009/27) for deposit. Neem leaves have been used to prepare aqueous extracts. Neem leaves were collected and then freeze-dried using a previously published extraction method (Khillare and Shrivastv, 2003). Neem leaves were briefly ground in distilled water before being filtered. The filtrate was centrifuged at 5000 rpm for 10 minutes at 4° C, and the supernatant was collected. Fat-soluble components were removed by washing with chloroform (1:1; v/v) and then centrifuging the filtrate at 3000 rpm for 20 minutes. The aqueous phase (upper phase) was collected, lyophilized, and stored for storage at -20°C until use. Working concentrations of LNLP (50, 100, 250, 500 and 1000 mg/ml) were freshly prepared prior to use.

Chemical properties of the components of LNLP

To determine the presence of lipids, proteins, and carbohydrates in the components of LNLP, the chemical composition of these substances was studied. The previously described procedure (Hawk, 1965) was used to prepare Fehling's solution for carbohydrate content determination. Fehling's solution was prepared by mixing copper sulfate (34.65 g in 500 ml) with an alkaline tartrate (125 g potassium hydroxide, 173 g Rochelle's salt in 500 ml). To prevent spoilage, these solutions were stored separately in bottles with rubber stoppers and mixed in equal parts when necessary. For carbohydrate evaluation, LNLP was gradually added to the warm Fehling's solution and the mixture was heated between additions. In this case, the reduced copper oxide turns yellow or brownish red. Particle size appears to play a role in the colour difference of cuprous oxide precipitates under different conditions. Finer precipitates are yellow and coarser precipitates are red.

Animals

Wistar male albino rats weighing 200-220 g were collected from the department's rat colony and kept under standard conditions (26°±2°C, 45-55% relative humidity, and light/dark cycle 12:12 h) for 10 days. Animals were maintained on a standard diet with ad libitum access to water and acclimated to laboratory conditions for 48 h prior to the experiment to minimize non-specific stress. Prior to animal experiments, permission was obtained from the Institutional Animal Ethics Committee (IAEC), and animals were housed under standard conditions in an animal facility approved by the Committee for Control and Supervision of Experiments on Animals (CCSEA).

Acute Toxicity

An acute oral toxicity study was conducted according to OECD-404 guidelines (1987). Ten male albino rats were used in the experiment in each group. Group 1 served as a control group and the remaining groups received LNLP at various doses (250, 500 and 1000 mg/kg body weight, respectively). Each animal received an individual dose of LNLP orally. Animals were fasted the night before dosing. 3-4 hours after the dose, food was withdrawn. Rat mortality and behavioral changes were observed after a 24-hour treatment period. The acute toxicity LD_{50} was calculated as the statistical mean between the dose with 100% lethal outcome and the dose with no lethal outcome.

Experimental protocol

Antidiabetic analysis

To induce diabetes in male albino rats, a single intraperitoneal (IP) injection of a freshly prepared solution of alloxane monohydrate (120 mg/kg in saline) was given. Rats were selected for antidiabetic studies 48 h after induction if their fasting blood glucose levels were greater than or equal to 200 mg/dl. Levels were evaluated using an Accuchek one-contact active glucometer (Roche, USA). A total of 50 rats were randomly assigned to 5 groups of 10 each. Animals in Group I, serving as controls (non-diabetics), received vehicle, normal saline. Diabetic rats in group II received no treatment, diabetic rats in group III received glibenclamide 2.5 mg/kg, and diabetic rats in groups IV and V received graded doses of LNLP (50 and 100 mg/ kg, BW/ day, respectively). Daily oral administration was required for 28 days during each treatment period. Weight and blood glucose levels of the rats were measured weekly, and blood samples for hematological studies and serum for biochemical analysis were collected on days 14 and 28 after treatment.

Fasting blood glucose and body weight measurement

Over the course of the 28-day experiment, fasting blood glucose was measured at 7-day intervals using an Accuchek active blood glucose meter. The body weight of the animals was also measured using a weighing balance at 7-day intervals.

Hematological analysis and Serum preparation

Blood was drawn for a haematological evaluation on days 14 and 28 following treatment. Each rat was given a light ether anesthetic while 5 ml of fresh whole blood was drawn from it through the retro-orbital venous plexus. 5 ml of blood collected, of which 2 ml were used for the haematological evaluation. To analyze hematological parameters, blood was immediately drawn into tubes containing EDTA. Hemoglobin concentration (Hb), total red blood cells (RBC), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), total white blood cells (WBC), neutrophils, lymphocytes, eosinophils, monocytes, basophiles, and total platelet count are all measured using an automated hematology analyzer (Sysmex KX-21, Japan). In addition, the remaining 3 ml of blood was put into a sterile tube and allowed to coagulate for about 30 minutes. Clotted blood was centrifuged at 4000 rpm for another 10 minutes. Serum was placed into sample vials and stored at -20 °C before testing the insulin assay and serum protein concentrations.

Preparation of tissues homogenate

On day 28 of treatment, all rats were sacrificed after blood collection under light ether anesthesia. When all organs of all animals were collected, parts of the organs,

such as liver, skeletal muscle, and intestine, were removed and stored in a freezer at -20 °C. Liver and skeletal muscle were used for biochemical analysis of glycogen content and activity of carbohydrate metabolizing enzymes in the corresponding tissue samples. Intestinal samples were collected from animals, placed on ice, purified, and homogenized in aqueous potassium buffer (1 M, pH 7.4). Antioxidant enzymes and non-enzymatic antioxidant components were measured in the post-mitochondrial fraction (PMF) obtained by centrifuging the collected intestines at 10,000 rpm (4°C) for 10 minutes.

Biochemical assays

Serum chemistry profile

At 14 and 28 days after treatment, aliquots of each blood sample were placed in plain vials and centrifuged to obtain serum using the commercial kit (Excel Diagnostics Pvt. Ltd.). The sera were then used to evaluate lipid profiles including total cholesterol, triglycerides (TG), and high- and low-density lipoproteins (HDL and LDL) according to the manufacturer's recommended standard operating procedures. Serum insulin levels were measured using an ELISA kit specifically designed for rat insulin (Invitrogen Insulin Rat ELISA Kit) according to the manufacturer's recommendations, and the amount of insulin in serum was expressed as μIU/ml.

Glycogen content in liver and skeletal muscles

Liver and skeletal muscle glycogen levels were measured by standard method (Sadasivam and Manickam, 1996). Separately, skeletal and liver tissues were centrifuged at 8000 × g for 20 minutes while homogenizing at a tissue concentration of 100 mg/ml in hot 80% ethanol. The residue was collected, dried in a water bath, and extracted at 0 °C for 20 min by adding 5 ml of water and 6 ml of 52% perchloric acid solution. The collected material was separated into the supernatant after centrifugation at 8000 × g for 15 minutes. The recovered supernatant was placed in a graduated tube with 0.2 ml of distilled water for a volume of 1 ml. Graded standards were formed using 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 ml of working standard solutions. All these standards were then diluted with distilled water to a total volume of 1 ml. Anthrone reagent (4 ml) was added to each tube before heating the tube in a boiling water bath for 8 minutes. After cooling the tubes to room temperature, the colour intensity of the solution was measured from green to dark green at 630 nm. The amount of glycogen in tissue samples was determined from a standard curve plotted with a standard glucose solution and this amount was expressed as μg glucose/mg tissue.

Carbohydrate metabolic enzymes activities in liver and skeletal muscles

Glucose-6-phosphatase (G-6-Pase) activity

G-6-Pase activity in liver and skeletal muscle was assessed using standard protocol (Swanson, 1955). Individual tissues were homogenized in ice-cold 0.1 M phosphate buffered saline (pH 7.4) at a tissue concentration of 50 mg/ml. Add 0.1 ml of 0.1 M G-6-Pase solution and 0.3 ml of 0.05 M maleic acid buffer (pH 6.5) to a calibrated centrifuge tube and heat in a water bath to 37 °C for 15 minutes. To stop the reaction, 1 ml of 10% trichloroacetic acid was used, then the mixture was cooled on ice and centrifuged at 3000xg for 10 minutes. The enzymatic activity of each tissue was expressed as the amount of inorganic phosphate/g tissue released, which was determined by measuring the optical density at 340 nm.

Glucose-6-phosphate dehydrogenase (G-6-PDase) activity

G-6-PDase activity in liver and skeletal muscle was assessed according to recommended protocol (Langdon, 1966). Individual tissues were homogenized in ice-cold 0.1 M phosphate-buffered saline (pH 7.4) at a tissue concentration of 50 mg/ml. 0. 3 ml 1 M Tris chloride buffer (pH 7.5), 0.3 ml 2.5×10^{-2} M G-6-Pase, 0.1 ml of 2×10^{-3} M NADP and 0. 3 ml of 0.2M $MgCl_2$ and 0.3 ml of ice-cold tissue homogenate were added to the spectrophotometer cuvette. The absorbance change rate was measured at 340 nm. Enzyme activity is measured in units of the amount required to catalyze a reduction of 1 μM NADP per minute.

Hexokinase (HK) activity

Spectrophotometric evaluation of HA activity in liver and skeletal muscle tissues was performed (Chou and Wilson, 1975). The assay mixture contained 3.7 mM glucose, 7.5 mM $MgCl_2$, 11 mM thioglycerol and 45 mM HEPES buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); 0.9 ml of this mixture and 0. 03ml of 0.22M adenosine triphosphate was mixed well in a cuvette. Then, 0.1 ml of the tissue supernatant was placed in a cuvette and the absorbance was recorded. Enzyme activity was assessed by spectrophotometric measurement of HA-associated NADPH reduction at 340 nm.

Determination of intestinal enzymatic and non-enzymatic antioxidants activities

Hydrogen peroxide (H_2O_2) production

The H_2O_2 production was calculated using the Woff method (Woff, 1994) Basically, in 2.5 ml of 0.1 M potassium phosphate buffer (pH 7.4), 0. 250 ml iron ammonium sulphate, 0.1 ml sorbitol, 0.1 ml xylenol orange, 0.025 ml H_2SO_4 and 0.050 ml intestinal PMF were added. The reaction mixture was stirred thoroughly with shaking until foam formed and a light pink colour appeared. The reaction mixture was then incubated at room temperature for the next 30 minutes. Absorbance was measured at 560 nm using distilled water as a control. The resulting H_2O_2 was extrapolated from the standard H_2O_2 curve.

Thiobarbituric acid reactive substances

Malonic dialdehyde (MDA), a substance that reacts with thiobarbituric acid, was quantified in intestinal PMF. MDA was determined by measuring the thiobarbituric acid reactive complex (TBA) product or the pink colour of the product spectrophotometrically. Briefly, 0.4 ml of the intestinal PMF was combined with 0.5 ml of 30% TCA, and 0.5 ml of 0.75 % TBA prepared in 0.2 M HCl were added to 1.6 ml of Tris-KCl. The reaction mixture

was incubated in a water bath at 80° C for 45 minutes, cooled on ice and centrifuged at 4000 rpm for 15 minutes. The optical density of the resulting pink solution was calculated at 532 nm compared to a reference blank in distilled water. The amount of free MDA produced was used to calibrate intestinal PMF based on MDA production. MDA levels were calculated using the method of Varshni and Kale (1990). A molar extinction coefficient of 1.56×10^5 m/cm was used to calculate the amount of lipid peroxidation per unit/mg protein.

Reduced glutathione concentration

Decreased glutathione (GSH) in the intestine was calculated using the method of Jollow et al (1974) The assay is based on the oxidation of GSH with 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB). DTNB and glutathione (GSH) react to form yellow-coloured 2-nitro-5-thiobenzoic acid (TNB). Consequently, the concentration of GSH can be determined by measuring the absorbance at 412 nm. Briefly, 0. 5 ml of intestinal PMF was mixed with 0.5 ml of 4% sulphosalicylic acid (precipitating agent) and centrifuged at 4000 rpm for 5 minutes. Ellmann's reagent (0.04 g of DTNB in 100 ml of 0.1 M phosphate buffer, pH 7.4) was added to 0.5 ml of the resulting supernatant. Absorbance was measured at 412 nm using distilled water as a control prepared under the same conditions.

Total cellular thiols concentrations

Concentrations of protein and non-protein thiols were determined using the method of Sedlak and Lindsay (1968). A small portion of the intestinal PMF was treated with 6% concentration of trichloroacetic acid. After centrifugation, the supernatant and precipitate were dissolved in 2 ml of 0.4 M Tris-HCl buffer containing 10% SDS, then DTNB was added to the reaction mixture and the optical density was measured at 412 nm. A calibration curve was made using GSH as a standard and expressing protein and non-protein thiols in µmol/mg protein.

Superoxide dismutase (SOD) activity

The Misra and Fridovich method (1972) was used to calculate SOD activity levels. 100 ml of distilled water solution containing 100 mg of epinephrine was acidified with 0.5 ml of concentrated hydrochloric acid. This includes preventing oxidation of epinephrine at 480 nm in an alkaline environment using a UV spectrophotometer. 2.5 ml of 0.05 M carbonate buffer (pH 10.2) and 0.3 ml of 0.3 mM epinephrine were added to approximately 0.01 ml intestinal PMF. To evaluate the specific activity of SOD in the intestinal PMF, the rate of epinephrine autoxidation was measured at 30-second intervals in all groups. Considering inhibition of autoxidation as a unit of specific SOD activity, enzyme activity was expressed in arbitrary units.

$$\text{SOD activity (U/mg protein)} = \frac{\text{SOD activity (U/ml)}}{\text{Concentration protein (mg/ml)}}$$

Glutathione peroxidase (GPx) activity

According to Beutler et al (1963), the intestinal GPx activity was measured. The reaction mixture contained 0.5 ml of potassium phosphate buffer (pH 7.4), 0.1 ml of sodium azide, 0.2 mL of GSH solution, 0.1ml of hydrogen peroxide (H_2O_2), 0.5 ml of PMF and 0.6 ml of distilled water. After adding 0.5 ml trichloroacetic acid, centrifuged the mixture at 4000 rpm for 5 minutes, then the mixture was incubated in a 37°C water bath for 5 minutes. To 1 ml of the supernatant, 2 ml of K_2PHO_4 and 1 ml of Ellman's reagent were added and collected. Absorbance was measured at 412 nm using distilled water as a control and activity was measured in µg GSH consumption/min/mg protein.

Glutathione-S-transferase (GST) activity

The spectrophotometric method established by Haque et al (2003) was used to quantify GST activity. The reaction contained 0.1 ml of tissue homogenate, 1.67 ml of 0.1 M sodium phosphate buffer (pH 6.5), 0.2 ml of 1 mM GSH and 0.025 ml 1 mM 1-chloro-2,4-dinitrobenzene (CDNB). Spectrophotometric measurements of absorbance changes were performed at 340 nm, and GST activity was expressed as nmol of CDNB conjugate formed /min/mg protein using a molar extinction coefficient of $9.6 \times 10^3 M^{-1} cm^{-1}$.

Histopathology of liver and pancreas

Small pieces of liver and pancreas were taken from euthanized animals and properly fixed in 10% formalin. These tissues were processed and filled with paraffin. After staining 5 µm sections with hematoxylin and eosin, histopathological changes were observed under a light microscope.

Statistical analysis

Data were analyzed using a one-way analysis of variance (ANOVA) using the Graph Pad Prism software method, then all treated groups were compared to controls using either the Dennett's test or Turkey's multiple comparison test. SEM means (n=10) are shown. A significant difference between the experimental group and the control group is defined as $P \leq 0.05$.

RESULTS

The chemical properties of LNLP

Neem leaf extract were found to be carbohydrate based using the Fehling test. Carbohydrates with free or potentially free aldehyde or ketone groups can readily reduce certain metal ions, such as copper, bismuth, mercury, iron, and silver ions. The best and most widely used tests for sugar are based on this characteristic. For instance, black copper oxide becomes insoluble when blue copper hydroxide suspended in an alkaline medium is heated. However, in the presence of reducing agents such as certain sugars, cupric hydroxide is reduced to insoluble yellow or red copper oxide. Considering these characteristics, Fehling's test was used to study the chemical composition of Neem leaf extract. Reduction occurs when yellow or brown-red copper oxide is formed. Cuprous oxide precipitate varies in colour depending on the conditions, with fine-grained deposits being yellow and coarse-grained being red. This colour difference appears to be due to differences in particle size. Positive test results showed that neem leaf extract is mainly composed of carbohydrates.

Acute toxicity study

No adverse reactions or behavioral changes were noticed following the administration of any graded doses of LNLP during the entire experimentation period in the acute toxicity study. The fact that the overall ratio of food and water intake remained stable indicates that this LNLP did not affect the typical growth of the rats. According to the data obtained, the oral dose of LNLP had an LD_{50} of more than 1000 mg/kg.

Effect of LNLP on blood glucose and body weight of alloxan-induced diabetic rats

Hyperglycemia is induced in rats by alloxan monohydrate. Fasting blood glucose levels in treated rats were significantly ($P \leq 0.05$) lowered by LNLP compared to diabetic control and glibenclamide groups (Figure 1A). The reduction rate was comparable to the standard control group. Weight gain after 28 days was also statistically higher ($P \leq 0.05$) in the groups receiving graded doses of LNLP than in the control diabetic group (Figure 1B).

Effect of LNLP on haematological parameters in alloxan-induced diabetic rats

Induction of diabetes resulted in statistically significant reductions in PCV and Hb ($P \leq 0.05$) compared to normal control rats. However, during treatment with graded doses of LNLP, the values of these parameters increased significantly compared to the untreated diabetic group (Figure 2A and B). The RBC values of LNLP-treated rats and diabetic controls were not significantly different from each other (Figure 2C). Rats treated with graded doses of LNLP showed significant increases in MCHC compared to diabetic controls and glibenclamide-treated groups (Figure 2F). However, MCV and MCH values were not significantly different from each other (Figure 2D and E). Compared to diabetic control and glibenclamide-treated rats, platelet counts in LNLP-treated diabetic rats were significantly reduced ($P \leq 0.05$) and was comparable to normal controls (Figure 3A). Total white blood cell counts and differences were higher in control diabetic rats than in non-diabetic rats. Lymphocyte count and total white blood cell count (TWBC) decreased after LNLP administration (Figure 3B and C). Neutrophil counts in the untreated diabetic group were significantly increased compared to normal control and diabetic groups treated with graded doses of LNLP (Figure 3D). Eosinophil counts were significantly lower in untreated diabetic rats compared to healthy control and LNLP-treated groups (Figure 3F). The number of monocytes did not change significantly (Figure 3E).

Effect of LNLP on serum insulin and lipid profile of alloxan-induced diabetic rats

Figure 4 shows that the levels of TG, cholesterol and high-density lipoprotein (HDL) in the lipid profiles of animals with alloxan diabetes were significantly higher ($P \leq 0.05$) than those in the control group. Treatment of the diabetic group with 50 mg/kg and 100 mg/kg doses of glibenclamide and LNLP, respectively, significantly ($P \leq 0.05$) reversed the levels of these parameters to normal in the diabetic group (Figure 4B–D). With graded doses of LNLP-treated rats, there were no appreciable differences in the levels of low-density lipoproteins (LDLs) compared to the normal control and diabetic groups (Figure 4E).

Effect of LNLP on glycogen content and carbohydrate metabolic enzymes activities in liver and skeletal muscle of alloxan-induced diabetic rats

Comparing the animals from the alloxan-induced diabetic group to the animals from the non-diabetic control, there was a decrease in the amount of glycogen in the hepatic and skeletal muscle. Administering LNLP or glibenclamide to diabetic animals causes a significant ($P \leq 0.05$) recovery in the glycogen content towards the control level (Figure 5A). When compared to the control group, the induced diabetic animal showed a marked increase in G-6-Pase activity (Figure 5B) and a significant decrease in G-6-PDase and HK activity (Figure 5C and D). According to comparative analysis, giving diabetic animals graded doses of LNLP or glibenclamide resulted in significant protection and a return of these parameters to control group levels. The LNLP and glibenclamide groups did not differ significantly in the levels of these parameters.

Effects of LNLP on intestinal antioxidant enzymes and non-enzymatic antioxidants

An increase in H_2O_2 and MDA levels in the intestinal tissue was observed after diabetes was induced using alloxan monohydrate, according to the study. However, administration of LNLP at doses of 50 mg/kg and 100 mg/kg ($P \leq 0.05$) reduced the level of H_2O_2 produced and the amount of MDA in intestinal tissue (Figure 6A and B). Thiol levels and GSH levels were significantly (P 0.05) reduced in intestinal homogenate of diabetic untreated rats when compared with control. These parameters increased significantly ($P < 0.05$) after LNLP treatment at 100 mg/kg compared to untreated diabetic rats, but glibenclamide had the same effect (Figure 6C–E). In addition, untreated diabetic rats had significantly reduced ($P \leq 0.05$) activities of SOD, GPx and GST enzymes, whereas treated diabetic rats had significantly increased ($P \leq 0.05$) activities of these enzymes (Figure 7A-C).

Histopathology of the liver and pancreas

Histopathological studies revealed that all animal groups had altered liver and pancreas tissues (Figures 8 and 9). Histological examination of the livers of control rats revealed that they are composed of typical liver lobules, each formed by cords of hepatocytes diverging around the lobules from the central vein. Cell cords were separated by narrow blood sinusoids (Figure 8A). In rats with alloxan diabetes, periportal necrosis of hepatocytes was observed around the portal vein. Liver section displayed enlarged and clogged portal vessels as well as spots where inflammatory cells had been infiltrated. Hepatocytes were degenerated with irregular shape, turbid swelling, and vacuolization of the cytoplasm (Fig. 8B). However, these changes were less pronounced in diabetic rats given a graded dose of LNLP and conventional glibenclamide. Sections from the glibenclamide and graded LNLP dose groups showed partially normal aligned hepatocytes and normal hepatocytes with less central venous congestion,

indicating restoration of normal liver architecture (Figure 8C-E).

Normal acinar cells and islet β cells were observed in pancreatic slices of the control group. Nearby acinar cells are darker in colour than islets. Acinar cells are pyramidal cells with an apical basal nucleus and eosinophilic cytoplasm (Figure 9A). Under diabetes control, both endocrine and exocrine components show pathological changes. Almost all enlarged acinar cells have small vacuoles and the epithelium of the interlobular duct had a flat surface. Islet β-cells are usually small and sparse and show fibrosis and cellular rearrangement (Figure 9B). Glibenclamide and LNLP were administered at doses of 50 and 100 mg/kg body weight/day to diabetic rats. This resulted in a moderately normal proportion of acinar cells and smaller, slightly abnormal islet β-cells (Figure 9C-E). Unlike the control group, these treatment groups showed signs of cell regeneration in the islets of Langerhans. Reduced β-cell damage, more symmetrical vacuoles, and an increase in the number of islets indicate markedly improved cell damage. Acinar cells underwent less pronounced atrophic changes and there was a clearer separation between exocrine and endocrine components. The group of diabetic rats treated with LNLP (100 mg/kg daily body weight) was considered to achieve the greatest improvement (Figure 9E).

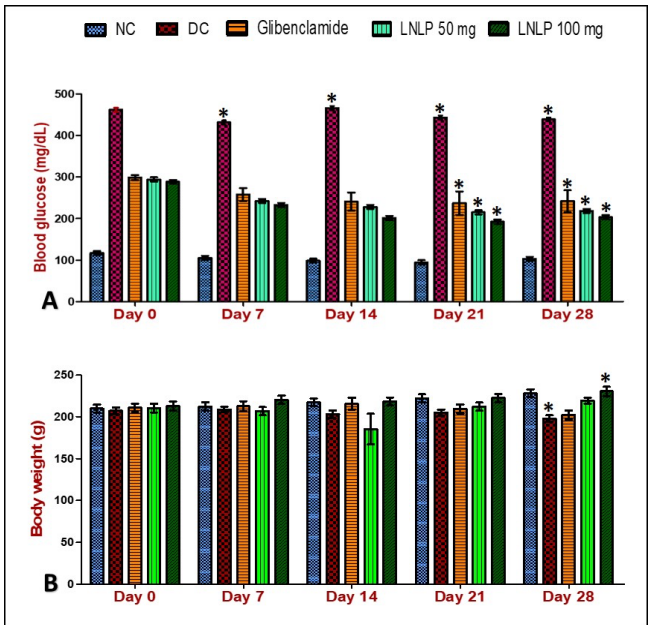

Figure 1: Effect of LNLP on alloxan-induced diabetic rats' (A) blood glucose and (B) body weight. For each group, the values are the mean SEM, with N=10. * P ≤ 0.05 in comparison to the experimental and control rats.

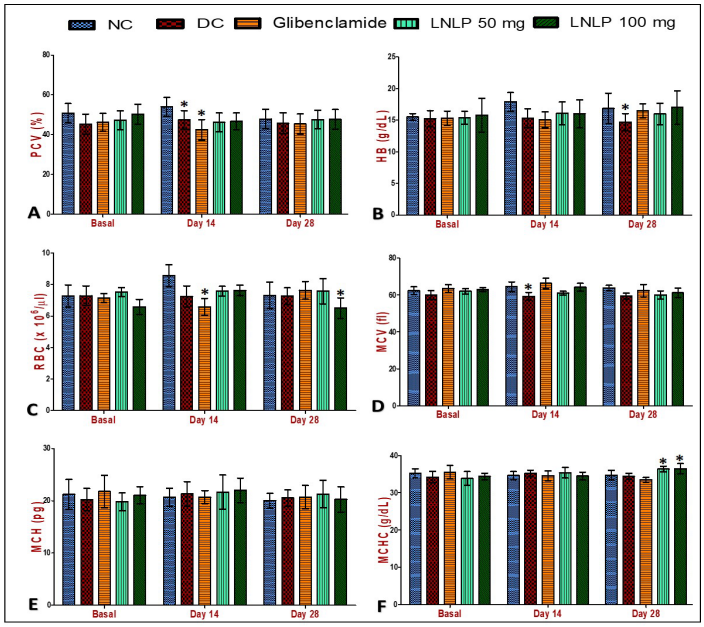

Figure 2: Effect of LNLP on (A) PCV (B) HB levels, (C) RBC, (D) MCV, (E) MCH, and (F) MCHC in alloxan-induced diabetic rats. Values for each group are the mean SEM of N=10. *P ≤ 0.05 compared with control and experimental rats.

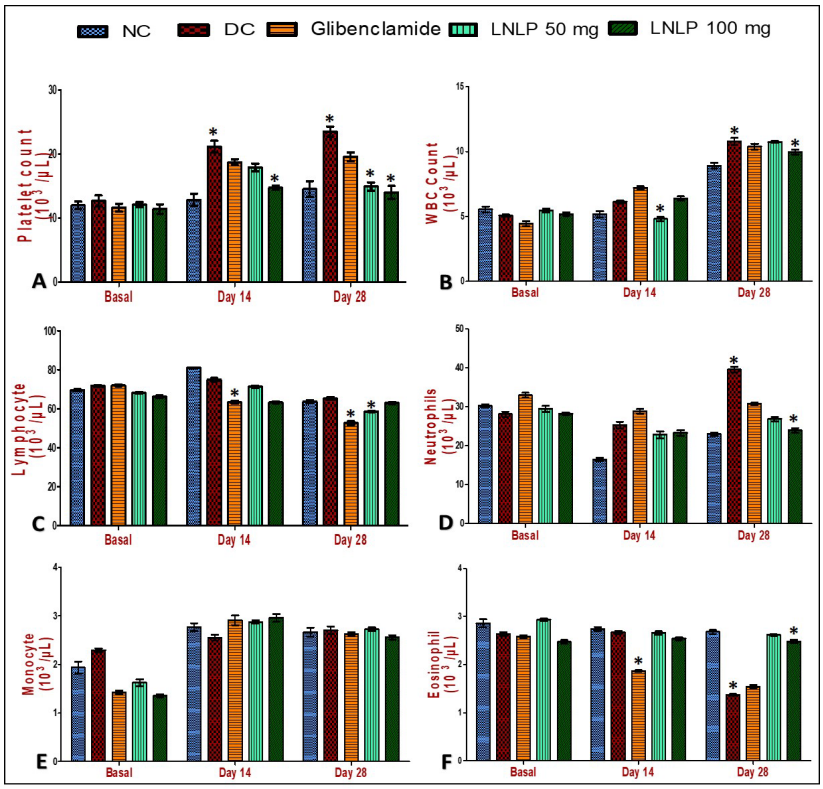

Figure 3: Effect of LNLP on (A) platelet counts, (B) leukocyte counts, and (C-F) differential leukocyte counts (lymphocytes, neutrophils, monocytes, eosinophils) in alloxan-induced diabetic rats. Values are mean SEM, N=10 for each group. *P ≤ 0.05 compared with control and experimental rats.

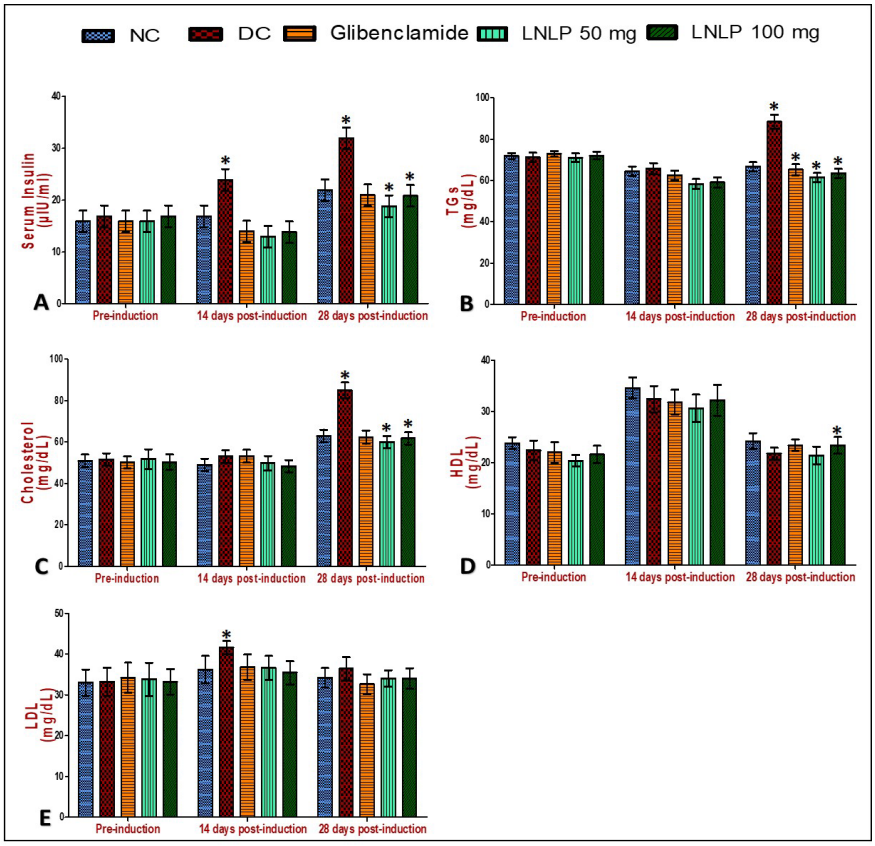

Figure 4: Effects of LNLP on (A) serum insulin and (B-E) lipid (TG, cholesterol, HDL, and LDL) profiles of alloxan-induced diabetic rats. Values are mean SEM, N=10 for each group. *P ≤ 0.05 compared with control and experimental rats.

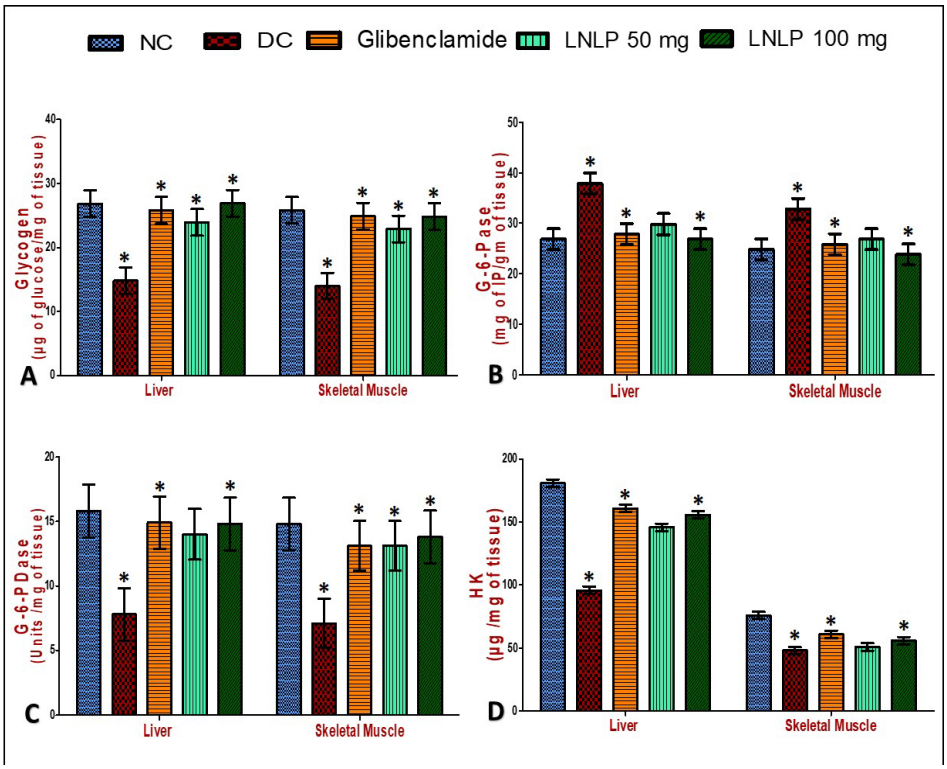

Figure 5: Effects of LNLP on (A) glycogen levels and (B-D) carbohydrate metabolism enzymes (G-6-Pase, G-6-PDase and HK) in alloxan-induced diabetic rats in liver and skeletal muscle. Values are mean SEM, N=10 for each group. *P ≤ 0.05 compared with control and experimental rats.

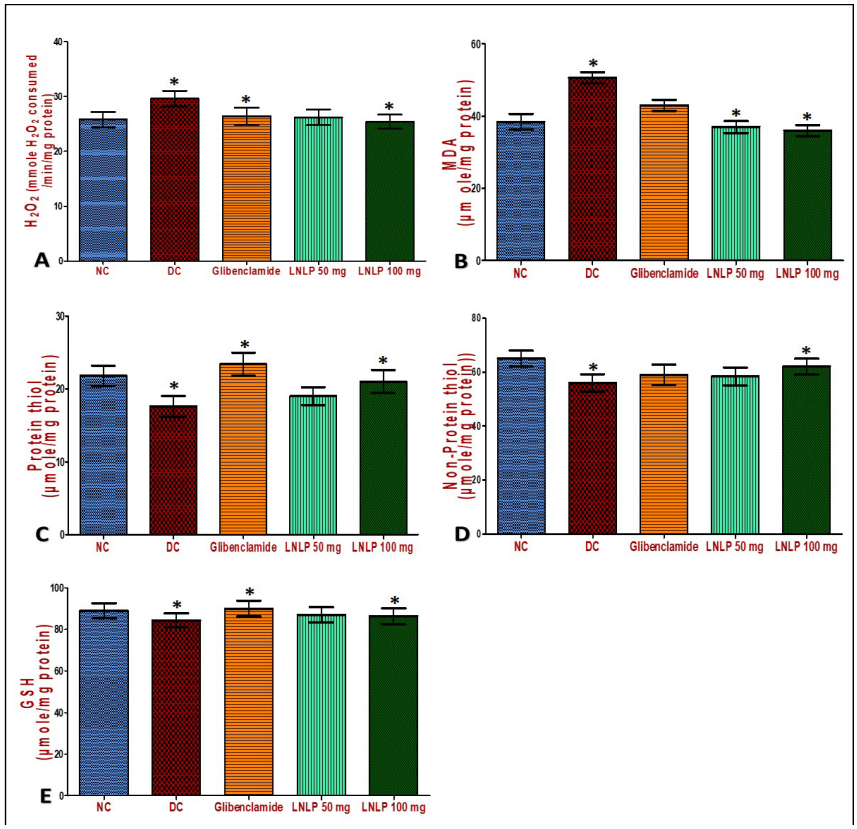

Figure 6: Effect of LNLP on (A) H_2O_2 levels, (B) MDA content, (C) protein thiols, (D) non-protein thiols, and (E) GSH concentrations in intestinal tissues of alloxan-induced diabetic rats. Values are mean SEM, N=10 for each group. *P ≤ 0.05 compared with control and experimental rats.

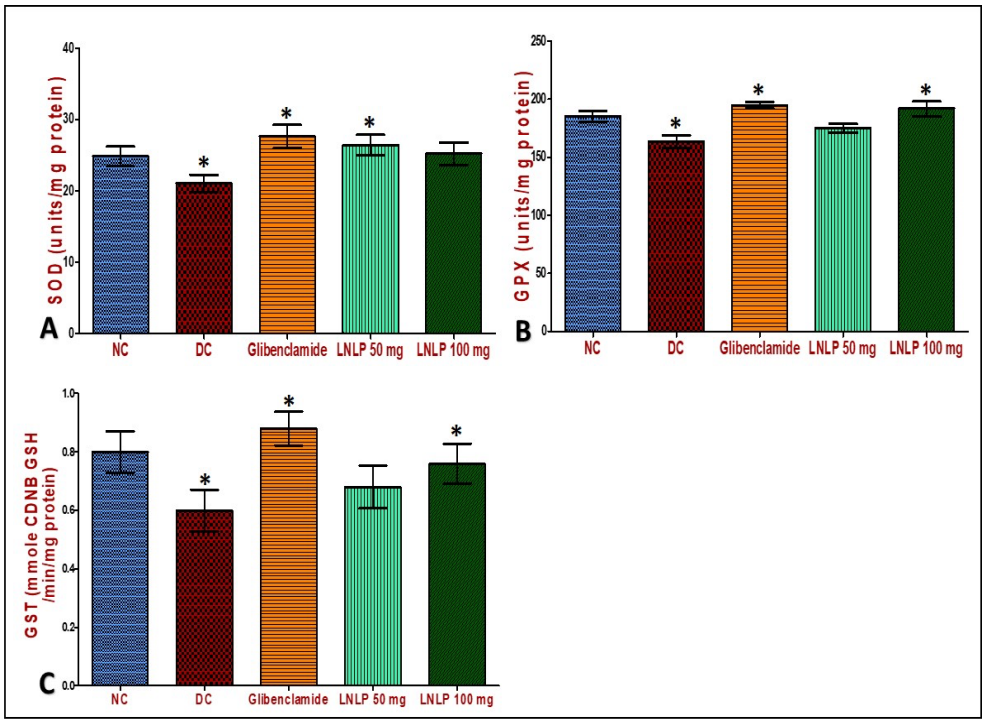

Figure 7: Effect of LNLP on (A) SOD, (B) GPx, and (C) GST activity of alloxan-induced diabetic rats in intestinal tissue. Values are mean SEM, N=10 for each group. *P ≤ 0.05 compared with control and experimental rats.

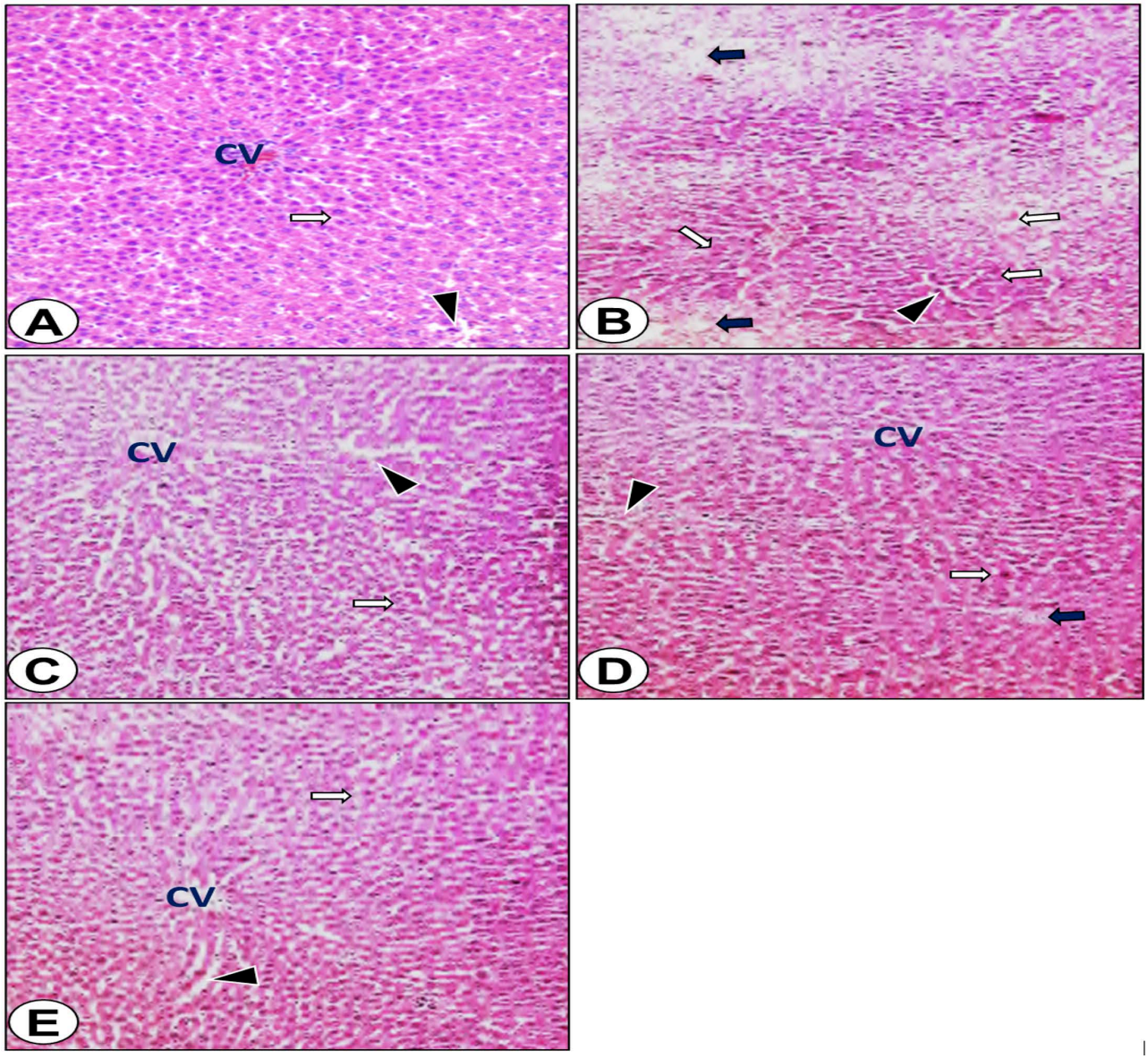

Figure 8: Photomicrographs of liver sections from rats with alloxan diabetes. (A) Normal hepatocytes (arrow) surround a normal control rat liver showing the central vein (CV) and hepatic sinusoids. Radial arrangement around the hepatic cord is also normal. (B) Hepatic sinusoids (black arrowhead) and increased vacuolation (arrows) are seen in the livers of diabetic control rats, with cloudy swelling, patchy necrosis (black arrow), and congestive CV of the hepatocyte cytoplasm. (C) In diabetic rats given glibenclamide, normal liver architecture was restored, and normal hepatocytes (arrows) arranged in normal sheets or cords around the CV. (D) and (E) Treatment of diabetic rats with LNLP at doses of 50 mg and 100 mg/kg partially restored the normal arrangement of hepatocytes (arrows) by improving liver structure and reducing pathological changes (arrows). Congested CV, hydropic degeneration, and degranulated cytoplasm of hepatocytes are illustrations of moderate liver effects. Hematoxylin and eosin (H&E) staining (200x).

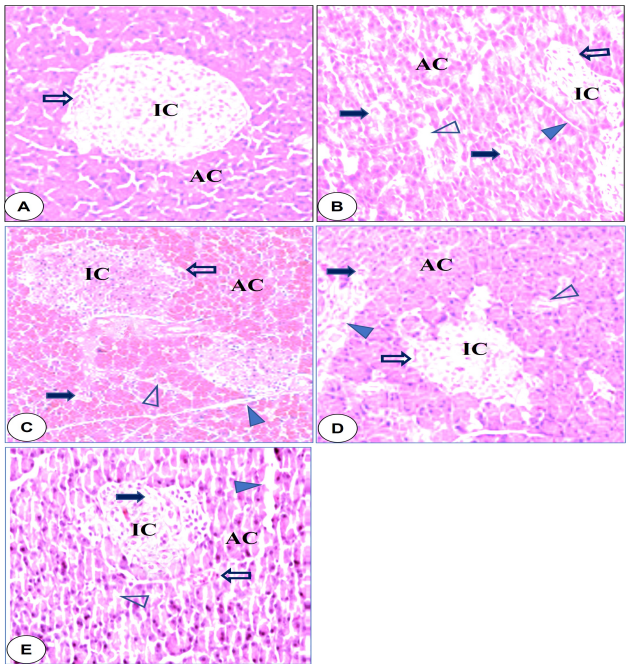

Figure 9: Photomicrographs of pancreatic sections from rats with alloxan diabetes. (A) The intralobular and mesangial ducts containing pancreatic secretions are almost normal in the control group, and normal exocrine acini (hollow arrows) are also visible. Acinar cells (AC) with prominent nuclei were in lobules and stained strongly. Islet cells (IC) are visible within the AC and are encased in a fine capsule. (B) Diabetes control islets come in a varying size and are sparsely distributed. Almost all acinar cells have small vacuoles (black arrows) and compressed epithelium (black arrowhead) lining the interlobular ducts. Islet β cells are fibrosis and almost completely disorganized. (C) In the glibenclamide group, interstitial islets of various sizes are visible (hollow arrows). Acinar cells can be seen among the islet cells. The stain of the islet was paler than that of the adjacent acinar cells. Both the intralobular (hollow arrowhead) and interlobular (black arrowhead) ducts are wider. After administration of 50 mg/kg and 100 mg/kg LNLP (D and E), respectively, the islets of Langerhans in diabetic rats showed almost normal structural characteristics. Acinar cells showed fewer atrophic changes and better separation of exocrine and endocrine components was observed. Remnants of islet cells are restricted to the periphery of the islets and are sparsely distributed (hollow arrows), and hyalinization can be seen in the center of the islets. Hematoxylin and eosin staining (200x).

DISCUSSION

In modern medicine, treating diabetes without side effects remains a challenge. The search for suitable hypoglycemic agents has focused on plants used in traditional medicine. In part, this is because natural products, usually in the form of natural fresh juices, pastes, or dry powders, tend to offer promising treatment options (Rates, 2001). Even though the limit dose was maintained, there was no mortality for 14 days in this study, suggesting that LNLP is non-toxic and may be safe at up to 1000 g/kg body weight. Substances with LD_{50} values above 50 mg/kg are non-toxic and therefore the tested extracts are considered safe. In this study, alloxan-induced hyperglycemia was used to evaluate the antidiabetic and antioxidant functions of LNLP. Alloxan is one of the common diabetes inducers often used to evaluate its antidiabetic potential. The hypoglycemic and weight-increasing effects seen in LNLP-treated diabetic rats can be attributed to increased glucose metabolism and signs of hypoglycemic properties. On the other hand, after oral administration of graded doses of LNLP, weight gain increased significantly from day 21 onward. This indicates that effective glycemic control or reversal of gluconeogenesis is a mechanism by which LNLP significantly improves overall health and metabolic processes.

Weight preservation in this study is similar to other studies in induced diabetic rat models that have shown encouraging results in terms of glucose reduction, overall pancreatic health and hypoglycemic potential in experimental animals (Shailey and Basir, 2012, Shrivastava *et al.,* 2012, Satyanarayana *et al.,* 2015, McCalla *et al.,* 2015). However, LNLP administration reduced blood glucose levels to the normal range, presumably by increasing insulin sensitivity. It has been reported treatment with neem leaf extract lowered blood sugar levels in Charles Foster rats that developed diabetes due to a high-fat diet, demonstrating the extract's potential in treating diabetes (Shrivastava *et al.,* 2012). However, the exact mechanism of action that causes hypoglycemia has not yet been elucidated. However, a literature review suggests that glycosides, terpenoids and flavonoids present in neem leaf and seed extracts have a cumulative effect on these actions (Chattopadhyay, 1999). However, administration of LNLP reduced blood glucose to normal levels, presumably by improving insulin sensitivity, which is consistent with the above reference study where blood glucose increased and the same levels returned to control levels in diabetic diabetic-induced rats, indicating that LNLP has anti-diabetic potential.

In this study, we found that untreated diabetic rats had significantly lower PCV, Hb, and MCHC values, indicators of anemia, 28 days after diabetes induction. An indication that LNLP contains bioactive principles that can stimulate the formation or secretion of erythropoietin, a substance in the blood that stimulates red blood cell production, is a significant increase in PCV, Hb and MCHC values after administration of LNLP in diabetic rats (Ezeigwe *et al.,* 2020).The increased platelet count in diabetic rats and the subsequent decrease in these values

after treatment may be related to the ability of LNLP to make platelets more insulin-sensitive, which in turn leads to a decrease in platelet aggregation. According to a report by Uko et al. (2013) diabetes study participants with higher platelets and TWBC also had higher blood sugar levels. The anti-inflammatory properties of LNLP may help alleviate the low-grade inflammatory state present in diabetes due to insulin resistance, as evidenced by the reduction of TWBC and lymphocyte counts in LNLP-treated diabetic rats (Vozarova *et al.,* 2002).The results of this study confirm the results of other studies on hematological parameters and suggest a reduction of increased glycation and oxidative stress processes in blood cells (McCalla *et al.,* 2015, Okpe *et al.,* 2019). An important biochemical abnormality in DM is hyperglycemia with dyslipidemia characterized by elevated TG, TC, and LDL levels and decreased HDL (Schofield *et al.,* 2016). The finding of this study that alloxan-induced diabetes significantly increased TC and total TG levels supports the hypothesis that DM is associated with dyslipidemia. However, after treatment with LNLP, the altered serum lipid profile was significantly improved, further confirming the ability of LNLP to reduce the abnormal lipid metabolism commonly associated with DM.

The amount of glycogen in various tissues directly reflects insulin activity. This is because insulin stimulates glycogen synthase and inhibits glycogen phosphorylase, both promote intracellular glycogen storage. In this experiment, addition of either LNLP or glibenclamide to diabetic rats significantly increased glycogen in both liver and skeletal muscle by stimulating glycogen synthase and inhibiting glycogen phosphorylase, which may result from elevated insulin levels (Jensen and Lai, 2009) or, in part, beneficial effects that may be attributed to several mechanisms such as insulin sensitization, glycogen synthesis, inhibition of carbohydrate metabolizing enzymes, and antioxidant activity (Ezeani *et al.,* 2017). The main objective of this study was to measure the activity of important enzymes involved in carbohydrate metabolism in both the liver and skeletal muscle of alloxan-diabetic rats. The gluconeogenesis enzyme, G-6-Pase, is important for supplying glucose to other organs during conditions of diabetes, prolonged fasting, or starvation. Insufficient insulin levels may be responsible for the elevated G-6-Pase levels found in these tissues of diabetic rats, and reduced levels of G-6-Pase activity in response to LNLP or glibenclamide supplementation suggests improved insulin secretion that regulates inhibition of key enzymes in gluconeogenesis (Petersen and Shulman, 2018). Depletion of liver and muscle glycogen has been observed in diabetic animals due to reduced enzymatic activity of HK (Murray *et al.,* 2000). After addition of LNLP or glibenclamide to diabetic rats, liver and skeletal muscle HK activities were significantly altered, indicating that the hypoglycemic effect observed was a result of increased glucose utilization at both skeletal muscle and liver levels. The fact that administration of LNLP to diabetic rats restored G-6-PDase activity to near-normal levels suggests that administration of LNLP promotes carbohydrate transport through the hexose monophosphate pathway (Abdel-Rahim *et al.,* 1992). By releasing superoxide radicals (O_2), hydroxyl radicals (OH) and hydrogen peroxide (H_2O_2), administration of alloxane triggers an oxidative stress (ROS) cascade that damages and eventually destroys cells (Lenzen and Munday, 1991). Increases in HO and MDA levels, significant decreases in GSH, protein and non-protein thiol levels, and activities of antioxidant enzyme systems in diabetic control rats in this study all indicate that oxidative stress may have occurred, and these antioxidants levels were increased in the intestinal homogenates of LNLP-treated rats, suggesting that LNLP has antioxidant properties that protect the pancreas from free radicals (Shailey and Basir, 2012). Diabetes-associated enzyme glycation or H_2O_2 inactivation may lead to reduced SOD activity (Sozmen *et al.,* 2001) because of depletion from excessive use of these enzymes to combat the free radicals produced by hyperglycemia. This suggests that the ability to prevent antioxidant inactivation may be responsible for the regenerated activity of antioxidant enzymes in intestinal homogenates of LNLP-treated rats and that the hypoglycemic effect of LNLP may be involved in and mediated by modulation of cellular antioxidant defence system.

In this study, improvement of histological tissue recovery of liver and pancreas confirmed the anti-diabetic properties of LNLP. Increased vasculature in pancreatic islets approaching levels seen in normal control rats may account for increased β-cells, better insulin secretion and improved glycemic control. In addition to improving liver and skeletal muscle glycogen levels, alloxan-induced moderate suppressive effects in diabetic rats essentially confirmed the changes observed in biochemical assays. This provides another explanation for the regenerative activity of pancreatic β cells in LNLP-treated diabetic rats (Barthel and Schmoll, 2003). The regenerative effect demonstrated with LNLP supplementation can be interpreted as an expression of recovery towards functional improvement of hepatocytes and normalization of liver function marker enzymes, suggesting an antioxidant role by stabilizing action at the membrane level towards normal hepatocyte function (Chattopadhyay, 2003). Restoration of significant structural changes in liver histology may be a result of restoration of glucose control rather than a direct effect of LNLP on the liver cells themselves.

CONCLUSION

In our study, the positive effects of effective antioxidant and antidiabetic activity were comparable to those of the standard drug glibenclamide, indicating that LNLP is the best drug because it is a natural product that can help diabetic patients with dyslipidemia and oxidative stress. More research is needed to identify the active compounds of LNLP with antihyperglycemic and antioxidant activities and the precise mechanisms of their action before they can be stabilized as therapeutically effective hypoglycemic agents.

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