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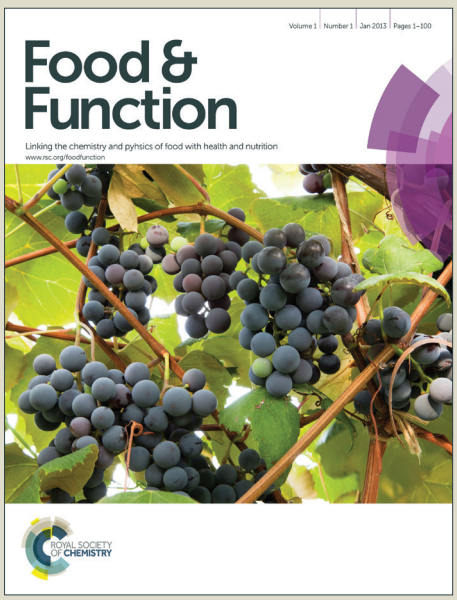

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Morus nigra leaf extract improves glycemic response and redox profile in the liver of diabetic rats

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ABSTRACT

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by hyperglycemia and alterations in carbohydrate, lipid, and protein metabolism. DM is associated with increased oxidative stress and pancreatic beta cell damage, which impair the production of insulin and the maintenance of normoglycemia. Inhibiting oxidative damage and controlling hyperglycemia are two important strategies for the prevention of diabetes. The pulp and leaf extracts of mulberry (*Morus nigra L.*) have abundant total phenolics and flavonoids, and its antioxidant potential may be an important factor for modulating oxidative stress induced by diabetes. In this study, DM was induced by intraperitoneal injection of alloxan monohydrate (135 mg/kg). Female Fischer rats were divided into four groups: control, diabetic, diabetic pulp, and diabetic leaf extract. Animals in the diabetic pulp and diabetic leaf extract groups were treated for 30 days with *M. nigra L.* pulp or leaf extracts, respectively. At the end of treatment, animals were euthanized and liver and blood samples were collected for analysis of biochemical and metabolic parameters. Our study demonstrated that treatment of diabetic rats with leaf extracts decreased superoxide dismutase (SOD)-catalase (CAT) ratio and carbonylated protein levels by reducing oxidative stress. Moreover, the leaf extract of *M. nigra L.* decreased matrix metalloproteinase (MMP)-2 activity, increased insulinemia, and alleviated hyperglycemia-induced diabetes. In conclusion, our study found that the leaf extract of *M. nigra L.* improved oxidative stress and complications in diabetic rats, suggesting the utility of this herbal remedy in the prevention and treatment of DM.

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1. INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by hyperglycemia and alterations in carbohydrate, lipid, and protein metabolism.[1] This disorder decreases antioxidant enzyme activities[2] and causes oxidative stress-induced damage to pancreatic beta cells,[3] leading to impairments in the production of insulin and maintenance of normoglycemia. Similarly, hyperglycemia contributes to the maintenance of oxidative stress through the generation of RONS (reactive oxygen and nitrogen species) or by altering the redox balance;[4] this creates a vicious self-sustaining cycle known as glucotoxicity. Individuals with diabetes usually have elevated levels of triglycerides, low-density lipoprotein, and total cholesterol owing to reduced high-density lipoprotein levels,[5] resulting in lipotoxicity. Glucotoxicity and lipotoxicity may be responsible for the majority of micro- and macrovascular complications induced by diabetes.[6]

Matrix metalloproteinases (MMPs) act as effectors of extracellular matrix remodeling in physiological and pathological conditions[7] and as modulators of inflammation.[8] In addition, MMPs are involved in oxidative stress-related cell damage.[9] Diabetes-induced changes in blood vessels may enhance the remodeling activity of MMPs.[10,11] Zhou et al. (2005) found that MMP-2 levels increased during the development of DM and that its inhibition reduced blood glucose level in diabetic rats.[12] Therapeutic targets that inhibit MMPs can improve insulin resistance and oxidative stress. Thus, the identification of novel therapeutic targets that can improve the redox status may lead to better glycemic control and may minimize late complications of diabetes.

Herbal medicine has been used to treat chronic diseases, such as diabetes, and may be effective in improving glycemic control.[13,14] The pharmacological activities of Traditional Chinese Medicine is well known, and herbal remedies have been shown to have anti-hyperglycemic properties.[15,16] For example, a *Morus* species or mulberry as it is popularly known, has been used traditionally as an anti-diabetic herbal medication.[17,18] The fruits, leaves, and roots of *Morus* species contain large amounts of active components that have multiple therapeutic effects, including anti-hyperglycemic properties such as reducing blood glucose levels and increasing insulin sensitivity, and has few adverse effects.[19,20] The anti-diabetic effects of *M. alba* are well established,[21-23] but the effects of *M. nigra L.* are not well characterized. *M. nigra L.* contains higher levels of secondary metabolites, mainly phenolic compounds,[24] total phenolics and

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flavonoids than the levels in *M. alba* and *M. rubra*,[25] showing excellent antioxidant potential. Based on the chemical composition of *M. nigra*, it could be suggested that antioxidant potential may be an important factor for modulating diabetes-induced oxidative stress. Therefore, we conducted a comparative study examining the effects of chronic treatment with pulp and leaf extracts of *M. nigra L.* in diabetic rats on metabolic parameters and redox status since oxidative stress is also an important factor in the establishment of diabetic complications and worsening of the disease.

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2. Material and Methods

2.1. Botanical Material

Mulberry tree specimens were collected in 2011 in the city of Ouro Preto, Minas Gerais, Brazil. The specimen was identified as *M. nigra,* voucher number OUPR 27087, by Dr. Viviane R. Scalon and deposited in the Herbarium José Badini – Universidade Federal de Ouro Preto (UFOP).

2.2. Preparation of fruit pulp and leaf extracts of *M. nigra*

Immediately after it was collected, the fruit was pressed, filtered, and stored at -80°C in order to obtain the pulp**.** Leaf hydroethanolic extract was prepared as previously described by Grance *et.al*[26] with some modifications. To isolate the leaf extract of *M. nigra L.*, the leaves were air dried at 50ºC and powdered. Approximately 1000 g of the dried powder was extracted with distilled water and 70% alcohol (1:1 ratio) for 24 h at room temperature. Vacuum filtration and evaporation of the solvent in a rotovap yielded a concentrated mass of green leaves. This concentrate had a dark green color and a sweet smell. The *M. nigra L.* extract was dissolved in filtered water and administered at 500 mg/kg.[27-28]

2.3. Total polyphenol determination

Folin-Ciocalteu reagent was used to quantify total polyphenols in the plant extracts, as previously described by George *et al*.[29] Briefly, 2.5 mL of Folin reagent diluted in distilled water (1:10) was added to 500 µL of the diluted sample or a standard solution of gallic acid at room temperature. The absorbance at 760 nm relative to the blank (distilled water) was determined. All analyses were performed in triplicate. The total polyphenol content was expressed in milligrams of gallic acid equivalent (GAE) per 100 g of pulp or leaf extract of *M. nigra L*.

2.4. Antioxidant capacity *in vitro*

Antioxidant capacity was measured as the capability of the sample to scavenge the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). Sequestration of DPPH by fruit pulp or leaf extracts of *M. nigra L.* was performed as previously described by Brand-Williams *et al*[30] with modifications. A standard curve was prepared using the reference antioxidant trolox (6-hydroxy-2,5,7,8-tetrameticromo-2-carboxylic acid). Antioxidant

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activity was defined as the reduction in absorbance of the DPPH radical at 515 nm; the percentage inhibition was calculated according to the formula below:

% Scavenging activity = $(1- A_{Sample\ 515}/ A_{Control515}) \times 100$

2.5. UPLC-DAD-ESI-MS analyses

Analyses were performed using an ultra-performance liquid chromatography **(**UPLC) Acquity (Waters, Milford, MA, USA)-coupled diode array detector (DAD) and ion trap mass spectrometer equipped with an electrospray ionization (ESI) operated in the following conditions: capillary voltage, 3500 V; positive and negative ion mode; capillary temperature, 320°C; source voltage, 5 kV; vaporizer temperature, 320°C; corona needle current, 5 mA and sheath gas and nitrogen, 27 psi. Analyses were run in the full scan mode (100-2000 u). ESI-mass spectrometry (MS/MS) analyses were performed with an UPLC Acquity (Waters) with helium as the collision gas, with collision energy set to 30 eV.

Chromatographic separation was carried out on Acquity UPLC^{TM} BEH RP-18 column (1.7 μm, 50 × 2 mm i.d.; Waters). The eluent phase consisted of water 0.1 % formic acid (solvent A) and acetonitrile 0.1 % formic acid (solvent B). The elution time was 0-11 min, with a linear gradient from 5% to 95% B. The flow rate was 0.3 ml min^{-1}, and the sample injection volume was 4.0 μL. The ultraviolet (UV) spectra were registered from 190 to 450 nm.

MS analysis was performed on quadrupole instrument fitted with an electrospray source in the negative mode (Ion spray voltage: -4 kV; orifice voltage -60 V).

2.6. Animals protocols

Female albino Fischer rats (The Laboratory of Experimental Nutrition of the UFOP), approximately 120 days old and weighing approximately 200 g, were used for this study. During the experimental period, the rats were maintained in a well-ventilated environment with controlled temperature, ventilation, and humidity. They had access to water and commercial rat chow *ad libitum*. This work was conducted in accordance with the international standards of animal protection and the ethical principles of the National Council of Animal Experimentation (CONCEA) and was approved by the Ethics Committee on Animal Use (CEUA) of the UFOP, protocol #2012/19.

To induce diabetes, we intraperitoneally injected 135 mg/kg alloxan (alloxan monohydrate, Sigma-Aldrich, St Louis, MO, USA) dissolved in 0.2 mL sterile saline.

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Control animals received 0.2 mL sterile saline only. Three days after alloxan administration, animals were fasted for 12 h, and blood samples were collected and blood glucose levels (Accu-Chek® Active, Roche) were measured. Animals with glucose levels above 300 mg/dL (16 mmol/L) were considered diabetic. Animals were divided into four groups: control group (C) received 1.0 mL water, diabetic group (D) received 1.0 mL water, group DP received 1.0 mL pulp of *M. nigra L.*, and group DL received 1.0 mL leaf extract of *M. nigra L.*, daily for 30 days.

2.7. Biochemical analysis

Glucose, total cholesterol, high-density lipoprotein (HDL) cholesterol, triacylglycerols, urea, creatinine, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) levels were determined by colorimetric methods using Labtest Diagnóstica S.A. (Lagoa Santa, MG, Brazil). Insulin levels were measured using a rat/mouse insulin enzyme-linked immunosorbent assay (ELISA) kit (Merck, Kenilworth, NJ, USA), according to the manufacturer's protocol.

2.8. Euthanasia and Tissue Collection

After 30 days of treatment, the animals were fasted for 8 h and anesthetized by isoflurane inhalation. Blood was collected through the brachial plexus into polypropylene tubes with or without 15 µL of Glistab® anticoagulant (Labtest, Cat. 29) in order to obtain plasma or serum, respectively. The blood was centrifuged at 10,000 g for 15 minutes, and the supernatant (plasma or serum) was stored at 4°C. Hepatic tissue was perfused with saline and collected immediately after euthanasia and stored at -80°C.

2.9 Determination of antioxidant defenses

2.9.1 Catalase activity

Catalase (CAT) activity was determined by measuring the conversion of hydrogen peroxide (H_2O_2) to molecular oxygen and water as previously described by Aebi (1984).[31] In brief, 100 mg of hepatic tissue was homogenized in phosphate buffer (pH 7.4) and then centrifuged at 10000 g for 10 min at 4 °C. Supernatant (10 µL) was mixed with K_2HPO_4 solution (50 µL), milli-Q water (40 µL, Millipore, Bedford, Massachusetts, USA), and 2.5 mM H_2O_2 (900 µL), and absorbance was measured at 240 nm at 25°C. Concentrations of H_2O_2 and samples were chosen such that the

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degradation rate was linear at 0.5, 1, 2, and 3 min. Hydrolysis of 1 μmol of H_2O_2 per min was equivalent to one unit (U) of catalase.

2.9.2 Superoxide dismutase activity

Superoxide dismutase (SOD) activity was determined using a superoxide dismutase assay kit (Cayman Chemical, Ann Arbor, MI, USA), based upon the principle of xanthine oxidase activity. In brief, 100 mg of hepatic tissue was homogenized on ice in 1 ml of 20 mM HEPES buffer, pH 7.2, containing 1 mM EGTA, 210 mM sucrose, and 70 mM mannitol. The homogenate was centrifuged at 12,000 g for 10 min at 4°C, and 10 μL of the supernatant was added to xanthine oxidase solution. The plate was incubated on a shaker at room temperature for 20 min, and the absorbance was measured at 450 nm with a Biotek (Winooski, VT, USA) ELx808 plate reader.

2.9.3 Total glutathione concentration and oxidized glutathione (GSSG)/ reduced glutathione (GSH)

To determine the levels of total GSH, 100 mg of hepatic tissue was homogenized in 5% sulfosalicylic acid solution. After homogenization, samples were centrifuged at 10,000 g for 10 min at 4 °C, and the supernatant was analyzed using a Sigma kit that employed a kinetic method based on the reduction of DTNB [5,5'-dithiobis(2-nitrobenzoic *acid*)] to TNB [5-thio- 2-nitrobenzoic acid]. Samples were measured spectrophotometrically at 412 nm, and a solution of reduced FSH (G4251; Sigma) was used to produce a standard curve. Total GSH is expressed in nanomole per mL of sample.

2.10. Determination of oxidative stress markers

Carbonylated Protein

Protein oxidation by reactive oxygen species (ROS) can lead to the formation of carbonyl derivatives. Carbonylated proteins were measured according to the method described by Levine *et al*.[32] In brief, 400 mg of tissue was homogenized in 100 mM phosphate buffer (pH 6.7) and 1 mM EDTA. After homogenization, samples were centrifuged at 10,000 g for 10 min at 4°C. The supernatant was collected, and proteins were precipitated using trichloroacetic acid (TCA) (10%), 2,4-dinitrophenylhydrazine (DNPH), and HCl at room temperature for 30 min. The precipitate was resuspended in 10% TCA and centrifuged at 4700 g for 5 min at 4°C. The precipitate from this step was washed twice with ethanol–ethyl acetate (1:1 v/v), then dissolved in 6% SDS, and

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centrifuged at 18,000g for 10 min at 4°C. Supernatant absorbance was read at 370 nm. Results are expressed as nanomoles of carbonyl groups per milligram of protein. The total protein was determined using the bicinchoninic acid (BCA) assay.

2.11 Western blot analysis

Equal amounts of protein in radioimmunoprecipitation assay (RIPA) buffer with protease inhibitor (measured using the BCA assay, according to the manufacturer's protocol) were resolved on 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride (PVDF) membranes (Immun-Blot® 0.22mm Bio-Rad, Hercules, CA, USA), and incubated with mouse monoclonal anti-MMP-2 (Millipore MAB3308) or anti-GAPDH antibodies (Cell Signaling, Beverley, MA, USA, 2118). Protein bands were visualized with enhanced chemiluminescence (ECL) prime reagent (GE Healthcare, Chalfont St. Giles, UK, RPN2232) and imaged by either exposing to autoradiography film or using a charge-coupled device (CCD)-based imager (Carestream 4000 MM Pro Image Station, Carestream, Rochester, NY, USA).

2.12 Gelatin zymography

MMP-2 and MMP-9 activities were detected using gelatin zymography, as previously described.[33] In brief, tissue extracts were diluted with 10 mM HEPES (pH 7.4) and mixed with non-reducing loading buffer. Samples were run on 8% polyacrylamide gels copolymerized with 2 mg/ml gelatin. Gels were rinsed in 2.5% Triton X-100 (3 × 20 min) and incubated for 18 h at 37°C in buffer containing 50 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$ and 0.05% NaN_3 (pH 7.5). Gels were stained with 0.05% coomassie brilliant blue G-250 for 3 h and destained in 4% methanol:8% acetic acid solution.

2.13 Statistical analysis

The data were analyzed by Kolmogorov-Smirnov to test for normality, and all data showed a normal distribution. All values are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using one-way analysis of variance (ANOVA), with the Tukey post-hoc test used to test for differences between groups. Prism 5.0 (GraphPad, La Jolla, CA, USA) was used to perform the analysis. Differences were considered significant when $p < 0.05$.

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3. RESULTS

UPLC-DAD-ESI-MS analysis

The *Morus* pulp and leaf extract preparations were analyzed by UPLC-DAD-MS/MS. Phenolic substances were identified in the *Morus* pulp, including flavonoid heterosides (Figure 1, Table 1). In the leaves, isomers of caffeoylquinic acid and flavonoid heterosides were identified (Figure 2 and Table 2).

In vitro assays: polyphenol content and DPPH radical-scavenging activity

For *M. nigra L*, the polyphenol content in the leaf extract was greater than the pulp (Figure 3A). The ability of three different dilutions of leaf extract and pulp to neutralize DPPH radical was determined. The capacity of the leaf extract to neutralize the DPPH radical was greater than the pulp in all evaluated dilutions (Figure 3B).

In vivo assays

Biochemical parameters analysis

Plasma glucose levels significantly increased and serum insulin levels significantly decreased in diabetic group animals relative to controls, demonstrating the establishment of experimental diabetes (Figure 4 panels A and B). The final weight of diabetic rats was significantly less than controls (Table 3). In addition, the concentration of biomarkers of liver damage (ALT and AST) and urea were increased in diabetic rats relative to control. Regarding the lipid profile, the diabetic animals showed a decrease in total cholesterol level; but this decrease was in the HDL-cholesterol fraction, and there was a 55% increase in the concentration of triacylglycerol relative to controls. Treatment with pulp did not alter any of the biochemical parameters measured in comparison with the diabetic group. However, leaf extract treatment decreased AST and ALT concentrations by 47% and 33%, respectively, in diabetic rats. Leaf extract treatment significantly decreased the concentration of urea, and there was a 23% reduction in triglyceride level in the diabetic rats (Table 3). Treating diabetic rats with leaf extract significantly reduced blood glucose and significantly increased the serum insulin level compared with untreated diabetic animals (Table 3).

Antioxidant Profile

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To measure antioxidant levels in hepatic tissue, we evaluated the activity of total SOD and CAT (Figure 4). SOD activity increased (Figure 5A) and CAT activity decreased (Figure 5B) in diabetic animals compared to controls, leading to an increase in the SOD/CAT ratio (Figure 5C). Therefore, diabetic animals featured an oxidative profile. Treatment with pulp or leaf extract effectively reversed this oxidative profile, significantly decreasing the SOD/CAT ratio compared to the diabetic group. The SOD/CAT ratio in pulp and leaf extract treated diabetic animals was similar to the control group.

Regarding GSH metabolism in hepatic tissue, there was no difference in total concentration of GSH in any of the evaluated groups (Figure 6A). However, we did observe a decrease in the ratio GSH/GSSG in diabetic animals when compared to non-diabetic animals. Treatment with *M. nigra L.* leaf had a tendency to increase this ratio (GSH/GSSG), although this difference was not statistically different (Figure 6B).

Diabetes-induced oxidative damage

In order to measure oxidative damage to proteins, we evaluated carbonylated protein levels in hepatic tissue. The concentration of carbonylated protein in the liver of diabetic animals was greater than controls (Figure 7), and treatment with the pulp and leaf extract effectively reduced carbonylated protein levels.

MMP-2 and MMP-9

MMPs are known for their important role in extracellular matrix remodeling. MMP-2 protein levels and activity increased in the livers of diabetic rats, and leaf extract treatment effectively decreased the expression and activity of MMP-2 in diabetic rats (Figures 9 and 8B). There was no difference in MMP-9 activity among any of the experimental groups (Figure 8D). Figures 8A and 8C show representative examples of the zymography gels.

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4. DISCUSSION

The main finding of our study is that treatment with leaf extract from *M. nigra L.* reduced glucose and increased serum insulin levels relative to control in an animal model of diabetes. Moreover, leaf extract treatment improved the lipid profile and antioxidant status and attenuated levels of liver damage markers.

The use of antioxidants can reduce the hepatic insult under hyperglycemic conditions, and it can affect glucose metabolism pathways. Plants containing flavonoids and other polyphenolic compounds have been shown to have antioxidant potential and may effectively reduce hyperglycemia mediated by oxidative stress and liver injury.[34] Studies have been conducted to identify plants with hypoglycemic and anti-diabetic activities that may ameliorate complications of DM.[35] In this regard, medicinal plants, such as *M. nigra L.*, are widely used in many countries. Almost all parts of the plant have pharmacological activities,[36,37] and various biologically active metabolites have been isolated and identified from different *M.* species.[38] Moreover, studies have revealed that the fruit and leaves of *Morus* plants contain many bioactive components, such as alkaloids, anthocyanins, and flavonoids.[39] However, it is known that *M. nigra* can grow in a wide range of climatic, topographical, and soil conditions, which can affect its chemical composition.[40] Thus, characterizing the extract is extremely important to validate the biological results. The flavonoids identified in this study from the extracts of *M. nigra L.* leaves and fruits were previously identified in extracts of this species.[39-42] This is the first study to compare the phytochemical composition and biological effects of pulp and leaf extracts of *M. nigra L.* The biological activities of the pulp and leaf extracts were different, and this may be due to differences in their phytochemical compositions. There were more phenolic compounds in the leaf extract than the pulp extract, demonstrating that the constituents of the extracts differed both quantitatively and qualitatively. These differences may explain the higher *in vitro* antioxidant capacity of the leaf extract, demonstrated by the neutralization of DPPH radicals, than pulp extract. Caffeoylquinic acids are phenolic compounds with promising anti-diabetic activity.[41] The caffeoylquinic acids identified in *M. nigra L.* leaf extract, but not in pulp, in our study may have reduced blood glucose levels and increased insulin serum concentration in diabetic animals. Diabetes is normally associated with dyslipidemia,[41] and we found that leaf extract treatment decreased serum triacylglycerol concentration. Moreover, treatment with this extract decreased the

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levels of ALT and AST, suggesting that it effectively protected the liver from damage induced by oxidative stress, which is a characteristic of diabetes. The SOD and CAT enzymes constitute the first line of cellular antioxidant defense. SOD activity increased and CAT activity decreased in untreated diabetic rats in our study. SOD is a key enzyme of the antioxidant defense system, catalyzing the dismutation of superoxide radicals to produce H_2O_2.[42] Its overexpression, however, is harmful to cells.[43] An increase in SOD activity accompanied by a decrease in CAT activity has been associated with elevated levels of H_2O_2 and accompanying oxidative damage.[44] Both the pulp and leaf extract treatments effectively restored the SOD/CAT balance in diabetic rats, thereby reducing SOD activity, increasing CAT activity, minimizing the deleterious effects of H_2O_2 accumulation, and restoring the redox balance of the tissue.

Another indicator of oxidative stress is the oxidized/reduced GSH ratio.[45] GSH levels decrease and those of GSSG increase in response to diabetes-induced oxidative stress,[46] as evidenced by decreased GSH/GSSG ratio in diabetic animals. Treatment with *M. nigra L* leaf extract tended to increase the GSH/GSSG ratio. We suggest that the decreased consumption of GSH can be attributed to an increase in the efficiency of the enzyme system for the neutralization of reactive species. The levels of carbonylated protein, a protein oxidation marker increased in diabetic animals and treatment with both the pulp and leaf extracts effectively minimized protein damage, as evidenced by the decrease in the concentration of carbonylated protein. These results suggested that treatment with *M. nigra L.* attenuated diabetes-induced oxidative stress. MMPs are associated with inflammatory process[47] and are overexpressed in diabetes.[11,48] Some natural products have been shown to inhibit MMPs.[49,50] *M. nigra L.* leaf extract effectively decreased MPP-2 protein expression and activity. These results can be explained by the presence of phenolic compounds in leaf extracts; these phenolic compounds can inhibit MMP.[47] Taken together, our results suggested that *M. nigra L.* leaf extract improved the antioxidant profile in the liver, decreased serum glucose levels, and increased serum insulin levels in diabetic rats (Figure 10). The different properties of the pulp and leaf extracts can be attributed to their different phytochemical composition. We have demonstrated for the first time that treatment with leaf extract is more efficacious than that with the pulp and highlighted the importance of understanding the properties of *M. nigra L.* leaf extracts.

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5. CONCLUSION

Our study showed that treatment of the diabetic rats with the leaf extract decreases MMP-2 levels and SOD/CAT ratio, consequently reducing carbonylated protein levels and oxidative stress. Moreover, the leaf extract of *M. nigra L.* allevaited hyperglycemia induced by diabetes. Treatment with *M. nigra* can improve oxidative stress and complications due to diabetes.

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Figure Captions

Figure 1. Ultra-performance liquid chromatography-diode array detector-electrospray ionization-mass spectroscopy (UPLC-DAD-ESI-MS) profile of *Morus nigra L.* pulp. Conditions: CHS130 100 RP-18 column (1.7 µm, 50 × 3 mm i.d.). 1. Cianidin 3-*O*-glucoside; 2. Cianidin 3-*O*-glucosyl-ramnoside; 3. Quercetin 3-*O*-glucoside / Quercetin 7-*O*-glucoside; 4. Quercetin 7-*O*-glucoside / Quercetin 3-*O*-glucoside

Figure 2. UPLC-DAD-ESI-MS profile of *Morus nigra L.* leaf extract. Conditions: CHS130 100 RP-18 column (1.7 µm, 50 × 3 mm i.d.). 1. 3-*O*-caffeoylquinic acid; 2. 4-*O*-caffeoylquinic acid; 3. 5-*O*-caffeoylquinic acid; 4. Luteolin-7-*O*-gentiobioside; 5. 6-Hydroxy-luteolin-7-*O*-rutenoside; 6. Quercetin-3-*O*-furanosyl-2''-ramnosyl

Figure 3. Antioxidant capacity of *Morus nigra L.* A: Polyphenol concentration (*values are expressed in mg of gallic acid in 100 g of pulp or leaf extract). B: Capacity to neutralize 2,2-diphenyl-1-picrylhydrazyl (DPPH), a measure of antioxidant activity, of pulp and extract of *Morus nigra* and the reference antioxidant Trolox, in different dilutions and concentrations, respectively, at 30 min.

Figure 4. **A**: Plasma glucose levels (mg/dL). **B**: Serum insulin levels (pmol/L). Control (C), Diabetic (D), Diabetic treated with pulp (DP) and Diabetic treated with leaf extract (DL). One way analysis of variance (ANOVA) with Tukey's post-hoc test. $p<0.05$ for statistical significance. The same letters indicate no significant difference between the indicated groups.

Figure 5. Antioxidant profile. ***(A)*** Liver superoxide dismutase (SOD) levels (U/mL) after 30 days of orally administered treatment. ***(B)*** Liver catalase levels (CAT) (µmol/mL) after 30 days of treatment. ***(C)*** The SOD/CAT ratio in C, D, DP and DL groups. One way ANOVA with Tukey's post-hoc test. $p<0.05$ for statistical significance. The same letters indicate no significant difference between the indicated groups.

Figure 6. Redox status. **A**: Liver total glutathione (GSH) levels (total GSH nmol/mL) after 30 days of treatment; **B**: Liver GSH/glutathione disulfide (GSSG) ratio (total GSH nmol/mL) after 30 days of treatment. Groups analyzed were C, D, DP and DL, One way ANOVA with Tukey's post-hoc test. $p<0.05$ for statistical significance. The same letters indicate no significant difference between the indicated groups.

Figure 7. Carbonylated proteins levels (U/mg protein) after 30 days of treatment. Groups analyzed were C, D, DP and DL, One way ANOVA with Tukey's post test. $p<0.05$ was considered statistically significant. The same letters indicate no significant difference between the indicated groups.

Figure 8. **A**: Representative image of matrix metalloproteinase (MMP)-2 bands on the gel. B: MMP-2 activity in hepatic homogenate, 30 µg protein per well. C: Representative image of MMP-9 bands on the gel. D: MMP-9 activity in hepatic homogenate, 30 µg protein per well. C, D, DP and DL, HT-1080 (Std.) One-way

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ANOVA with Tukey's post-hoc test. $p<0.05$ for statistical significance. The same letters indicate no significant difference between the indicated groups

Figure 9. **A**: Representative image of MMP-2 on x-ray film. **B**: Western blot for MMP-2, 30 µg protein per well, in hepatic homogenates. C, D, DP and DL, HT-1080 (Std.) One way ANOVA with Tukey's post-hoc test. $p<0.05$ for statistical significance. The same letters indicate no significant difference between the indicated groups.

Figure 10. Proposed model for the antioxidant effect of leaf extract from *Morus nigra*. MPP: matrix metalloproteinases, SOD/CAT: Superoxide dismutase/catalase, GSSG/GSH: oxidized/ reduced glutathione, CP: carbonylated protein. (□) increased; (□) decreased. Oxidative stress induces hyperglycemia, and hyperglycemia sustains oxidative stress, increasing oxidative damage and creating a vicious cycle.

Table Captions

Table 1. Flavonoids identified in the pulp *Morus nigra* by UPLC-DAD-ESI-MS

Table 2. Flavonoids identified in the leaf extract *Morus nigra* by UPLC-DAD-ESI-MS

Table 3. Biochemical parameters. The results are expressed as mean ± SEM, C=Control, D=Diabetic, DP=Diabetic treated with pulp and DL=Diabetic treated with Leaf extract. One-way ANOVA with Tukey's post-hoc test. $p<0.05$. The same letters indicate no significant difference between the indicated groups.

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Figure 1

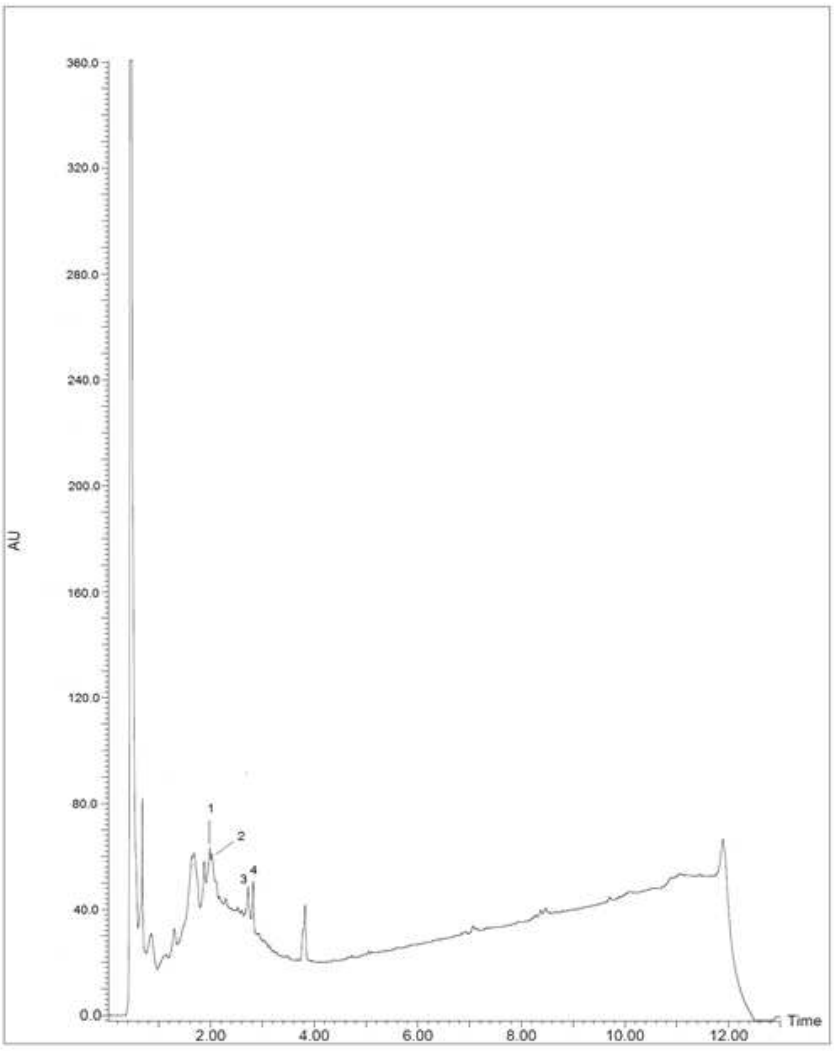

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Figure 2

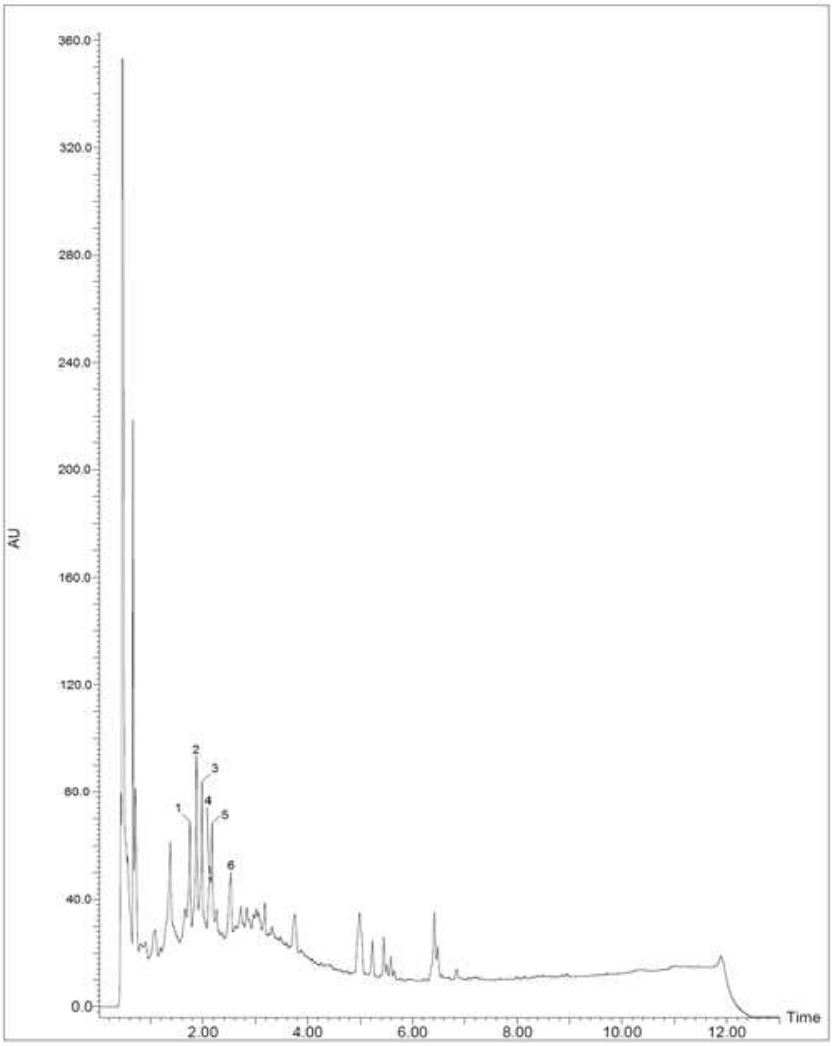

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Figure 3

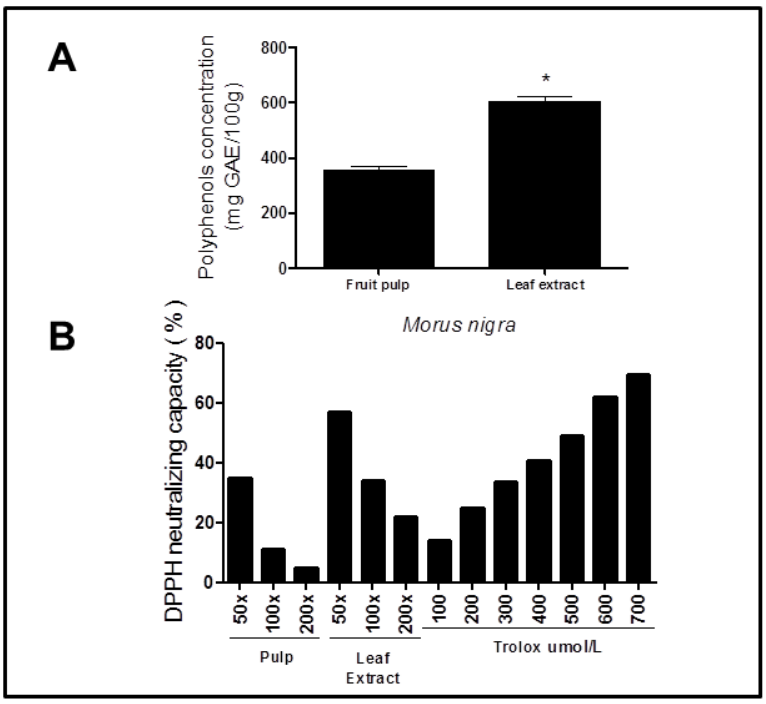

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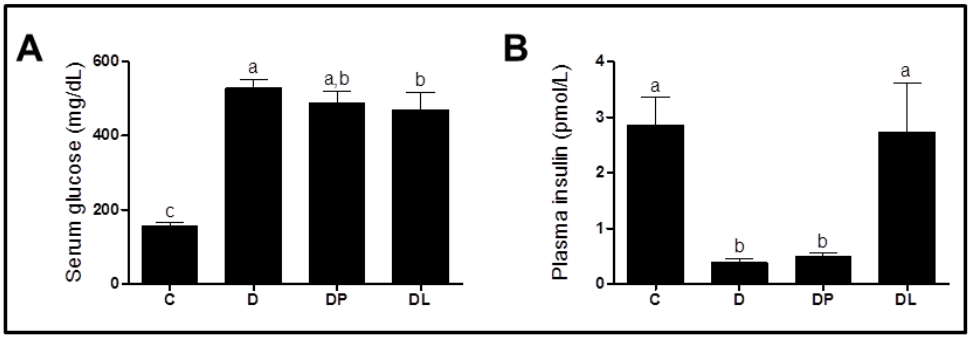
Figure 4



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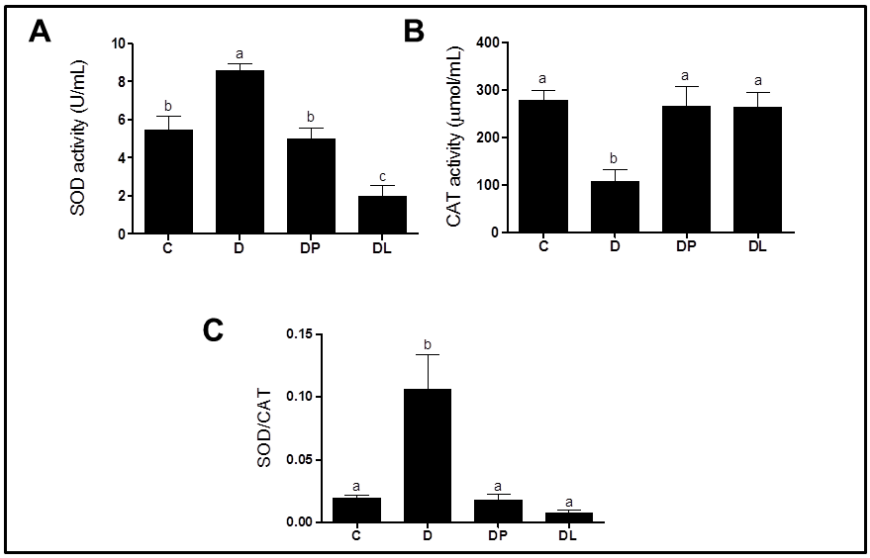

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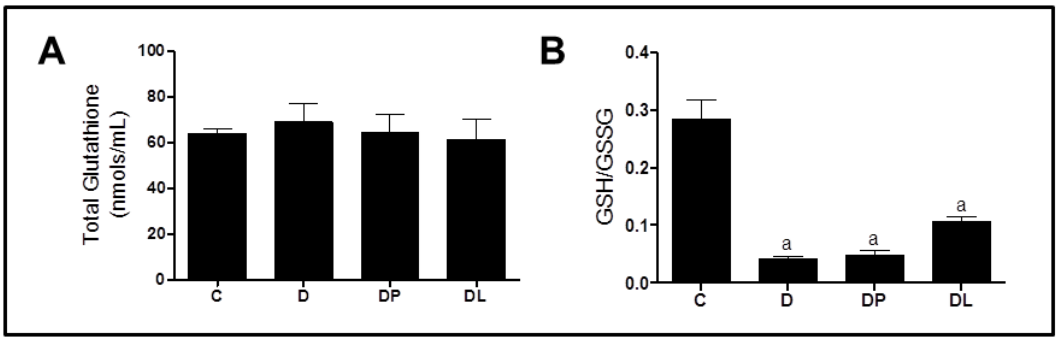
Figure 6



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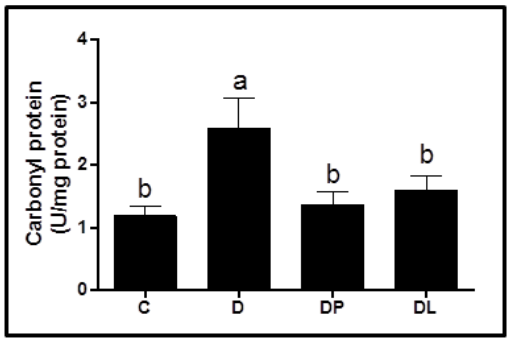

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Figure 8

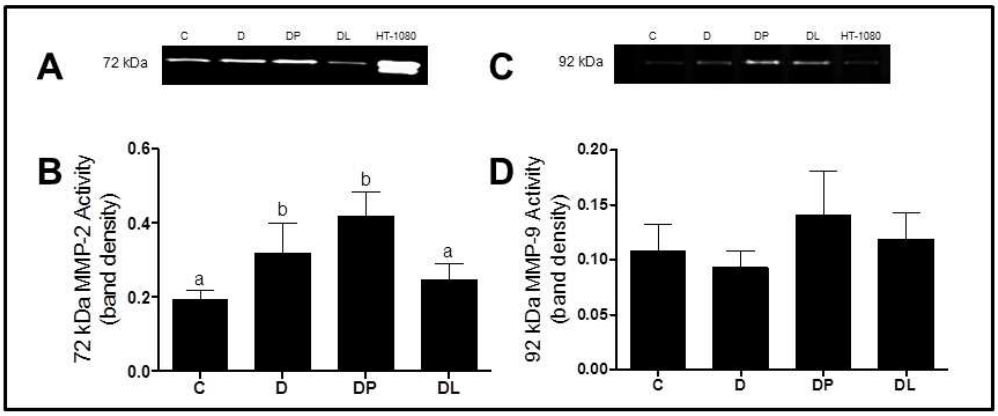

Figure 9

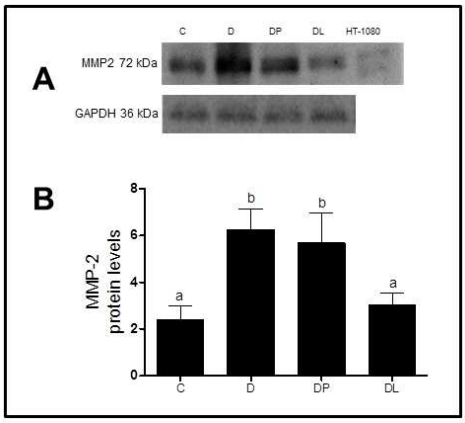

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Figure 10

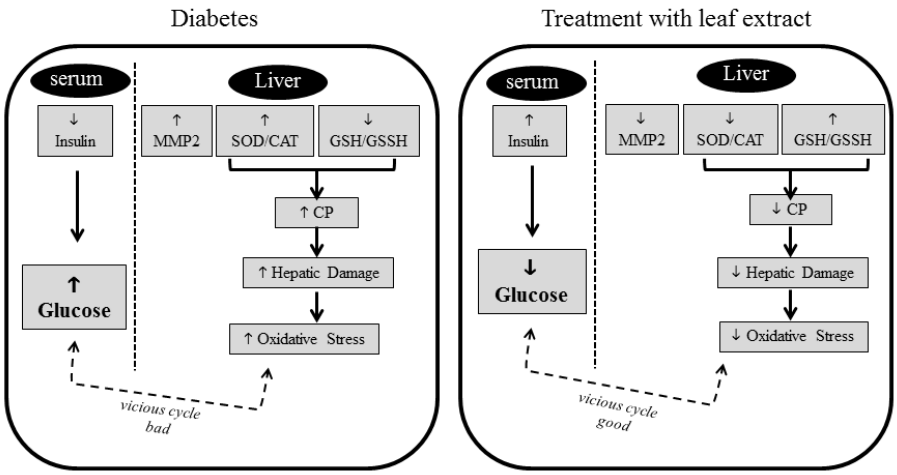

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Table 1

Peak	Compound	RT (min)	UV (nm)	LC-MS $[M-H]^-$ (*m/z*)	LC-MS $[M+H]^+$ (*m/z*)
1	Cianidin 3-*O*-glucoside	1.99	280.1	447.32	449.38 (287.0)
2	Cianidin 3-*O*-glucosyl-ramnoside	2.10	281.1	593.21	595.42 (449.1; 287.1)
3	Quercetin 3-*O*-glucoside/ Quercetin 7-*O*-glucoside	2.71	255.1; 359.1	463.53 (301.1)	465.35 (303.2)
4	Quercetin 7-*O*- glucoside/ Quercetin 3-*O*-glucoside	2.82	254.1; 358.1	463.25 (301.0)	465.48 (303.4)

Table 2

Peak	Compound	RT (min)	UV (nm)	LC-MS $[M - H]^-$ (*m/z*)	LC-MS $[M+H]^+$ (*m/z*)
1	3-*O*-caffeoylquinic acid	1.76	323.1	353.38 (191.1; 179.0; 134.8)	355.39
2	4-*O*-caffeoylquinic acid	1.88	321.1	353.38 (191.1; 179.0; 135.2)	355.72
3	5-*O*-caffeoylquinic acid	1.99	323.1	353.38 (190.8; 179.0; 135.0)	355.92
4	Luteolin-7-*O*-gentiobioside	2.15	255.1; 328.1	609.37 (447.2; 285.3)	611.44
5	6-Hydroxy-luteolin-7-*O*-rutenoside	2.17	265.3; 327.8	609.58 (301.0)	611.51
6	Quercetin-3-*O*-furanosyl-2''-ramnosyl	2.53	264.1; 357.8	579.21 (433.8; 301.0; 277.0)	581.72

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Table 3

Morphometric and Biochemical Parameters	Groups (*Average± Std. Error*)			
	C	**D**	**DP**	**DL**
Initial weight (g)	187.10±5.78	181.50±5.03	182.70±5.42	182.90±4.42
Final weight (g)	196.71±6.81^{a}	165.61±4.14^{b}	163.82±11.10^{b}	162.85±12.28^{b}
Liver (g)	7.22±0.38	8.02±0.35	7.21±0.37	7.36±0.47
ALT (U/mL)	39.40±3.92	170.95±17.05^{a}	182.65±12.37^{a}	114.42±25.65
AST (U/mL)	70.76±5.49^{b}	182.72±28.08^{a}	173.42±25.27^{a}	96.03±33.51^{b}
Creatinin (mmol/L)	66.25±3.60	71.57±4.92	92.17±16.97	73.51±8.79
Urea (mmol/L)	6.91±0.28^{c}	22.11±1.4^{a}	20.30±0.99^{a,b}	17.37±0.48^{b}
Total cholesterol (mmol/L)	1.85±0.13^{a}	1.42±0.09^{a,b}	1.09±0.16^{b}	0.96±0.18^{b}
HDL cholesterol (mmol/L)	1.08±0.07^{a}	0.64±0.02^{b}	0.62±0.06^{b}	0.55±0.04^{b}
Atherogenic fraction (mmol/L)	0.69±0.03	0.60±0.08	0.38±0.10	0.56±0.11
Triacylglycerol (mmol/L)	1.15±0.20	1.79±0.23	1.67±0.31	1.37±0.27

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