Research Article

Hepatoprotective Effect of *Naravelia zeylanica* Against Streptozotocin-Induced Oxidative Damage in Wistar Rats

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ABSTRACT

Oxidative stress plays a significant role in the development of Diabetes Mellitus and its vascular complications. Although diabetic hepatopathy is less common, it is one among the targeted organ related to diabetes. The present study was designed to elucidate the hepatoprotective efficacy of methanolic extract of *Naravelia zeylanica* (MNZY) in STZ-induced diabetic rats. Wistar rats were induced for diabetes by single injection of STZ (55 mg/kg i.p.). The rats were randomly divided into six groups of 5 animals each. Animals were treated with MNZY (100 and 200 mg/ kg b.w) and glibenclamide (5 mg/ kg b.w) for 45 days respectively. Serum biomarkers such as aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) were assessed to determine hepatic injury. Malondialdehyde (MDA), reduced glutathione (GSH) and ascorbic acid levels were measured to assess the free radical activity in liver. Hepatic antioxidant activities of glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) were also determined. The results revealed that MDA content, ALT, AST and ALP levels of diabetic rats were found to be significantly increased ($p < 0.05$) as compared to the control rats due to STZ induction. Besides, a significant amelioration in antioxidant enzymes like SOD, CAT, GPx and the non-enzymatic antioxidants such as reduced glutathione (GSH) and the ascorbic acid levels were significantly increased ($p < 0.05$) in MNZY treated diabetic rats. These results indicate that MNZY exerts hepatoprotective activity in experimental diabetic rats.

Keywords: Oxidative stress, free radical, antioxidant, hepatoprotective, *Naravelia zeylanica.*

INTRODUCTION

Diabetes Mellitus is a major metabolic disorder and a leading cause of morbidity in the world[1]. It is shown to be associated with increased oxidative stress that arises due to the disturbances in homeostatic phenomenon between increased production of free radicals or reduced antioxidant defenses[2]. The role of oxidative stress in the pathogenesis of diabetes mellitus is not only by free radical generation[3] but also due to non-enzymatic protein glycosylation and auto-oxidation of glucose, which weakens the antioxidant enzymes and formation of peroxides[4]. The overproduction of free radicals and accumulation of lipid peroxidation by-products can also lead to diabetic complications. Therefore, to reduce oxidative stress, by preventing or delaying the progression or reverting the complications of diabetes becomes the primary goal of antioxidant treatment. Several plant products are known to exhibit potent antioxidant activities in the prevention and treatment of diabetes[5,6].

Naravelia zeylanica (NZY) belonging to family Ranunculaceae is found to be distributed throughout India mainly in warm regions of Eastern Himalayas, Assam, Bengal, Bihar and greater parts of Deccan Peninsula[7].

In Indian system of medicine Ayurveda, NZY has been employed in the treatment of pitta, helminthiasis, dermatopathy, leprosy, inflammation, wound and ulcer[8]. Medicinal plants are the good sources of antioxidants and free radical scavengers, as they protect against ROS-induced oxidative stress/damage. Recently, natural therapies have gained importance as they have been shown to regulate the oxidative stress related complications of diabetes[9]. Natural products with antidiabetic and antioxidant properties are in increasing demand to attenuate induced oxidative stress and its complications[10].

Hence, it is recommended that treatment with natural antioxidants may be a useful approach to manage diabetes and its complications[11]. Therefore, the present investigation was aimed to elucidate the protective nature of MNZY on oxidative stress and antioxidant defense system in a chemically induced diabetic model.

MATERIALS AND METHODS

Drugs and Chemicals

Streptozotocin and Glibenclamide were obtained from ProLab Marketing Pvt. Ltd. (New Delhi).

The chemicals and solvents utilized for the study were obtained from Hi-Media (Mumbai) and were of analytical grade.

Plant Materials and Preparation of Extract

The plant has been collected from the hills of Munnar, Iddikki districts, Kerala, India. Botanical identification was performed by Prof. Jayaraman, Director, Plant Anatomy Research Centre, Chennai, India (Ref. no. PARC/2011/914). The dried whole plant powder (100g) of

NZY was extracted sequentially using various organic solvents in increasing order of polarity (hexane, dichloromethane, ethyl acetate and methanol)[12] at room temperature and was concentrated using reduced pressure.

Experimental Animals

Male Wistar rats (150 – 200 g) were purchased from King's Institute, Chennai. The animals were maintained under laboratory condition in the animal house, with five rats per cage at 18±2°C in a light controlled room (12 h dark/12 h light) and were provided with commercial pellet diet with free access to water ad libitum. The study protocol was approved by the Institutional Animal Ethical Committee (52/IAEC/2011) with the recommendations for the proper care and use of laboratory animals.

Induction of Diabetes

Diabetes was induced in Wistar rats (150-200 g) by administering a single dosage (i.p) of Streptozotocin (STZ) at a concentration of 55 mg/kg b.w. in a freshly prepared 0.1M cold citrate buffer (pH 4.5)[13]. Fasting blood glucose was measured periodically. Blood glucose levels above 250 mg/dL were considered as diabetic and used for the study.

Experimental Design

Streptozotocin induced diabetic rats with marked elevation in fasting blood glucose levels (above 250 mg/dL) were prioritized for the study. The experimental animals were randomly divided into six groups of five animals each. Group I: Control rats (5% CMC); Group II: Control rats + MNZY (200 mg/kg b.w.); Group III: Diabetic rats (STZ- induced); Group IV: STZ- induced+ MNZY (100 mg/kg b.w.); Group V: STZ- induced+ MNZY (200 mg/kg b.w.); Group VI: STZ-induced+ Glibenclamide (5 mg/kg b.w.)[13].

Biochemical Analysis

At the end of the study, the experimental animals were fasted overnight, anaesthetized and sacrificed by cervical decapitation[14]. The blood was collected and centrifuged to separate the serum and the biochemical parameters were analyzed. The activities of AST, ALT and ALP were determined in the serum using commercially available kits as per the manufacturer's instructions.

Preparation of Tissue Homogenate

The liver tissues from the control and experimental rats were excised and rinsed with ice-cold saline. The tissue homogenates were prepared using 0.1 M Tris-HCl buffer[15], pH 7.4 at 4°C, in a homogenizer with a Teflon pestle for 3 min. The homogenate was centrifuged at 3,000 g for 10 min at 4°C. The supernatant was used for the biochemical parameters. The protein concentration was measured using Lowry's method[16].

Estimation of Lipid Peroxidation

Lipid peroxidation (LPO) was assessed by measuring the concentration of malondialdehyde (MDA)[17]. Briefly, 0.2 mL of tissue homogenate, 0.2 mL of SDS, 1.5 mL of acetic acid and 1.5 mL of TBA were added. The mixture was made up to 4 mL with water and boiled in a water bath at 95°C for 60 min. After cooling, 1 mL of water and 5 mL of n-butanol/pyridine mixture were added and shaken vigorously. Pink chromogen (secondary product of LPO) formed was measured at 535 nm. 1, 1', 3, 3'-tetra methoxy propane was used as a standard. The level of lipid peroxides were expressed as n moles of MDA/mg of protein.

Estimation of Superoxide Dismutase

Superoxide dismutase (SOD) was estimated as per Misra and Fridovich (1972)[18]. Briefly, the tissue homogenate (0.1 mL) was mixed with reaction mixture containing sodium carbonate (1 mL, 50 mM), nitroblue tetrazolium (0.4 mL, 25 mM), and hydroxylamine hydrochloride (0.2 mL, 0.1 mM). Enzyme reaction was initiated by adding 0.2 ml of NADH (780 µM) and stopped precisely after 1 min by adding 1 ml glacial acetic acid. The absorbance was read at 560 nm. The enzyme activity was expressed as units/mg protein.

Estimation of Glutathione Peroxidase

The glutathione peroxidase (GPx) activity was determined as reported earlier[19]. 0.2 mL tissue homogenate was added to the reaction mixture containing 0.2 mL of EDTA, 0.1 mL of sodium azide, 0.1 mL of H_2O_2, 0.2 mL of reduced glutathione and 0.4 mL of phosphate buffer and incubated at 37°C for 10 min. The reaction was stopped by the addition of 0.5 mL of TCA and centrifuged at 2000 rpm. To the supernatant, 3 mL of disodium hydrogen phosphate and 1.0 mL of DTNB were added and the absorbance was measured at 420 nm immediately. The enzyme activity was expressed as µmoles of glutathione oxidized/min/mg of protein.

Estimation of Catalase

The catalase (CAT) activity was determined according to the method of Takahara et al., (1960)[20]. Briefly, to 0.2 mL of tissue homogenate, 1.2 ml of Phosphate buffer and 1.0 mL of H_2O_2 solution was added. The decrease in the absorbance was measured at 240 nm at 30 sec intervals for 3 min. The activity of enzyme was expressed as µmoles of H_2O_2 consumed/min/mg of protein.

Estimation of Reduced Glutathione

Reduced glutathione (GSH) was determined by using the methodology of Sedlak and Lindsay (1968)[21]. Briefly,0.5 mL tissue homogenate was mixed with 0.2 M Tris buffer with pH of 8.2 and then mixed with 0.1 mL of 0.01 M Ellman's reagent, (5,5'-dithiol-(2-nitro-benzoic acid)) (DTNB) and centrifuged at 3000 g for 15 min. The absorbance was read at 412 nm. The amount of glutathione was expressed as µg/100 g of tissue[22].

Estimation of Ascorbic Acid

Ascorbic acid was estimated by the method of Omaye[23]. Ascorbic acid was oxidized to form dehydroascorbic acid and ketoglutaric acid. These products were treated with 2, 4-dinitrophenylhydrazine (DNPH) to constitute the derivative of bis-2, 4-dinitrophenylhydrazine.

This compound in the presence of strong sulphuric acid undergoes rearrangements to form a product that can be measured at 520 nm.

Histopathology of Liver

Animals were euthenized with mild ether anesthesia and dissected[24].

Liver was excised, washed with saline and a small portion of the organ was quickly fixed in 10% formalin.

The tissue was processed, embedded and sectioned using microtome.

Thin sections of liver (5-micron) were mounted on slide and stained with Hematoxylin & Eosin. The slides were then examined and photographed.

Statistical Analysis

All data were expressed as mean ± SEM. Statistical analysis was performed by one-way ANOVA followed by post hoc test for multiple comparisons. $p < 0.05$ was considered as significant.

RESULTS

Effect of MNZY on Body Weight

The average weight gained by the animals during the experimental period of 45 days were represented in Table 1.

A significant reduction in weight was observed in diabetic rats ($p< 0.05$) as compared to control.

Oral administration of MNZY (100 and 200 mg/kg b.w.) to diabetic rats significantly increased the weight ($p<0.05$) during the experimental period as compared with the diabetic group.

However the glibenclamide treated diabetic rats exhibited the maximum weight gain as compared with the other treated groups.

Effect of MNZY on Aspartate Transaminase (AST), Alanine Transaminase (ALT) and Alkaline Phosphatase (ALP) Activity

The effect of MNZY on serum biomarkers of liver toxicity in experimental rats were depicted in Figure 1. The activities of ALP, AST and ALT were significantly elevated in STZ-induced diabetic rats ($p< 0.05$) as compared with control rats. Treatment with MNZY and glibenclamide showed significant reduction ($p<0.05$) in the ALP, AST and ALT activities in diabetic rats as compared with untreated diabetic rats. The effect of MNZY was on par with that of glibenclamide treated diabetic rats. But there was no significant difference between both the doses of MNZY.

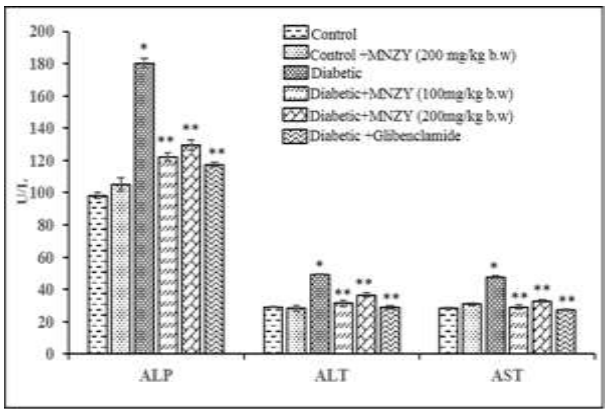

Figure 1: Effect of MNZY on ALP, ALT and AST activities in experimental rats. Data were analyzed by one-way ANOVA followed by post-hoc test to indicate difference among groups. Values are expressed as mean ±SEM (n=5). **, p<0.05* compared with the control group, ***, p<0.05* compared with the diabetic group.

Effect of MNZY on Lipid Peroxidation

The levels of MDA in liver of experimental rats were represented in Figure 2. A significant increase in MDA was observed in diabetic rats ($p< 0.05$) as compared with control. The elevation of MDA levels in diabetic rats could be attributed to the oxidative stress induced by STZ. Administration of MNZY and glibenclamide significantly decreased ($p< 0.05$) the level of MDA in diabetic rats as compared to diabetic group.

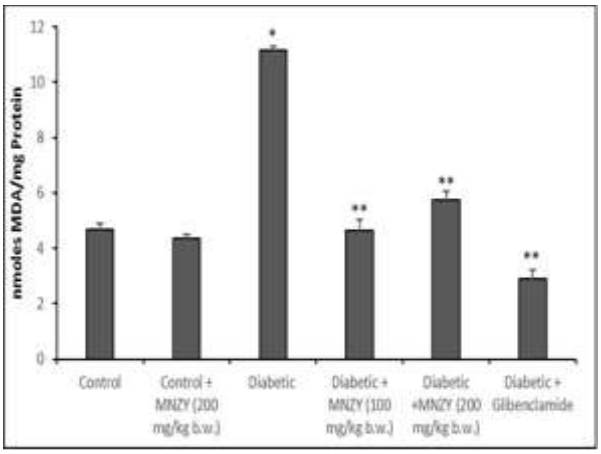

Figure 2: Effect of MNZY on liver MDA level in control and diabetic rats. Data were analyzed by one-way ANOVA followed by post-hoc test to indicate difference among groups. Values are expressed as mean ±SEM (n=5). **, p<0.05* compared with the control group, ***, p<0.05* compared with the diabetic group.

Effect of MNZY on Antioxidant Enzymes

The activity of antioxidant enzymes such as CAT, SOD and GPx in liver of experimental rats were represented in Table 2. A significant reduction ($p< 0.05$) in the activities of CAT, SOD and GPx were observed in the liver of diabetic rats as compared with control.

The decreased levels of CAT, SOD and GPx in the diabetic rats were significantly restored (*p< 0.05*) by MNZY treatment as compared with diabetic rats. The effect of MNZY was on par with diabetic rats treated with glibenclamide.

Table 1: Effect of MNZY on body weight of experimental animals

Experimental Groups	Body weight (g)			
	Initial weight (0^{th} day)	Final weight (45^{th} day)	ΔMean weight = (Final weight-Initial weight)	%Δweight gain = (Mean weight/Initial weight) × 100
Control	164.2±2.65	189.8±9.56	25.6±9.7	15.6±6.12
Control + MNZY (200mg/kg b.w)	163±3.6	185±3.1	22±216	13..58±1.4
Diabetic	166.2±3.02	111.6±3.4*	–54.6±3.07*	–32.84±1.7*
Diabetic + MNZY (100mg/kg b.w)	165.6±3.5	212.4±4.3**	46.8±3.2**	28.3±2.1**
Diabetic + MNZY (200mg/kg b.w)	167±3.57	199.6±6.71**	32.6±4.1**	19.44±2.3**
Diabetic + Glibenclamide	168±2.8	216.8±1.7**	48.8±3.8**	29.22±2.7**

Values are expressed as mean ± SEM (n=5) *, *p< 0.05* compared with control group, **, *p<0.05* compared with diabetic group.

Table 2: Effect of MNZY on CAT, SOD and GPx Activities

Experimental Groups	CAT (μM of H_2O_2 consumed/min/mg protein)	SOD (U/mg protein)	GPx (μmol of GSH utilized/min/mg protein)
Control	2.815±0.102	43.055±1.654	6.725±0.118
Control + MNZY (200 mg/kg b.w.)	2.74±0.12	40.82±1.18	5.96±0.12
Diabetic	0.761±0.076*	26.79±1.102*	1.172±0.21*
Diabetic + MNZY (100 mg/kg b.w.)	2.66±0.134**	41.145±1.105**	4.42±0.248**
Diabetic +MNZY (200 mg/kg b.w.)	2.47±0.169**	31.57±1.656**	3.19±0.472**
Diabetic + Glibenclamide	2.845±0.081**	45.925±1.105**	6.318±0.192**

Values are expressed as mean ± SEM (n=5) *, *p< 0.05* compared with control group, **, *p<0.05* compared with diabetic group.

Table 3: Effect of MNZY on Ascorbic acid and GSH Activities

Experimental Groups	Ascorbic acid (μg/g protein)	GSH (μg of GSH consumed/min/mg protein)
Control	1.77±0.39	59.995±4.05
Control + MNZY (200 mg/kg b.w.)	1.63±0.07	56.845±1.08
Diabetic	0.43±0.09*	24.675±2.84*
Diabetic + MNZY (100 mg/kg b.w.)	1.64±0.27**	56.955±1.04**
Diabetic +MNZY (200 mg/kg b.w.)	1.52±0.32**	38.69±1.34**
Diabetic + Glibenclamide	1.72±0.02**	58.775±0.89**

Values are expressed as mean ± SEM (n=5) *, *p< 0.05* compared with control group, **, *p<0.05* compared with diabetic group.

Effect of MNZY on GSH and Ascorbic Acid

The concentration of GSH and ascorbic acid in liver of experimental rats were represented in Table 3.

The level of GSH and ascorbic acid were significantly reduced (*p<0.05*) in diabetic rats as compared with the control. The reduction in these levels were reverted to near normal by oral administration of MNZY.

However, diabetic rats treated with glibenclamide exhibited maximal effect.

Histopathology and Microscopic Examination of Liver

The liver tissue of control and MNZY treated non- diabetic rats showed a normal hepatocyte with vesicular nuclei (Figure 3 A & B). On the other hand, the liver of diabetic rats showed hepatocyte with congested nuclei with vacuolation and unclear sinusoid (Figure 3 C).

The liver morphology of diabetic rats treated with MNZY low dose and high dose (Figure 3 D&E) exhibited normal architecture compared to the control rats with smaller glycogen granules. These pathomorphological alteration observed in diabetic rats treated with MNZY was on par with diabetic rats treated with glibenclamide (Figure 3 F) in exhibiting similar effects.

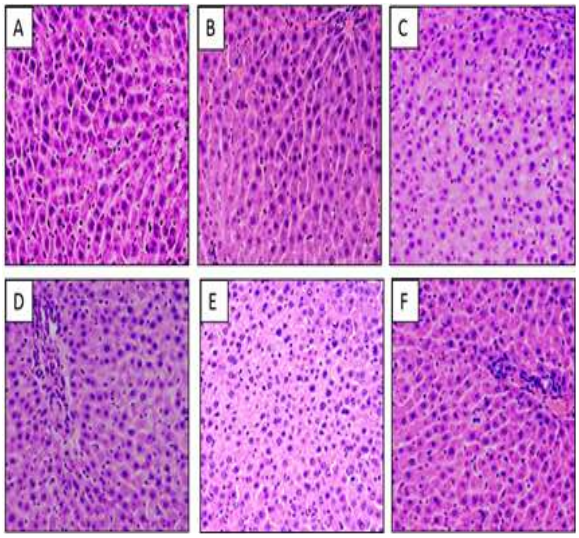

Figure 3: Histopathology of liver tissue of experimental rats (A) Section of liver tissue of control rat showing normal hepatocytes, (B) Section of rat liver tissue of MNZY (200 mg/kg) treated control rats, (C) Section of diabetic rat liver tissue showing damaged hepatocytes with congested nuclei, (D & E) Section of liver tissues from MNZY (100 & 200 mg/kg respectively) treated diabetic rats showing near normal hepatocyte with vesicular nuclei, (F) Section of liver tissue of glibenclamide treated diabetic rat showing normal architecture.

DISCUSSION

Reactive oxygen species and free radical generation are suggested to be a potent donor involved in various types of disorders including diabetes[24]. The elevated free radical production or reduced antioxidant defense mechanism might play a significant role in diabetic complication due to increased oxidative stress[25]. In a living organism, antioxidant enzymes as well as non-enzymatic antioxidants form the first line of defense against ROS-induced oxidative damage.

Reduction in oxidative stress levels were observed in experimental animals treated with plant extract or compounds[26].

A severe loss in body weight is a characteristic feature in STZ-induced diabetes that might exhibit most of the diabetic complications through oxidative stress[27].

In diabetic rats, a decrease in body weight occurs due to the loss or degradation of structural proteins owing to diabetes which contribute to the body weight[28].

Although, weight loss is one of the main signs of diabetes, but its mechanism is not well understood[29].

In the present study, administration of MNZY significantly ($p< 0.05$) increased the body weight of diabetic rats during treatment suggesting that MNZY protect body weight loss which can be attributed to its ability to reduce oxidative damage[30].

Hematological screening which is in clinical practice is the liver function test (LFTs) that is commonly used to screen for liver disease. These tests are used to monitor the progression of a known disease and the effects of potentially hepatotoxic drugs[31]. Aspartate transaminase (AST), Alanine transaminase (ALT) and Alkaline phosphatase (ALP) are the most common LFTs used to screen liver toxicity in a diseased condition like diabetes. The damage to liver tissue causes the release of these enzymes into the circulatory system which is due to cellular infiltration and functional disturbances in the cell membrane[32]. Administration of MNZY to diabetic rats significantly ($p<0.05$) decreased the activities of ALP, AST and ALT to near normal which was as comparable with that of glibenclamide. Our data shows that MNZY, to a certain extent, protects the structural integrity of the liver by preventing the intracellular enzyme leakage caused by the stress induced by STZ[30]. Similar observations were reported with various plants with proven hepatoprotective property, particularly in *Costus pictus*[33] and *Ichnocarpus frutescents*[34].

The increase in ROS production and cellular lipid peroxidation due to the oxidative stress eventually plays a significant role in the development of diabetes mellitus[35]. In the present study, a significant ($p< 0.05$) increase in MDA concentration was observed in diabetic rats and this level was reverted to near normal in MNZY treated diabetic rats. The increased peroxidation in diabetic conditions might be due to the oxidative stress in the cell as a result of depletion of antioxidant systems. Oxidative stress is a major determinant of antioxidant levels as it could increase the generation of free radicals[36]. SOD and CAT are the two major antioxidant enzymes that scavenge the free radicals generated due to increased oxidative stress *in vivo*. In the present study, the activities of SOD, CAT and GPx were significantly ($p< 0.05$) decreased in diabetic rats. A decline in the activity of these antioxidants leads to the formation of superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), which in turn generate hydroxyl radicals (-OH), resulting in initiation and propagation of lipid peroxidation[37]. SOD catalyzes the dismutation of O_2^- into H_2O_2 which gets converted to H_2O by CAT or GPx[38]. The MNZY treated diabetic rats showed

an increase in the activities of SOD, CAT and GPx thereby reverting back to normal levels. These results revealed that MNZY may contain free radical scavenging activity and exert a beneficial effect on pathological changes caused by reactive oxygen species. Several plants have been reported for free radical scavenging activity by increasing SOD, CAT and GPx levels[39].

The non-enzymatic antioxidant namely, GSH and ascorbic acid play a vital role in preventing the cells from oxidative damage similar to enzymatic antioxidants. GSH is a tripeptide and is reported to be essential for recycling of other antioxidants like vitamin E and vitamin C[40] and acts as a substrate for GPx which is involved in preventing the deleterious effect of oxygen radicals[41]. In the present study, liver of diabetic rats exhibited decreased GSH level indicating increased utilization of free radicals leading to increase in lipid peroxidation. On administration of MNZY, diabetic rats showed increase in GSH level indicating reduction in lipid peroxidation. Ascorbic acid is involved in recycling process of other vitamins such as the conversion of tocopheroxyl radicals to tocopherol[42]. In the present study, the ascorbic acid level was decreased in diabetic rats which might be due to excessive oxidation and lack of regeneration from their radical form to a reduced form. Administration of MNZY to diabetic rats increased the level of ascorbic acid to near normal indicating decreased utilization of antioxidant. These results are in line with findings reported from another plant with similar effect namely *Mollugo nudicaulis*[43] and *Chrysanthemi flos*[24].

The excessive production of free radicals play an important role in the development of STZ-induced experimental diabetes[44]. Natural antioxidants from plant sources have been suggested to have beneficial effects in the treatment of oxidative stress[45]. In the present study, the protective effect of MNZY on STZ related cytotoxicity was examined in the liver tissue of experimental diabetic rats. After oral administration of MNZY, the liver injury in STZ-induced diabetic rats was reduced when compared to diabetic rats.

The results of the present study revealed that administration of MNZY to diabetic rats profoundly increased the antioxidant status and decreased the elevated serum biomarkers of liver tissue damage. Therefore, MNZY might prove to have a prophylactic effect against diabetic complications and ameliorates diabetic hepatopathy through its antioxidant potential.

CONCLUSION

In conclusion, the present investigation shows that MNZY has hepatoprotective activity against lipid peroxidation caused by the production of reactive oxygen species thereby enhancing the antioxidant enzymes in liver tissue. Further detailed investigations are required to elucidate the exact mechanisms by which MNZY elicits its modulatory properties and the active phytoconstituent responsible for the aforesaid action.

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