

Original Research Article

Cytoprotective, Antihyperglycemic and Antioxidative Effect of Naringenin on Liver and Kidneys of Swiss Diabetic Mice

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Received: 30/09/2016

Revised: 15/10/2016

Accepted: 21/10/2016

ABSTRACT

Long-term effects of diabetes include serious health complications of liver and kidneys such as diabetic hepatopathy, a glycogen load on hepatocytes, and nephropathy, the damage to the glomerular capillaries. Polyphenols such as flavonoids have shown to possess effective antioxidant properties. In this study, the effect of naringenin as an antitoxic, antioxidative, and antihyperglycemic agent was studied and compared with standard L-ascorbic acid and metformin in diabetic Swiss albino mice. Acute toxicity, blood glucose, glycosylated hemoglobin, serum hepatic marker enzymes, antioxidant enzyme status both in vitro and in vivo, lipid peroxidation, histological examination, and transmission electron microscopy studies were conducted in streptozotocin-induced diabetic mice. Diabetes was induced by intraperitoneal injections with streptozotocin. Significantly higher levels of blood glucose and glycosylated hemoglobin; elevated levels of serum hepatic marker enzymes; lower levels of the antioxidative enzymes; and raised levels of malondialdehyde were seen. Hyperglycemia also affected the liver and kidney tissues, as seen under both light and transmission electron microscope in diabetic mice. Administration of intraperitoneal injections of naringenin to the diabetic mice for a period of 28 days prevented oxidative stress and lowered blood glucose and glycosylated hemoglobin including the serum hepatic marker enzymes and lipid peroxidation. Acute toxicity studies revealed naringenin to be less toxic. Further, histopathological and electron microscopy studies revealed the antioxidative nature of naringenin. These results suggested the positive effects of naringenin in mice, proving its further use in the management of diabetic hepatopathy and nephropathy.

Keywords: Diabetes, Naringenin, Streptozotocin, Acute toxicity, Oxidative stress.

INTRODUCTION

Diabetes occurs when the body is not able to produce enough insulin or is unable to use insulin.^[1] International Diabetes Federation estimates 415 million people worldwide, or 8.8% of adults aged 20-79 with diabetes, which is set to escalate as high as 642 million people by 2040.^[2] Oxidative stress is found out to be a major cause for the development of diabetes.^[3] The progression of diabetes has been associated with the increase in the production of free radicals^[4] and leads to a

decrease in the ability of defense by various antioxidants.^[5] Many oral antidiabetic drugs are available but these can have side effects, with the most common ones being hypoglycemia, diarrhea, weight gain, and vomiting, and the rare ones being severe hypoglycemia, edema, and heart failure.^[6] Therefore renewed attention towards natural medicines. Flavonoids are naturally occurring compounds in fruits, vegetables, nuts, herbs, cocoa, and grain seeds, which have shown to effectively scavenge free radicals.^[7] Naringenin is one such flavonoid

(belonging to the class flavonones) found in tomatoes and citrus fruits, especially grapefruit and grapes, that has potential antioxidative, [8] prevents hyperinsulinemia, [9] regulates hypertension. [10] However, in-depth studies with liver and kidneys are inadequate, and hence in the present study we have tried to find the antihyperglycemic and antioxidative role of naringenin on liver and kidneys in streptozotocin (STZ)-induced Swiss albino mice.

MATERIALS AND METHODS

2.1 Chemicals

(\pm)Naringenin (95%), streptozotocin, metformin, 1-chloro-2,4,dinitro benzene (CDNB), pyrogallol, and 2,2-diphenyl-1-picryl hydrazyl (DPPH) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA); L-ascorbic acid was purchased from Sisco Research Laboratory Pvt. Ltd. (SRL), India. Glycosylated hemoglobin kit obtained from Medsource Ozone Biomedicals Pvt. Ltd. (Faridabad, India). STZ was stored at 2–4 °C and naringenin at 4–8 °C protected from direct sunlight. All other chemicals were procured from Merck Co. (Mumbai, India), Sisco Research Laboratories (Mumbai, India), HiMedia (Mumbai, India), and local standard suppliers and were of analytical grade.

2.2 Experimental Animals

Male Swiss albino mice (Balb/C strain), weighing 25–30 g were used in the study. All the procedures were reviewed and carried out in accordance with the Institutional Ethics Committee (animal models), North-Eastern Hill University, Shillong, India, guidelines. Mice were housed in polypropylene cages and maintained at a temperature of 25±2 °C with a 12-hour day-night cycle. They were provided a standard laboratory feed obtained from Amrut Laboratory, Pune, India, and water was fed *ad libitum*.

2.3 Experimental induction of diabetes mellitus

Prior to injections, mice were fasted for a period of 6 h. Diabetes was then induced by a single high dose of

intraperitoneal injection of STZ [120 mg/kg body weight (b.w.)] dissolved in 0.1 M citrate buffer (pH 4.5), [11] followed by another 1ml of 5% glucose after half an hour to prevent the sudden shock of initial hypoglycemia because of marked release of insulin from pancreas due to STZ toxicity. [12] After 72 h, fasting blood glucose levels were checked with SD Check glucometer (SD Biosensor Inc., South Korea). Mice with blood glucose concentration of and above 200 mg/dl were selected and used for the experiments.

2.4 Experimental design

The mice were divided into the following groups with each group comprising a total of six mice each:

Group A: Normal mice that received only citrate buffer [10 ml/kg body weight (b.w.) with pH 4.5]

Group B: Diabetic mice induced by STZ injections intraperitoneally

Group C: Diabetic mice treated with naringenin in 0.5% carboxymethyl cellulose intraperitoneally every alternate day for a period of 28 days

Group D: Diabetic mice treated with L-ascorbic acid (50 mg/kg b.w.) intraperitoneally every alternate day for a period of 28 days

Naringenin concentration was kept at 50 mg/kg b.w., as many studies had taken this as the standard concentration. [13,14] At the end of the experimental period, the mice were fasted for 6 h. Prior to sacrifice by cervical dislocation, blood samples were collected from each mice from the retro-orbital sinus under anesthesia. [15] Liver and kidney tissues were excised and washed with homogenization buffer (pH 7.4) to remove excess blood and debris, and were stored at -80 °C until further analysis.

2.5 Preparation of the liver and kidney tissues

The method for homogenization was adopted from John M. Graham. [16] Liver and kidney tissues were weighed and thawed at 4 °C with the homogenization medium (pH 7.4) containing 0.25 M sucrose as an osmotic balancer, 1 m

Methylenediaminetetraacetic acid (EDTA), and 10 mM HEPES-NaOH. The obtained homogenates were then centrifuged to obtain mitochondrial and cytosolic parts, respectively. Total protein content for the determination of the specific activities of the enzymes was determined by the Bradford method [17] using bovine serum albumin (BSA) as the standard.

2.6 Acute toxicity studies

Organisation for Economic Co-operation and Development (OECD) guidelines were followed for the toxicity studies. [18] Female mice were used for the study because females were generally more sensitive toward lethal dose 50% (LD₅₀) studies. [19] Six groups were made containing six female mice in each group. Following limit test procedure, [18] increasing concentration of naringenin was intraperitoneally injected as listed below:

Group A: Control mice with 0.5% carboxymethyl cellulose

Group B: Naringenin dose 400 mg/kg b.w.

Group C: Naringenin dose 800 mg/kg b.w.

Group D: Naringenin dose 1,200 mg/kg b.w.

Group E: Naringenin dose 1,600 mg/kg b.w.

Group F: Naringenin dose 2,000 mg/kg b.w. Food and water was provided *ad libitum*. Animals in all groups were observed for 0 h, 2 h, 4 h, 6 h, 8 h, and 24 h. LD₅₀ value was determined by following the arithmetic method described by Ghosh. [20]

$$LD_{50} = \frac{\text{Maximum dose (100% dead)}}{\frac{\text{Product } (a \times b)}{\text{Number of animals in each group}}},$$

where a is the dose difference and b is the mean mortality

2.7 Biochemical studies

2.7.1 Blood glucose and glycosylated hemoglobin determination

Blood glucose was determined by the glucose oxidase-peroxidase (GOD-POD) method using Coral diagnostic kit (Goa, India). Glycosylated hemoglobin was assayed in the blood by the cation exchange method using commercial diagnostic kit

(Medsource Ozone Biomedicals Pvt. Ltd., Faridabad, India). Four groups of mice (six mice in each group) were selected and doses were intraperitoneally injected on every alternate day for a period of 28 days.

Group A: Control mice administered with citrate buffer, pH 4.5

Group B: STZ induced diabetic untreated mice

Group C: Diabetic mice administered with 50 mg/kg b.w. naringenin

Group D: Diabetic mice administered with 50 mg/kg b.w. metformin.

Metformin was administered in mice as described by Zhang. [21] Mice were starved overnight and blood was collected from retro-orbital plexus under anesthesia.

2.7.2 Serum hepatic marker enzymes determination

Activities of serum glutamate-oxalate-transaminase (SGOT) and serum glutamate-pyruvate-transaminase (SGPT) were determined by modified International Federation of Clinical Chemistry (IFCC) method and alkaline phosphatase (ALP) was determined using the para-nitrophenylphosphate (pNPP) method. Blood was collected from retro-orbital sinus. All the marker enzymes were determined using Coral diagnostic kit (Coral Clinical Systems, Goa, India) and mean activities were expressed in terms of units/liter (U/L).

2.7.3 Antioxidant activity determination in liver and kidney tissue

Activity of superoxide dismutase (SOD) both Mn-SOD and Cu/Zn-SOD was assayed by the method of Marklund and Marklund. [22] The change in absorbance was read at 420 nm against the blank on a spectrophotometer and was expressed as units/milligram/min protein.

Catalase (CAT) activity was assayed by the method of Hugo Aebi. [23] In this assay, the rate of decomposition of hydrogen peroxide (H₂O₂) was measured at an absorbance of 240 nm and expressed as units/milligram/min protein.

peroxidase (Gpx) activity was determined by the method of Rotruk et al.

[24] In this method, the rate of glutathione oxidation by H₂O₂ as catalyzed by the Gpx present in the supernatant is determined. The color that develops is read against the reagent blank at 412 nm and the activity of Gpx was expressed in microgram of reduced glutathione (GSH) consumed/min/mg protein.

The activity of glutathione-S-transferase (GST) was assayed by 1-chloro-2, 4, dinitro benzene (CDNB) method. [25] The conjugation of GSH with 1 chloro, 2-4 dinitrobenzene (CDNB), a hydrophilic substrate, was observed spectrophotometrically at 340 nm and was expressed as micromoles of CDNB formed/min/mg protein.

2.7.5 In vitro determination of antioxidant activities

DPPH (2,2-diphenyl-1-picryl hydrazyl) assay was evaluated by the method of McCune & Johns. [26] The progress of the reaction was followed on the Cary 50 Bio UV–Vis spectrophotometer at 517 nm. Sample concentration providing 50% of inhibition (IC₅₀) was calculated from the graph. Ascorbic acid was used as standard.

Following formula was used:

$$\text{Inhibition of DPPH radical (\%)} = \frac{C - T}{C} \times 100$$

where C = absorbance of control and T = absorbance of test.

The ferric reducing antioxidant power (FRAP) assay was performed according to the method of Benzie and Strain. [27] Absorbance was taken at every 15 sec interval until 4 minutes. The test was completed in triplicate and vitamin C (ascorbic acid) was used as standard antioxidant.

2.7.6 Histological studies

After 28-day treatment, both the liver and kidneys were excised after sacrifice by cervical dislocation. Conventional techniques of hematoxylin-

eosin (HE) staining were used for histological studies. [28] Briefly, sections (4–5 µm thick) were cut from the tissue-embedded paraffin blocks using a rotary microtome (Leica, Germany) and stained with hematoxylin followed by eosin. This was then examined by bright field microscopy [Olympus BX51].

2.7.7 Transmission electron microscopy (TEM)

Liver and kidney tissues were excised from the mice after sacrifice and washed repeatedly with phosphate buffer, pH 7.4. Ultra-thin sections (50–60 nm) were generated with a Reichert Ultracut S microtome and examined with a JEM-100 CX II transmission electron microscope. Images were collected with a CCD camera system.

2.7.8 Statistical analysis

All the results were expressed as mean ± S.E.M. of the observation made on six mice in each group. Data were analyzed using one-way analysis of variance (ANOVA) using the statistical package IBM SPSS Software package for Windows (Version 19.0). Post-hoc testing was assessed with Tukey's multiple range test. P<0.05, P<0.01, and P<0.001 were regarded as statistically significant depending on the experiment performed.

RESULTS

3.1 Acute toxicity studies

The mean mortality (b) was found by taking the number of dead animals in the given interval and adding it with the number of dead animals in the upper interval and dividing the result by 2. The mean mortality (b) in each group of animals was multiplied with the differences between doses for the same interval and the product was noted. The sum of the product was divided by the number of animals in a group and the resultant quotient was subtracted from the dose which killed all the animals (least lethal dose) in the group to calculate LD₅₀.

Table 1: Arithmetic method of determination of LD₅₀ of Naringenin. n = number of animals in each group; LD₅₀ = 2,000 -(5,600/6) = 600 mg/kg body weight

Groups	Dose (mg/kg)	Number of dead animals (n=6)	Dose difference (a)	Mean mortality (b)	Product (aXb)
1	400	0	0	0	0
2	800	2	400	1	400
3	1200	4	400	3	1200
4	1600	5	400	4.5	1800
5	2000	6	400	5.5	2200
				TOTAL	5600

LD₅₀ values of naringenin was 600 mg/kg body weight. 0% mortality was found at doses 100 mg/kg body weight and also 100% mortality was found in the dose of 2,000 mg/kg body weight.

3.2 Biochemical studies

3.2.1 Blood glucose and glycosylated hemoglobin determination

In table 2, diabetic untreated (Group B) mice showed significantly ($P<0.001$) higher mean levels of blood glucose and

glycosylated hemoglobin when compared with normal untreated (Group A) mice. In contrast, mice treated with naringenin and metformin exhibited significantly lower mean levels ($P<0.001$) of blood glucose and glycosylated hemoglobin when compared with diabetic untreated (Group B) mice. No significant differences were noted between the values obtained in naringenin-treated (Group C) mice and those in metformin treated (Group D) mice.

Table 2: Mean blood glucose levels and glycosylated hemoglobin percentages in Swiss albino mice. Values represented in mean \pm SEM, n = 6.

Groups	Mean blood glucose [mg/dl]	Glycosylated hemoglobin [% Hb]
Group A [Normal untreated]	93.85 \pm 4.01 ^b	7.071 \pm 0.271 ^b
Group B [Diabetic untreated]	469.50 \pm 10.97 ^a	15.048 \pm 0.525 ^a
Group C [Diabetic + naringenin-treated]	121.50 \pm 7.06 ^b	9.408 \pm 0.423 ^{a,b}
Group D [Diabetic + vitamin C-treated]	118.83 \pm 5.40 ^b	8.575 \pm 0.320 ^b

^a Statistically significant difference ($P<0.001$) when compared with Group A values.

^b Statistically significant difference ($P<0.001$) when compared with Group B values.

^c Statistically significant difference ($P<0.01$) when compared with Group D values.

3.2.1 Serum hepatic marker enzymes determination

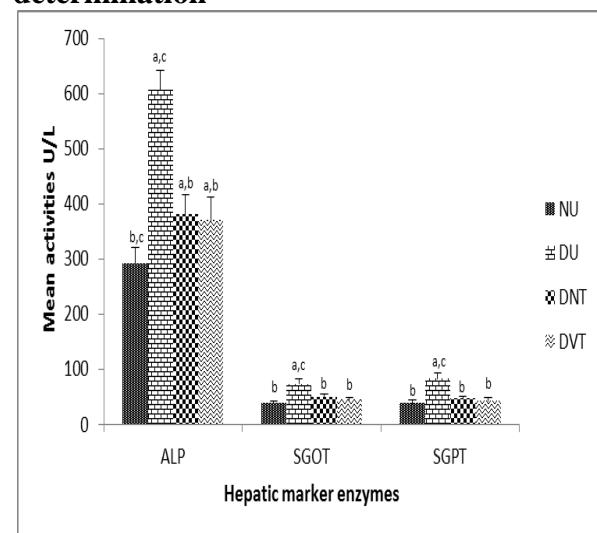


Figure 1: Mean activities of hepatic marker enzymes in serum of Swiss albino mice. Values represented in mean \pm SEM, n=6. The enzyme activities are expressed as units/liter. NU – normal untreated, DU – diabetic untreated, DNT – diabetic naringenin treated, and DVT – diabetic vitamin C treated.

^a Statistically significant difference ($P<0.01$) when compared with Group A values.

^b Statistically significant difference ($P<0.01$) when compared with Group B values.

The mean activities of serum GOT, GPT, and ALP were found to be significantly higher ($P<0.001$) in DU group mice than in NU group mice. While mice in DNT- and DVT-treated groups exhibited significantly lower levels ($P<0.001$) of these enzymes than DU mice, the mean activities of ALP were still significantly higher ($P<0.01$) than NU mice, although in case of SGOT and SGPT, the mean activities were not found to be statistically significant.

3.2.2 Enzymatic antioxidants determination

3.2.2.1 Antioxidative enzyme activities in liver

Table 3 shows that significantly lower ($P<0.001$) mean activities of these enzymatic antioxidants were observed in liver tissue of diabetic untreated (Group B) mice than those in normal untreated (Group A) mice. Conversely, significantly higher

($P<0.001$) mean activities of these enzymes were observed in diabetic mice treated with naringenin (Group C) and vitamin C (Group D) than those in diabetic untreated (Group B) mice. Remarkably, there was no significant difference between naringenin-

treated (Group C) and vitamin C-treated (Group D) mice. However, the mean values of all the enzymes were significantly lower than those of untreated normal mice, and naringenin- and vitamin C-treated mice.

Table 3: Mean activities of superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and glutathione-s-transferase (GST) in liver of control and experimental group of Swiss albino mice.

Groups	Superoxide dismutase	Catalase (CAT)	Glutathione peroxidase (GPx)	Glutathione-s-transferase (GST)
	Cu/Zn-SOD	Mn-SOD		
Group A [Normal untreated]	0.089 \pm 0.002 ^b	0.100 \pm 0.005 ^b	0.112 \pm 0.005 ^b	5.173 \pm 0.380 ^b
Group B [Diabetic untreated]	0.044 \pm 0.002 ^a	0.054 \pm 0.003 ^a	0.058 \pm 0.002 ^a	1.839 \pm 0.220 ^a
Group C [Diabetic + naringenin-treated]	0.078 \pm 0.004 ^b	0.090 \pm 0.002 ^b	0.089 \pm 0.001 ^{a,b}	4.673 \pm 0.306 ^b
Group D [Diabetic + vitamin C treated]	0.083 \pm 0.003 ^b	0.094 \pm 0.001 ^b	0.093 \pm 0.001 ^{a,b}	4.839 \pm 0.235 ^b

Values represented in mean \pm SEM, n=6. The enzyme activities are expressed as: SOD and catalase – units/milligram protein/min, glutathione peroxidase – micromoles of reduced glutathione consumed/min/mg protein, glutathione-s-transferase – micromoles of 1-chloro,2,4 dinitrobenzene formed/min/mg protein.

^a Statistically significant difference ($P<0.001$) when compared with Group A values.

^b Statistically significant difference ($P<0.001$) when compared with Group B values.

3.2.2.2 Antioxidative enzyme activities in kidney

Table 4: Mean activities of superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and glutathione-s-transferase (GST) in liver of control and experimental group of Swiss albino mice.

Groups	Superoxide dismutase	Catalase (CAT)	Glutathione peroxidase (GPx)	Glutathione-s-transferase (GST)
	Cu/Zn-SOD	Mn-SOD		
Group A [Normal untreated]	0.073 \pm 0.002 ^b	0.081 \pm 0.002 ^b	0.096 \pm 0.002 ^b	4.390 \pm 0.351 ^b
Group B [Diabetic untreated]	0.043 \pm 0.002 ^a	0.041 \pm 0.002 ^a	0.047 \pm 0.001 ^b	1.273 \pm 0.187 ^b
Group C [Diabetic + naringenin-treated]	0.066 \pm 0.003 ^b	0.076 \pm 0.003 ^b	0.074 \pm 0.001 ^{a,b}	3.890 \pm 0.334 ^b
Group D [Diabetic + vitamin C treated]	0.069 \pm 0.001 ^b	0.077 \pm 0.003 ^b	0.078 \pm 0.001 ^{a,b}	4.056 \pm 0.289 ^b

Values represented in mean \pm SEM, n=6. The enzyme activities are expressed as: SOD and catalase – units/milligram protein/min, glutathione peroxidase – micromoles of reduced glutathione consumed/min/mg protein, glutathione-s-transferase – micromoles of 1-chloro,2,4 dinitrobenzene formed/min/mg protein.

^a Statistically significant difference ($P<0.001$) when compared with Group A values.

^b Statistically significant difference ($P<0.001$) when compared with Group B values.

Table 4 shows that significantly ($P<0.001$) lower mean activities of these enzymatic antioxidants were observed in kidney tissue of diabetic untreated (Group B) mice than those in normal untreated (Group A) mice. Conversely, significantly higher ($P<0.001$) mean activities of these enzymes were observed in diabetic mice treated with naringenin (Group C) and vitamin C (Group D) than those in diabetic untreated (Group B) mice. Remarkably, there was no significant difference between naringenin treated (Group C) and vitamin C treated (Group D) mice. However, the mean values of all the enzymes were significantly

lower than those of untreated normal mice, and naringenin- and vitamin C-treated mice.

3.2.3 Malondialdehyde (MDA) concentrations in liver and kidney tissues of Swiss albino mice

Figure 2 depicts that the mean levels of malondialdehyde (MDA) in liver and kidney tissues of diabetic untreated (Group B) mice was significantly higher ($P<0.001$) than in normal untreated (Group A) mice. Remarkably, while the mean levels of liver and kidney tissue MDA in diabetic mice treated with naringenin (Group C) and in those treated with vitamin C (Group D) were significantly lower ($P<0.001$) than in

diabetic untreated (Group B) mice, these levels were not significantly different to those in normal untreated (Group A) mice. There was no significant difference between the mean values in Group C and Group D mice.

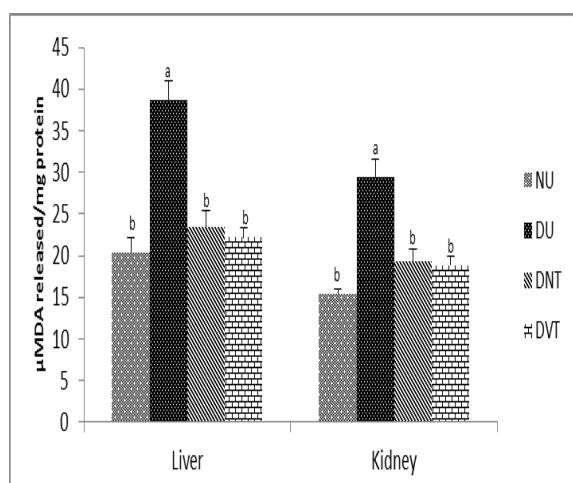


Figure 2: Mean levels of MDA in liver and kidney tissues of Swiss albino mice. Values represent the mean \pm SEM, n=6. NU – normal untreated, DU – diabetic untreated, DNT – diabetic naringenin treated, and DVT – diabetic vitamin C treated. Statistical analysis: one-way analysis of variance [ANOVA] with post-hoc testing.

^aStatistically significant difference ($P<0.001$) when compared with NU values.

^bStatistically significant difference ($P<0.001$) when compared with DU values.

3.2.4 In-vitro activity of antioxidative enzymes in Swiss albino mice

3.2.4.1 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

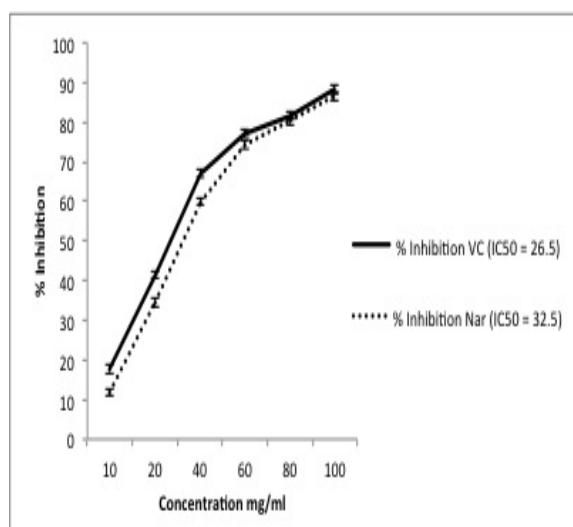


Figure 3: Concentration vs. % Inhibition of naringenin and vitamin C (ascorbic acid). Each point obtained from three experiments performed in triplicates (mean \pm SEM).

The radical scavenging activity of naringenin and vitamin C was calculated at 517 nm as shown in Figure 3. From the inhibition curve, the IC₅₀ values (mean \pm SEM) were found out to be 26.5 ± 1.51 μ g/ml for vitamin C and 32.5 ± 1.05 μ g/ml for naringenin.

3.2.4.2 Ferric reducing antioxidant power (FRAP) assay:

The trend for ferric ion reducing activities of naringenin and ascorbic acid is shown in Figure 4. Values are expressed in mean \pm SEM. Each value represents the mean of three replications.

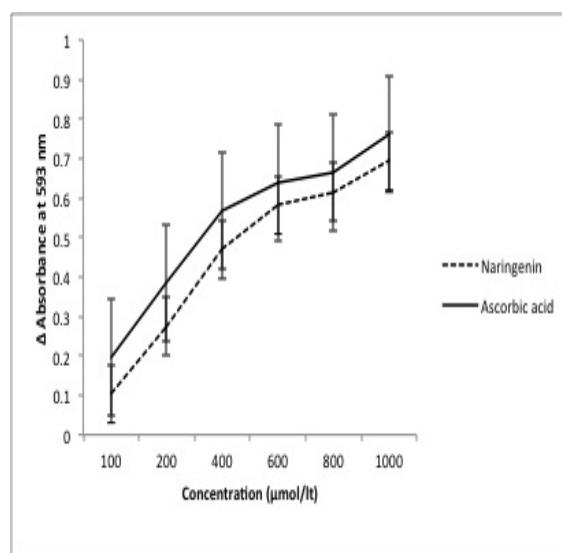


Figure 4: Concentration vs. Absorbance change (ΔA) of naringenin and ascorbic acid (vitamin C).

3.2.5 Histopathological examination of tissues in Swiss albino mice

3.2.5.1 Histopathological examination of liver tissue in Swiss albino mice

The histoarchitecture of the liver tissue in all the four groups of Swiss albino mice is shown in Figure 5. Normal hepatocytes and normal central vein were seen in the liver tissue of normal untreated (NU) mice. Glycogenated nucleus, sinusoidal enlargement, fatty vacuole in small numbers, fewer hepatocytes and distended central vein with mild red blood cells congestion were observed in diabetic untreated (DU) mice. Interestingly, regeneration of hepatocytes with reduction in vacuolation, mild sinusoidal dilation, and

near normal hepatocytes was observed in both naringenin-treated (DNT) and vitamin

C (mild RBC congestion observed)-treated (DVT) mice.

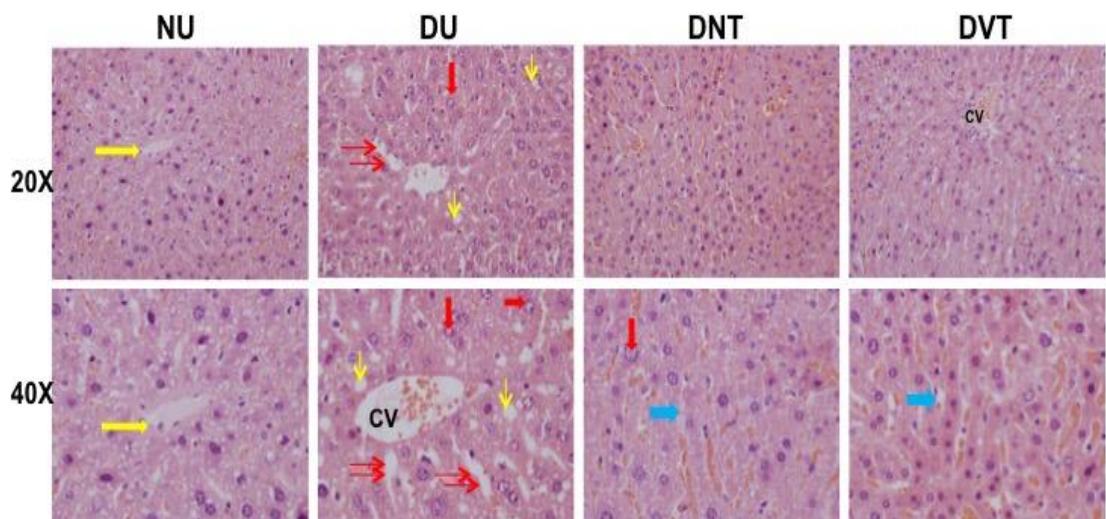


Figure 5: Histoarchitecture of liver tissues of Swiss albino mice. Hematoxylin and eosin (H&E) staining with 20X and 40 X magnification.

3.2.5.2 Histopathological examination of kidney tissue of Swiss albino mice

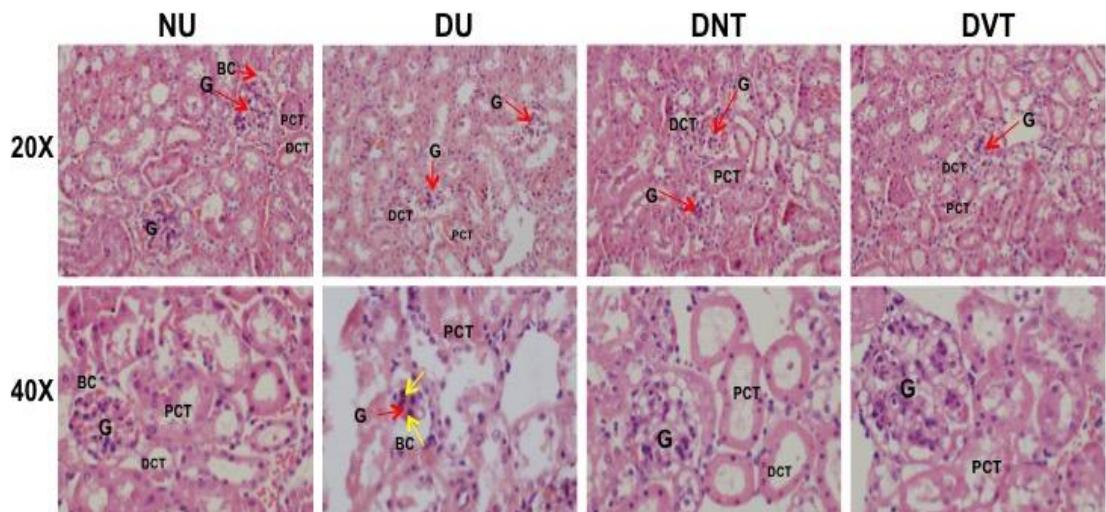


Figure 6: Histoarchitecture of kidney tissues of Swiss albino mice. Hematoxylin and eosin (H&E) staining with 20X and 40X magnification.

The histoarchitecture of the kidney tissue in all the four groups of Swiss albino mice is shown in Figure 6. Normal kidney tissue with average size glomerulus (G), normal Bowman's capsule (BC), proximal convoluted tubules (PCT), distal convoluted tubules (DCT), and no pathological abnormalities was observed in case of normal untreated (NU) mice. Hepatocellular cloudiness and swelling, inflammation in DCT, shrinkage of glomerulus, tubules expansion due to edema and segmental glomerular changes such as thickening of

the basement membrane, deposits in mesangium (thin yellow arrow), and hypercellularity were seen in diabetic untreated (DU) mice. Interestingly, restoration of near normal glomerular architecture but slightly bigger in size, normal basement membrane, and capillaries without any inflammatory cells were observed in both naringenin-treated (DNT) and vitamin C-treated (DVT) mice. Edema was also not observed in DNT and DVT mice.

3.2.6 Transmission electron micrographs of liver and kidney tissues

3.2.6.1 TEM of liver tissue

The electron micrographs of all the four groups of liver tissues of Swiss albino mice glomerular are shown in Figure 7. Normal mitochondria (M) and nucleus (Nu) with round or oval shaped hepatocytes in regular and symmetrical double membraned (thin arrows) structure, symmetrical rough endoplasmic reticulum with intact ribosomes (arrowheads) and presence of lipid droplets (L) with presence of granular matrix. Interestingly, the treated mice, diabetic with naringenin treated (DNT) and diabetic with vitamin C treated (DVT), showed only few swollen and disrupted mitochondria (*) and numerous recovered normal mitochondria (M), near normal (oval to round) nucleus (Nu) with some areas displaying uneven nuclear membrane (thin arrows), less dilation of endoplasmic reticulum compared with DU although still without any ribosomes (arrowheads), lipid droplets (L), and presence of granular matrix.

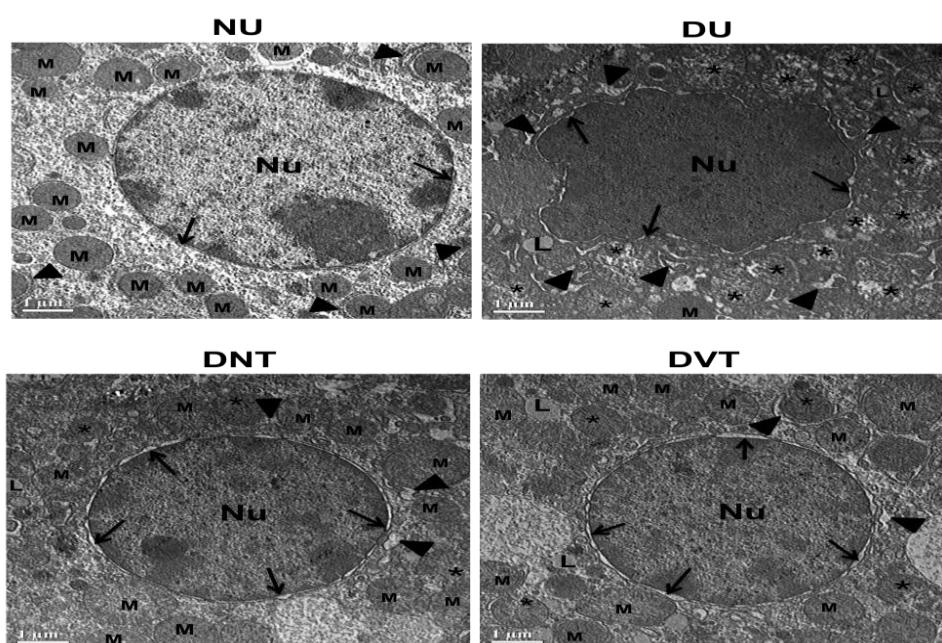


Figure 7: Representative photograph of electron microscopy showing the changes in hepatic cells in a section of liver tissue in Swiss albino mice. Magnification: 2,000 X.

3.2.6.2 TEM of kidney tissue

The electron micrographs of all the four groups of kidney tissues of Swiss albino mice glomerular urinary filtration barrier is shown in Figure 8. Normal foot processes (FP), podocytes (P), symmetrical glomerular basement membrane (BM), glomerular capillaries (GC), and slit diaphragm (arrowheads) were observed in normal untreated (NU) mice. Contrarily,

elongated and abnormal foot processes (*), abnormal thickening of the glomerular basement membrane (BM) and accumulation of debris in slit diaphragm, leading to a decrease in the number of slit pores (arrowheads) and alterations in podocytes integrity were observed in diabetic untreated (DU) mice. Remarkably, diabetic with naringenin treated (DNT) and diabetic with vitamin C treated (DVT) mice

showed some normal foot processes (FP) and some effaced (thick arrows) foot processes, segmental thickening of

glomerular basement membrane (BM), normal slit diaphragm (arrowheads), and clear area of glomerular capillary (GC).

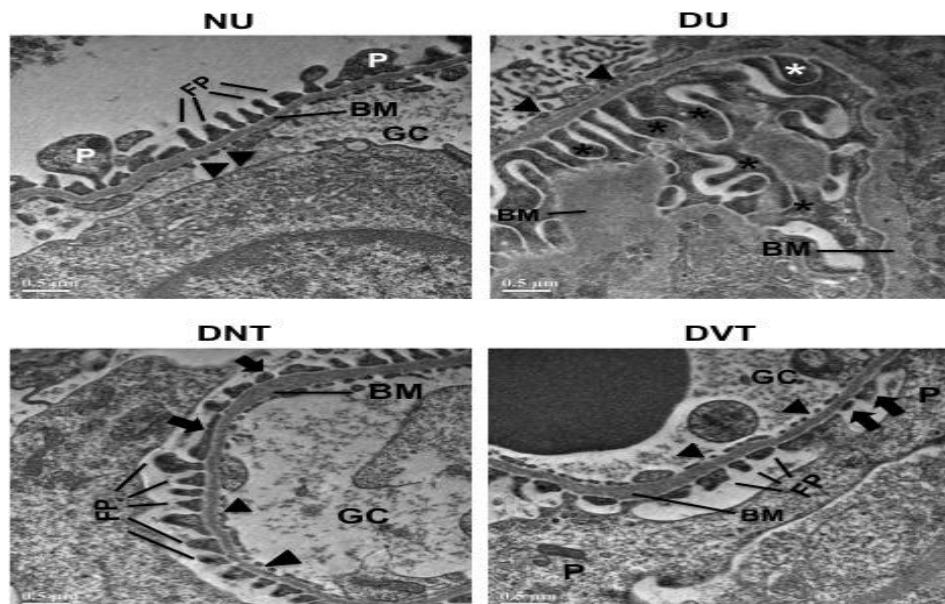


Figure 8: Representative photograph of electron microscopy showing the glomerular urinary filtration barrier in kidney tissue of Swiss albino mice. Magnification: 4,000X.

DISCUSSIONS AND CONCLUSION

The present investigation validated the antioxidative, antihyperglycemic, and protective role of naringenin in liver and kidney tissues against oxidative stress in STZ-induced diabetic damage. It has been well documented that STZ, a β -cytotoxic drug, is used for the induction of diabetes, [29] which causes the destruction of the β cells of the pancreas. [30] Hence, STZ was used in the present study.

Acute toxicity is the ability of the chemical to cause ill effect or death in a period of minutes, hours (24), or days (up to 2 weeks) after one oral or intraperitoneal administration. [31] Lethal dose 50 (LD₅₀) or median lethal dose is the statistically derived single dose of a substance that produces death in 50% of a population of test animals in which it is administered by any of the methods like oral, dermal, inhalation, intraperitoneal, or intravenous routes. [32] The acute toxicity study showed LD₅₀ value of naringenin to be 600 mg/kg body weight (Table 1). Thus, intraperitoneal administration of naringenin in mice is safe and very less toxic.

In this study, extremely high blood glucose levels were noted in diabetic untreated mice that after naringenin treatment decreased drastically (Table 2). Naringenin could possibly either stimulate residual pancreatic β cells to produce insulin [33] or the remaining hepatic tissues to increase the expression of insulin receptors in the remaining liver plasma membranes, [34] thus exerting antihyperglycemic action in diabetic mice.

In diabetes, glycated hemoglobin concentration serves as an indicator of average blood glucose concentration over a period of three months and is used as a diagnostic or screening tool for diabetes. [35] The decrease in hemoglobin in Table 2 may have been as a result of reduced blood glucose and stimulation of remaining pancreatic and hepatic tissues to increase insulin, [34] which then leads to decrease in the percentage of glycosylated hemoglobin. Also studies have shown that flavonoids did reduce the percentages of glycosylated hemoglobin due to its antioxidative nature hence inhibiting the glycosylation of hemoglobin. [36]

The liver plays a vital role in upholding the normal glucose levels during fasting as well as in the postprandial period. [37] Serum aminotransferases and acid phosphatases are elevated when there is damage to the liver, [38] which can be used in scrutinizing the progress during diabetic condition in mice. In the present study, naringenin-treated mice showed lower serum levels than diabetic mice (Fig. 1). This may be due to protective action of naringenin on liver tissues from damage by oxidative stress that leads to increase in insulin action.

SOD is considered a first-line defense against reactive oxygen species. Manganese SOD present and (Cu/Zn)-SOD present in mitochondria and cytosol respectively eradicate superoxide to hydrogen peroxide (H_2O_2). [39] The H_2O_2 is converted to water and oxygen by catalase. [40] Gpx, also does the similar work of detoxifying H_2O_2 to H_2O and O_2 in mitochondria. GST protects cells from reactive oxygen species and toxic substrates. [41] In this study, treatment with naringenin elevated the activities of antioxidants enzymes near normal levels. Studies have proved that, antioxidants reduces owing to overproduction of reactive oxygen species due to hyperglycaemia [42] and supplements of antioxidants does reduce ROS, and increase antioxidant enzymes and prevented diabetes mellitus. [43] Naringenin have showed to restore the levels of antioxidative enzymes in other study. [44] Naringenin therefore protects the liver and kidney cells from the oxidative stress due to its strong antioxidative nature.

Lipid peroxidation gives rise to a series of chain reaction and propagates the chain reaction leading to cell and cell membrane damage. [45] MDA has been used as a standard marker for tissue damage in lipid peroxidation studies. [46] In the present study, there is a rise in the level of MDA in diabetic mice brought it down significantly by naringenin. Naringenin reduces the level of MDA possibly by functioning as a free radical scavenger as it possess large

amounts of flavonoids [47] thereby decreasing the formation of free radicals and hence lipid peroxidation.

DPPH is a stable free radical and can easily accept an electron or hydrogen radical to become a stable diamagnetic molecule. [48] Naringenin showed a remarkable DPPH scavenging activity compared with the value obtained for vitamin C (Fig. 3). In FRAP assay, reduction of ferric – tripyridyltriazine to the ferrous complex can be measured. In both the cases, the absorbance increases with increasing concentration. [49] The ΔA of naringenin clearly increased, due to the formation of the Fe^{2+} -TPTZ complex with increasing concentration (Fig. 4).

The present study demonstrates that following treatment with naringenin, liver and kidney cells stained with H&E and observed under TEM displayed distinctly near normal architecture when compared to DU (Fig. 5, 6, 7 & 8). These suggest that naringenin may counteract the damage in liver and kidney tissues from free radical-mediated oxidative stress and restore the antioxidative enzymes. Polyphenols have shown to elevate the antioxidative enzymes in the liver hence enhancing their function [49] and when naringenin with Cd was administered to kidney tissues; it did decrease the lipid peroxidation level. [8]

CONCLUSION

In conclusion, naringenin clearly shows very less toxicity, antioxidative potential by enhancing the levels of antioxidative enzymes and protecting the liver and kidney tissues from oxidative stress. It also lowers and maintains the blood glucose and glycosylated hemoglobin levels showing its antihyperglycemic property.

ACKNOWLEDGEMENT

This work was supported by University Grants Commission, New Delhi, Government of India, for providing financial assistance under No. F. 4-9/ 2015 / DRS III (SAP-II) for which we are very thankful.

Declaration of interest: The authors report no declarations of interest.

REFERENCES

1. Alberti KGMM, DeFronzo RA, Zimmet P, (Eds.). International Textbook of Diabetes Mellitus. 2nd edition. Chichester, New York: John Wiley & Sons; 1997.
2. IDF Diabetes Atlas Seventh Edition. International Diabetes Federation;2015
3. Ceriello A. Oxidative stress and glycemic regulation. *Metabolism*. 2000; 49 (2 Suppl 1), 27-29.
4. Paneni F, Beckman JA, Creager MA, Cosentino, F. Diabetes and vascular disease: pathophysiology, clinical consequences, and medical therapy: part I. *Eur. Heart J.* 2013; 34 (31), 2436-2443.
5. Saxena AK, Srivastava P, Kale RK, et al. Impaired antioxidant status in diabetic rat liver. Effect of vanadate. *Biochem.Pharmacol.* 1993;45 (3), 539-542.
6. Foster DW. Diabetes mellitus. In Harrison's Principles of Internal Medicine (Isselbacher KJ, Braunwald E, Wilson JD, Martin JB, Fauci AS, Kasper DL eds.). 14th edition. McGraw-Hill, Inc (Health Professions Division); 1998.
7. Arts IC, Hollman PC. Polyphenols and disease risk in epidemiologic studies. *Am. J. Clin. Nutr.* 2005; 81, 317S-325S.
8. Renugadevi J, Milton Prabu S. Naringenin protects against cadmium-induced oxidative renal dysfunction in rats. *Toxicology*. 2009; 256, 128-134.
9. Mulvihill EE, Allister EM, Sutherland BG, et al. Naringenin prevents dyslipidemia, apolipoprotein-B overproduction, and hyperinsulinemia in LDL receptor-null mice with diet-induced insulin resistance. *Diabetes*. 2009; 58, 2198-210.
10. Alam MA, Kauter K, Brown L. Naringenin improves diet-induced cardiovascular dysfunction and obesity in high carbohydrate, high fat diet-fed rats. *Nutrients*. 2013; 5, 637-650.
11. Like AA, Rossini AA. Streptozotocin induced pancreatic insulitis: new model of diabetes mellitus. *Science*. 1976; 193, 415-417.
12. Kenneth K Wu, Youming H. Streptozotocin-induced diabetic models in mice and rats. *Curr.Protoc. Pharmacol.* 2008; 5.47.1-5.47.14.
13. Kannappan S, Anuradha CV. Naringenin enhances insulin-stimulated tyrosine phosphorylation and improves the cellular actions of insulin in a dietary model of metabolic syndrome. *Eur. J. Nutr.* 2010; 49 (2), 101-109.
14. Ortiz-Andrade RR, Sanchez-Salgado JC, Navarrete-Vazquez G, et al. Antidiabetic and toxicological evaluations of naringenin in normoglycaemic and NIDDM rat models and its implications on extra-pancreatic glucose regulation. *Diabetes Obes.Metab.* 2008; 10 (11), 1097-1104.
15. Ivorra MD, Paya M, Villar A, Hypoglycemic and insulin release effects of tormentic acid. A new hypoglycemic natural product. *Planta Med.* 1988; 54, 282-286.
16. John M Graham. Homogenization of mammalian tissues. *The Scientific World Journal* 2, 2002; 1626-1629.
17. Bradford MM. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. *Anal.Biochem.* 1976; 72, 248-254.
18. OECD Guidelines for Testing of Chemicals-425. Acute Oral Toxicity-Up-and-Down Procedure (adopted on 17th December 2001).
19. Lipnick RL, Cotruvo JA, Hill RN, et al. Comparison of the Up-and-Down, Conventiona LD₅₀ and Fixed Dose Acute Toxicity Procedures. *Food Chem. Toxicol.* 1995; 33, 223-231.
20. Ghosh MN. In Statistical Analysis, Fundamentals of Experimental Pharmacology. 2nd ed. Calcutta: Scientific Book Agency; 1984.
21. Zhang FX, Tan BKH. Effects of an ethanolic extract of *Gynuraprocurbens* on serum glucose, cholesterol and triglyceride levels in normal and Streptozotocin-induced diabetic rats. *Singapore Med. J.* 2000; 41, 1-7.
22. Marklund SL, Marklund G. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *Eur. J. Biochem.* 1974;47, 469-474.

23. Aebi H. Methods in enzymology. In: CatalaseIn Vitro, vol. 105. New York: Academy Press; 1984.
24. Rotruck JT, Pope AL, Ganther H.E. Selenium: biochemical role as a component of glutathione peroxidase. *Science*.1973; 179, 588-590.
25. Habig WH, Pabst MJ, Jakoby WB. Glutathione-S-transferases: the first enzymatic step in mercapturic acid formation. *J. Biol. Chem.* 1974;249, 7130-7139.
26. McCune LM, Johns T. Antioxidant activity in medicinal plants associated with the symptoms of diabetes mellitus used by the indigenous peoples of the North American boreal forest. *J. Ethnopharmacol.* 2002; 82 (2-3), 197-205.
27. Benzie IFF,Wai Y, Strain JJ. Antioxidant (reducing) efficiency of ascorbate in plasma is not affected by concentration. *J. Nutr. Biochem.*1999; 10, 146-150.
28. Kiernan JA. Histological and histochemical methods. Theory and practice. Oxford: Butterworth-Heinemann Publisher; 1999.
29. Ward DT, Yau SK, Mee AP et al. Functional, molecular, and biochemical characterization of streptozotocin-induced diabetes. *J. Am. Soc. Nephrol.* 2001;12 (4), 779–790.
30. Gupta S, Kataria M, Gupta PK, et al. Protective role of extracts of neem seeds in diabetes caused by streptozotocin in rats. *J. Ethnopharmacol.* 2004; 90, 185–189.
31. Senin R. Acute Toxicity Study [Internet]. 2006. Available from <<http://www.ccohs.ca/oshanswers/chem/casl/Ld50.html>>
32. Jaya Raj, MohineeshTiranth DDogra, Monika Pahuja, et al. Determination of median lethal dose of combination of endosulfan and cypermethrin in Wistar Rats. *Toxicol. Int.*2013; 20(Issue 1).
33. Annadurai T, Muralidharan AR, Joseph T, et al. Antihyperglycemic and antioxidant effects of a flavanone, naringenin, in streptozotocin-nicotinamide-induced experimental diabetic rats. *J. Physiol. Biochem.* 2012; 68:307-318.
34. Pinent M, Blay M, Bladé MC, et al. Grape seed-derived procyanidins have an anti-hyperglycemic effect in streptozotocin induced diabetic rats and insulinomimetic activity in insulin-sensitive cell lines. *Endocrinology*. 2004; 145, 4985-4990.
35. Alberti KGMM, Press CM. The biochemistry and complications of diabetes. In: Keen, H., Javve, J., (Eds.). *Complications of Diabetes*. London: Edward Arnold Publishers; 1982.
36. Manuel Y, Keenoy B, Vertommen J, et al. The effect of flavonoid treatment on the glycation and antioxidant status in type I diabetic patients. *Diabetes Nutr.Metab.*1999; 12, 256-263.
37. Duckworth WC, Hamel FG, Peavy DE. Hepatic metabolism of insulin. *Am. J. Med.* 1988; 85, 71-76.
38. Murray RK, Granner DK, Mayes PA, et al. Catabolism of proteins and of amino acid nitrogen. In: Harper's Biochemistry. 23rd ed. London: Prentice, Hall International Limited; 1993.
39. Evans JL, Goldfine ID, Maddux BA, et al. Are oxidative stress-activated signaling pathways mediators of insulin resistance and β -cell dysfunction? *Diabetes*.2003;52 (1), 1-8.
40. Johansen JS, Harris AK, Rychly DJ, et al. Oxidative stress and the use of antioxidants in diabetes: linking basic science to clinical practice. *Cardiovasc. Diabetol.*2005; 4 (1), 5.
41. Wu G, Fang YZ, Yang S, et al. Glutathione metabolism and its implications for health. *J. Nutr.* 2004; 134, 489-492.
42. Pimson C, Chatuphonprasert W, Jarukamjorn K. Improvement of antioxidant balance in diabetes mellitus type 1 mice by glutathione supplement. *Pak. J. Pharmaceut. Sci.* 2014; 27, 1731-1737.
43. Wang C, Li S, Shang DJ, Wang XL, et al. Antihyperglycemic and neuroprotective effects of one novel Cu-Zn SOD mimetic. *Bioorg. Med. Chem. Lett.* 2011; 21 (14), 4320–4324.
44. Renugadevi J, Prabu SM. Cadmium-induced hepatotoxicity in rats and the

- protective effect of naringenin. *Exp. Toxicol. Pathol.* 2010; 62, 171-181.
45. Gutteridge JM. Lipid peroxidation and antioxidants as biomarkers of tissue damage. *Clin. Chem.* 1995; 41, 1819-1828.
46. Shodehinde SA, Oboh G. Antioxidant properties of aqueous extracts of unripe *Musa paradisiaca* on sodium nitroprusside induced lipid peroxidation in rat pancreas *in vitro*. *Asian Pac J. Trop. Biomed.* 2013; 3 (6), 449-457.
47. Cavia-Saiz M, Bustos MD, Pilar-Izquierdo MC, et al. Antioxidant properties, radical scavenging activity and bio-molecule protection capacity of flavonoid naringenin and its glycoside naringin: a comparative study. *J. Sci. Food Agric.* 2010; 90, 1238-1244.
48. Soares JR, Dins TCP, Cunha AP, et al. Antioxidant activity of some extracts of *Thymus zygis*. *Free Radic. Res.* 1997; 26, 469-478.
49. Martín MÁ, Serrano AB, Ramos S, et al. Cocoa flavonoids up-regulate antioxidant enzyme activity via the ERK1/2 pathway to protect against oxidative stress-induced apoptosis in HepG2 cells. *J. Nutr. Biochem.* 2010; 21, 196-205.

How to cite this article: Sharma A, Patar AK, Bhan S. Cytoprotective, antihyperglycemic and antioxidative effect of naringenin on liver and kidneys of Swiss diabetic mice. *Int J Health Sci Res.* 2016; 6(11):118-131.
