Research Article

Nephroprotective effect of silymarin in hyperglycemia-induced oxidative stress in rats

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ABSTRACT

Background: Diabetes mellitus is a group of metabolic disorders characterized by hyperglycemia. Hyperglycemia is the etiological factor for oxidative stress-induced microvascular and macrovascular complications. Many animal experimental models and clinical trials have proved the antioxidant defense mechanism of flavonoids in ameliorating the progression of chronic diabetic complications. Hence, the objective of this study was to evaluate the nephroprotective effects of silymarin in alloxan induced Type I diabetes.

Methods: Male Wistar rats were divided into five groups of six each. Group I served as control. Group II, III, IV and V were diabetic rats. Group II diabetic rats received the vehicle. Groups III and IV were treated with 200 mg/kg and 400 mg/kg of silymarin, respectively. Group V was treated with glibenclamide (0.5 mg/kg). After 3 weeks, blood samples were collected from all the groups of animals to measure serum glucose, urea and creatinine. Lipid peroxidation study and histopathological study were conducted in the renal tissue to confirm the oxidative damage.

Results: The serum glucose, urea and creatinine significantly increased in untreated diabetic rats. In addition, there was a significant rise in lipid peroxidation with a glomerular atrophy and necrotic tubular epithelium in the renal tissue. The rise in serum glucose, urea and creatinine was ameliorated by silymarin. The renal tissue showed increased antioxidant levels, decreased lipid peroxides and only mild changes in glomeruli and tubules.

Conclusion: The results of this study indicate silymarin is an effective nutritional supplement to prevent complications of diabetes.

Keywords: Diabetes, Oxidative stress, Kidney, Silymarin

INTRODUCTION

Diabetes mellitus (DM) is a group of syndromes characterized by hyperglycemia, altered metabolism of carbohydrate, lipid and protein, resulting in microvascular and macrovascular complications.[1] DM is one of the most common non-communicable diseases. It is the fourth or fifth leading cause of death in most high-income countries. According to Diabetes Atlas published by International Diabetes Federation, there are 382 million people living with diabetes. By 2035, this will rise to 592 million. Diabetes caused 5.1 million deaths in 2013. Diabetes imposes a large economic burden on individuals, families, national health systems and countries. Type 2 DM accounts for 85-95% of all diabetes and Type 1 DM 5-10% of all diabetes. India has more diabetes than any other country in the world, although recent data suggest that China has even more.

The American Diabetes Association has proposed a generally accepted classification of DM into three Types: type 1A associated with beta cell destruction leading to absolute deficiency of insulin is immune mediated developing in childhood or early adulthood; Type 1B (idiopathic) is a non-immune form of DM frequently seen in minorities, especially Africans and Asians, with intermittent insulin requirements; Type 2 is associated with a relative insulin deficiency and insulin resistance; Type 3 diabetes is used to encompass diabetes caused by specific genetic defects (maturity-onset diabetes of the young), infections, other endocrine abnormalities and drug-induced diabetes.[2] Women who develop diabetes due to stress of pregnancy are classified as having gestational diabetes.

Hyperglycemia leads to increased production of reactive oxygen species (ROS). These free radicals are implicated in the complications of DM. Various animal studies have proved that hyperglycemia-induced oxidative stress causes a reduction in beta cell mass and impairment in its function.[3,4] Epidemiological studies, clinical trials and

animal experimental models have proved that dietary supplementation of antioxidants like vitamin E, vitamin C, etc., has reduced the incidence of oxidative damage related disorders such as ageing, cardiovascular diseases, diabetes, inflammation, and neurodegenerative disorder.[5] Ahmadvand reported that the coenzyme Q10 a natural antioxidant showed significant nephroprotective effect in diabetic rats compared with untreated diabetic animals.[6]

Flavonoids (more than 8000) constitute the largest and most important groups of polyphenolic compounds in fruits, vegetables, wine, tea and cocoa.[7] Recent attention has been focused on the potential use of flavonoids-based drugs for the prevention and treatment of oxidative stress-mediated diseases. Flavonoids can exert their antioxidant activity by various mechanisms, e.g., by scavenging or quenching free radicals, by chelating metal ions, or by inhibiting enzymatic systems responsible for free radical generation.

Silybum marianum L. (milk thistle), a member of *Carduus marianum* family, is an ancient medicinal plant which has been used for more than 2000 years for the treatment of liver and gall bladder disorders.[8] Silymarin is the active component of this herb that is a mixture of four flavonolignans namely silybin, isosilybin, silydianin and silychristin. Recently silymarin has received attention due to its other beneficial activities such as anticancer, chemoprotective, hypocholesterolemic, cardioprotective, neuroactive, neuroprotective and skin protective activities.[8] Besides the cytoprotective activity of silymarin mediated by its anti-oxidative and radical scavenging properties also new activities based on specific receptor interaction were discovered. These include stimulation of ribosomal RNA polymerase, and subsequent protein synthesis, leading to enhanced hepatocyte regeneration, pro-apoptotic and anti-angiogenic activity, inhibition of nuclear factor kappa B activity, inhibition of epidermal growth factor receptor – mitogen-activated protein kinase/extracellular regulated kinases ½ signaling, modulation of P-glycoproteins, etc., Few animal studies have proved the anti-diabetic activity of silymarin. Hence, the present study was done to explore the nephroprotective effect of silymarin against hyperglycemia-induced oxidative stress in diabetic rats.

METHODS

Study center

This study was undertaken at Central Animal House, Rajah Muthiah Medical College and Hospital, Annamalai University, Annamalai Nagar. All studies were conducted in accordance with the National Institute of Health "Guide for the care and use of Laboratory Animals" (NIH, 1985). The study was approved by the Animal Ethical Committee of Rajah Muthiah Medical College and Hospital (Registration No. 160/1999/[CPCSEA]) Annamalai University, Annamalai Nagar, Tamil Nadu, India (Proposal No.1017, dated 02-05-2013).

Materials

Chemicals and reagents

Silymarin (milk thistle) was purchased from Nature's Bounty Inc., Bohemia. NY 11716 U.S.A. Alloxan monohydrate (2,4,5,6-tetraoxypyrimidine-2,4,5,6-pyrimidinetetrone) was purchased from MP Biomedicals India Private Limited, Mumbai, Maharashtra. Biomedical and enzymatic kits for measuring lipid peroxidase and antioxidants enzymes were obtained from Sigma-Aldrich Chemicals and Mouli Enterprises. Blood glucose was determined using glucometer (strip test in one touch), obtained from Ramesh Surgical and Enterprises, Madurai. Serum urea and creatinine were determined by biochemical analyzer using commercial kits, obtained from Sigma-Aldrich Chemicals, Chennai.

Preparation of drug

Silymarin powder was dissolved in distilled water to make a solution of 50 mg/ml. Glibenclamide tablets were dissolved in distilled water to make a solution of 0.25 mg/ml.

Animals

Healthy adult male rats of Wistar strain weighing 230-250 g were used in the present study. They were purchased from the Central Animal House, Rajah Muthiah Medical College and Hospital, Annamalai University, Annamalai Nagar, Tamil Nadu, India. Animals were housed in polypropylene cages (28 cm × 22 cm × 14 cm) bedded with husk in groups of six under controlled environmental conditions (temperature - 23±2°C, humidity 65-70% and 12 hrs light/dark cycles) at Central Animal House, Rajah Muthiah Medical College and Hospital, Annamalai University. Animals were fed with standard pellet diet (VRK Nutritional Solutions, Baramati Agro Limited, Sangli, Maharashtra, India) and water *ad libitum*.

Induction of DM

Alloxan monohydrate powder was dissolved in distilled water to make a solution of 50 mg/ml.[9] The rats were fasted overnight, and hyperglycemia was induced by single SC injection of alloxan monohydrate (100 mg/kg).[9,10] The rats were maintained on 5% glucose solution for next 24 hrs to prevent hypoglycemia. The animals had access to food and water. The development of hyperglycemia in rats was confirmed by estimating fasting blood glucose at 48 hrs after alloxan monohydrate injection. The rats with fasting blood glucose level >150 mg/dl were considered as diabetic and were included in the study.

Study design

The rats were divided into five groups of six each (n=6):. Group I (n=6) normal control received 1 ml distilled water.

Group II (n=6) diabetic rats received 1 ml distilled water.
Group III (n=6) diabetic rats received silymarin 200 mg/kg (PO).
Group IV (n=6) diabetic rats received silymarin 400 mg/kg (PO).
Group V (n=6) diabetic rats received glibenclamide 0.5 mg/kg (PO).[11]

The drug treatment was carried out everyday morning using intragastric tube for 3 weeks after induction of DM.

Blood sampling

At the end of 7, 14 and 21 days of inducing DM fasting blood glucose levels were estimated in all the five groups using SD Code free glucometer. Blood samples were gathered by tail snipping method. On the 21st day of the experiment, blood samples were taken by retro-orbital puncture under intramuscular ketamine from all the groups of rats for biochemical analysis.

Serum preparation

The whole blood was collected from rats of each group in sterile, covered test tubes and labeled. After collection of the whole blood, it was allowed to clot undisturbed for 15-30 mins. The clot was removed by centrifuging at 1000-2000 $\times g$ for 10 mins in a centrifuge. The supernatant serum was obtained for biochemical analysis.

Tissue sampling

The animals were sacrificed by cervical dislocation and the kidneys from all the groups of rats were dissected out. They were processed for histopathological examination (HPE) and biochemical analysis.

Light microscopic study

For histopathological study the rat tissues were perfused with 10% formalin. The kidneys of the rats were excised immediately from the abdominal cavity and fixed in 10% neutral formalin, dehydrated in graded alcohol (80-100%), cleaned in xylene and embedded in paraffin. Then renal tissues were sliced into 3-5 μm pieces with a rotary microtone, deparaffinized in xylene, passed through varying grades of alcohol and finally stained with hematoxylin and eosin for histopathological assessment. The specimens were evaluated with a light microscope. All histopathological changes were examined by pathologist.

Biochemical analysis

Blood urea was estimated by urease method and serum creatinine was estimated by modified Jaffe's method.[12] Concentration of thiobarbituric acid reactive substances in the tissues was estimated by the method of Niehaus and Samuelsson.[13] Reduced glutathione in the tissues were estimated by the method of Ellman.[14] Superoxide dismutase (SOD) in the tissues was assayed by the method of Kakkar et al., and the activity of catalase (CAT) in the tissues were determined by the method of Sinha.[15,16]

Statistical analysis

Values of biochemical analysis were expressed as means±standard deviation for six rats in each group. The data were analyzed by Duncan's Multiple Range Test, using SPSS software version 17.0 (SPSS, Inc., Chicago, Illinois). Values not sharing a common superscript differ significantly at $p<0.05$.

RESULTS

Effect of silymarin on blood glucose levels

The glucose values were compared to values obtained for diabetic control rats and silymarin treated rats. In alloxan treated diabetic rats, the blood glucose levels remained significantly high on all the 3 weeks. Silymarin treated rats showed a significant reduction in blood glucose levels from 2nd week after induction. Silymarin treated rats showed maximum reduction in blood glucose level on 21st day at 400 mg/kg. In glibenclamide treated rats, blood glucose levels were significantly lower in all the 3 weeks compared to silymarin treated rats (Table 1).

Effect of silymarin on lipid peroxides (LPO) and antioxidants in kidneys

From this study it was observed that LPO level was significantly higher in diabetic rats compared to normal control rats. The diabetic rats treated with silymarin showed significant reduction in LPO. The antioxidant levels were significantly ($p<0.05$) higher in diabetic rats treated with silymarin in a dose-dependent manner compared with untreated diabetic rats. The LPO level in glibenclamide treated rats was significantly lower than the diabetic and silymarin treated rats. The glibenclamide treated rats also showed significant increase in antioxidant levels compared with diabetic rats (Table 2).

Effect of silymarin on serum urea and creatinine

The serum urea and creatinine levels were increased significantly in alloxan treated diabetic rats, compared to normal control rats at the end of 3rd week. The rats treated with silymarin after induction of diabetes showed significant ($p<0.05$) reduction in serum urea and creatinine in a dose-dependent manner compared to untreated diabetic rats. Glibenclamide treated rats also showed significant ($p<0.05$) reduction in serum urea and creatinine compared with diabetic rats (Table 3).

Table 1: Effect of silymarin on blood glucose levels in diabetic rats.

Groups	Day 1 (mg/dl)	Day 7 (mg/dl)	Day 14 (mg/dl)	Day 21 (mg/dl)
Normal control	76.00±3.35[d]	76.00±3.35[e]	76.00±3.35[e]	76.00±3.35[e]
Diabetic control	202.33±2.58[b]	184.83±4.83[a]	201.17±9.52[a]	206.00±10.95[a]
Diabetes+silymarin (200 mg/kg)	200.83±8.72[b]	176.67±4.13[b]	157.50±9.38[b]	127.33±1.63[b]
Diabetes+silymarin (400 mg/kg)	208.33±11.3[a]	171.17±2.99[c]	143.83±6.21[c]	118.67±3.01[c]
Diabetes+glibenclamide (0.5 mg/kg)	199.67±5.13[c]	155.33±3.44[d]	131.17±6.14[d]	108.83±2.04[d]

Values are expressed as means±SD for six rates in each group. Values not sharing a common superscript differ significantly at p≤0.05 (Dunken's test). SD: Standard deviation

Table 2: Effect of silymarin on LPO and antioxidants in kidneys of diabetic rats.

Group	SOD (unit/mg protein)	GSH (µg/mg protein)	CAT (µmol/mg protein)	LPO (mmole/100 g tissue)
Normal control	15.90±0.35[a]	31.72±0.86[b]	19.51±0.97[a]	3.55±0.36[d]
Diabetic control	5.11±0.58[e]	8.10±0.21[e]	3.38±0.48[e]	16.59±0.37[a]
Diabetes+silymarin (200 mg/kg)	11.12±0.71[d]	20.39±0.43[d]	17.92±0.72[c]	8.72±0.90[b]
Diabetes+silymarin (400 mg/kg)	15.30±0.46[b]	30.57±0.78[c]	17.72±0.66[d]	8.19±0.45[c]
Diabetes+glibenclamide (0.5 mg/kg)	13.86±0.12[c]	31.97±1.18[a]	18.24±0.64[b]	3.42±0.72[e]

Values are expressed as mean±SD for six rates in each group. Values not sharing a common superscript differ significantly at p≤0.05 (Dunken's test). SD: Standard deviation, LPO: Lipid peroxides, SOD: Superoxide dismutase, GSH: Glutathione, CAT: Catalase

Table 3: Effect of silymarin on serum urea and serum creatinine in diabetic rats.

Group	Urea (mg/dl)	Creatnine (mg/dl)
Normal control	26.92±1.77[e]	0.58±0.05[e]
Diabetic control	75.13±4.29[a]	1.78±0.09[a]
Diabetes+silymarin (200 mg/kg)	49.46±0.99[b]	1.65±0.11[b]
Diabetes+silymarin (400 mg/kg)	31.30±0.56[d]	0.64±0.04[d]
Diabetes+glibenclamide (0.5 mg/kg)	31.40±0.77[c]	0.72±0.02[c]

Values are expressed as mean±SD for six rates in each group. Values not sharing a common superscript differ significantly at p≤0.05 (Dunken's test). SD: Standard deviation
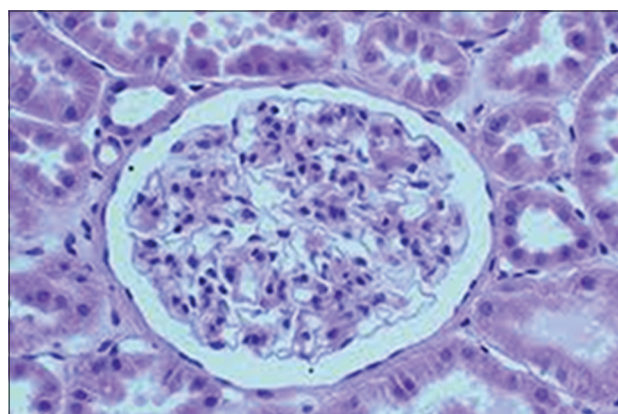

Figure 1: Photomicrograph of renal cortex of normal rats showing normal renal corpuscle and tubules with minimal congestion of cortical blood vessels (Group 1) H and E, ×200.

Histopathological study of renal cortex

Compared to the photomicrograph of renal cortex of normal rats (Figure 1), the diabetic rats showed renal cellular injury, necrotic tubular epithelium with widening of Bowman's capsule and the glomerular atrophy (Figure 2).

Diabetic rats treated with silymarin 200 mg/kg showed severe glomerular atrophy and necrosis of tubular epithelial cells (Figure 3).

However at silymarin 400 mg/kg, the renal cortex showed only mild swelling and congestion in the renal epithelium. There was no necrosis and the glomerular atrophy (Figure 4).

In diabetic rats treated with glibenclamide, the renal cortex showed moderate degeneration of renal epithelium, atrophied renal corpuscle and mild glomerular capillaries congestion (Figure 5).

DISCUSSION

The findings of the Diabetic Complications and Control Trial (DCCT) and The United Kingdom Prospective Diabetic Study support the idea that chronic hyperglycemia plays a causative role in the pathogenesis of diabetic micro and macrovascular complications.[1] Diabetic nephropathy is the leading contributor to end-stage renal disease. Hyperglycemia leads to increased production of ROS or superoxide in the mitochondria from glucose auto-oxidation, protein glycosylation and glucose metabolism via sorbital pathway. The ROS and glycosylation lead to disruption of cellular function, oxidative damage to
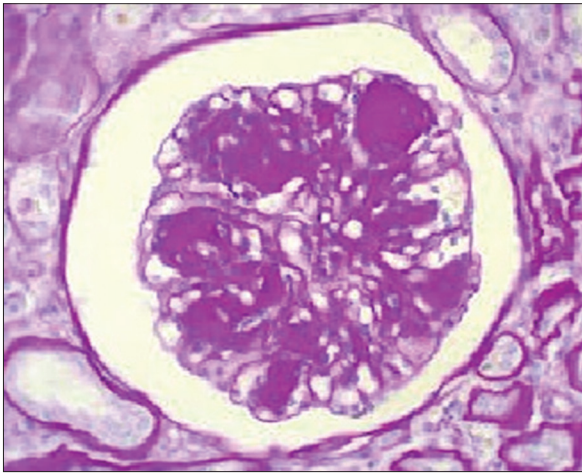
Figure 2: Photomicrograph of renal cortex of diabetic rats showing severe cellular injury, hydropic degeneration and swelling, glomerular atrophy and widening of the Bowman's capsule and necrotic tubular epithelium with pyknotic nuclei and acidophilic cytoplasm (Group 2) H and E, ×200.
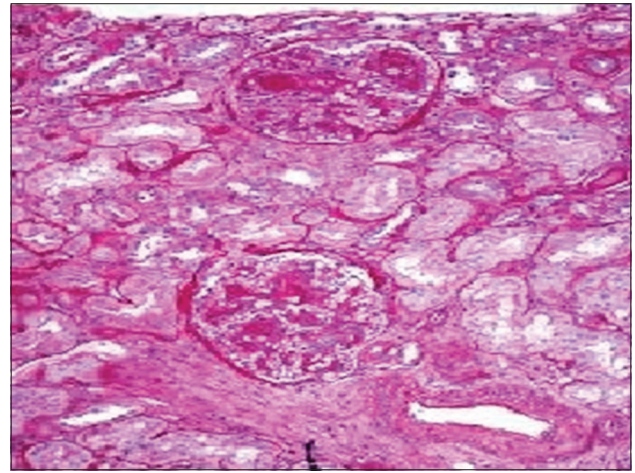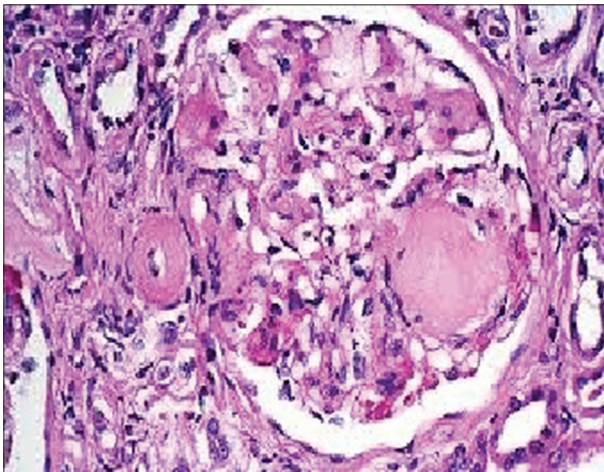
Figure 4: Photomicrograph of renal cortex of silymarin (400 mg/kg) treated rats showing mild cellular degeneration and swelling, mild fatty changes in the glomerular epithelial capillaries and mild changes in the glomerulus (Group 4) H and E, ×400.

Figure 3: Photomicrograph of renal cortex of silymarin (200 mg/kg) treated rats showing mild to moderate cellular degeneration, mild glomerular atrophy, and hyaline cast pinkish amorphous protein within the tubular lumen (Group 3) H and E, ×400.
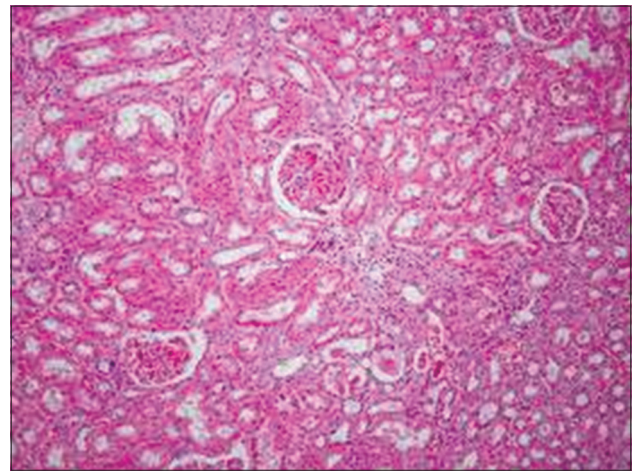
Figure 5: Photomicrograph of renal cortex of glibenclamide (0.5 mg/kg) treated rats showing moderate cellular hydropic degeneration, atrophied renal corpuscle and mild glomerular capillaries congestion (Group 5) H and E, ×200.

the membrane and other structures, susceptibility to LPO and exhaustion of antioxidant defences. Al-Enazi had reported rise in LPO in hepatic cells due to hyperglycemia induced by streptozotozin in rats.[17] In the present study also the diabetic rats showed an increase in LPO and decreased in antioxidants in the renal tissue. The oxidative damage to renal cell was further confirmed by increase in serum urea and creatinine in diabetic rats.

There are reports that natural antioxidants such as vitamin E, caffeic acid, lipoic acid, quercetin, melatonin and natural phenolic compounds have protective effects against hyperglycemia-induced oxidative stress.[18,19] Treatment of diabetic animals with silymarin significantly reduced LPO and increased the levels of SOD, glutathione reductase and

CAT. SOD protects tissues against oxygen free radicals by scavenging O_2. Thus, SOD can act as a primary defense against O_2 and prevents further generation of free radicals (Arivazhagan et al., 2000).[20] CAT protects tissues from highly reactive OH radicals (Sozmen et al., 2001). Silymarin by scavenging the free radicals and by raising the antioxidant activities prevented the renal damage and maintained the cell integrity. The DCCT demonstrated that the improvement of glycemic control reduced microalbuminuria (39%) and clinical nephropathy (54%).[21] Varzi et al. had reported that silymarin improved alteration in serum creatinine concentration in gentamicin treated dogs.[22] In the present study also silymarin treated diabetic animals showed a decrease in serum urea and creatinine, which confirms its nephroprotective effect. The cytoprotective action of silymarin against hyperglycemia-induced oxidative stress

was further revealed by HPE study. The histopathological study of renal tissue of diabetic rats showed the glomerular atrophy with necrosis of tubular epithelium. This damage was due to hyperglycemia. In silymarin treated diabetic rats there was restoration of glomerular structure, with only mild congestion in tubular epithelium.

CONCLUSION

This study proved that the silymarin had significant nephroprotective effect against the hyperglycemia-induced oxidative stress. Its good safety profile, easy availability and low cost are added advantages. Hence, silymarin may be added as an adjuvant therapy for preventing or slowing the progression of diabetic nephropathy.

ACKNOWLEDGMENT

REFERENCES

1. Triplitt CL, Reasner CA. Diabetes mellitus. In: Dipiro JT, Talbert RL, Yee GC, Matzke GR, Wells BG, Posey LM, editors. Pharmacotherapy - A Pathophysiologic Approach. 8th Edition, Volume 83. China: McGraw-Hill Companies; 2011:1255-302.
2. Martin NM, Bloom SR. Pancreatic endocrine disorders and multiple endocrine neoplasia. In: DA TM JD editors. Oxford Textbook of Medicine. 5th Edition. Volume 13.10. New York: Oxford University Press; 2010:1976-86.
3. Ihara Y, Toyokuni S, Uchida K, Odaka H, Tanaka T, Ikeda H, et al. Hyperglycemia causes oxidative stress in pancreatic beta-cells of GK rats, a model of type 2 diabetes. Diabetes. 1999;48(4):927-32.
4. Tokuyama Y, Sturis J, DePaoli AM, Takeda J, Stoffel M, Tang J, et al. Evolution of beta-cell dysfunction in the male Zucker diabetic fatty rat. Diabetes. 1995;44(12):1447-57.
5. Stahl W, Sies H. Antioxidant defense: vitamins E and C and carotenoids. Diabetes. 1997;46 Suppl 2:S14-8.
6. Ahmadvand H. Effects of coenzyme Q10 on hemoglobin A1C, serum urea and creatinine in alloxan-induced type 1 diabetic rats. Iran J Pharmacol Ther. 2012;11:64-7.
7. Ross JA, Kasum CM. Dietary flavonoids: bioavailability, metabolic effects, and safety. Annu Rev Nutr. 2002;22:19-34.
8. Morazzoni P, Bombardelli E. *Silybum marianum* (Carduus marianus). Fitoterapia. 1995;64:3-42.
9. Rao BK, Kesavulu MM, Giri R, Appa Rao C. Antidiabetic and hypolipidemic effects of *Momordica cymbalaria* Hook fruit powder in alloxan-diabetic rats. J Ethnopharmacol. 1999;67(1):103-9.
10. Kumar S, Kumar D, Deshmukh RR, Lokhande PD, More SN, Rangari VD. Antidiabetic potential of *Phyllanthus reticulatus* in alloxan-induced diabetic mice. Fitoterapia. 2008;79(1):21-3.
11. Lal VK, Gupta PP, Awanish P. Hypoglycemic effect of kyllinga triceps in STZ induced diabetic rats. J Diabetes Metab. 2012;5.6:1000203.
12. Allain CC, Poon LS, Chan CS, Richmond W, Fu PC. Enzymatic determination of total serum cholesterol. Clin Chem. 1974;20(4):470-5.
13. Niehaus WG Jr, Samuelsson B. Formation of malonaldehyde from phospholipid arachidonate during microsomal lipid peroxidation. Eur J Biochem. 1968;6(1):126-30.
14. Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys. 1959;82(1):70-7.
15. Kakkar P, Das B, Viswanathan PN. A modified spectrophotometric assay of superoxide dismutase. Indian J Biochem Biophys. 1984;21(2):130-2.
16. Sinha AK. Colorimetric assay of catalase. Anal Biochem. 1972;47(2):389-94.
17. Al-Enazi MM. Combined therapy of rutin and silymarin has more protective effects on STZ-induced oxidative stress in rats. J Appl Pharm Sci. 2014;4(01):021-8.
18. Balkis Budin S, Othman F, Louis SR, Abu Bakar M, Radzi M, Osman K, et al. Effect of alpha lipoic acid on oxidative stress and vascular wall of diabetic rats. Rom J Morphol Embryol. 2009;50(1):23-30.
19. Garfinkel D, Zorin M, Wainstein J, Matas Z, Laudon M, Zisapel N. Efficacy and safety of prolonged-release melatonin in insomnia patients with diabetes: a randomized, double-blind, crossover study. Diabetes Metab Syndr Obes. 2011;4:307-13.
20. Arivazhagan P, Thilakavathy T, Panneerselvam C. Antioxidant lipoate and tissue antioxidants in aged rats. J Nutr Biochem. 2000;11(3):122-7.
21. Powers AC. Diabetes mellitus. In: Kasper DL, Faver AS, Longo D, Branunald E, Hauser SC, Janesen JL, editors. Harrison's Principles of Internal Medicine. 16th Edition, Volume 323. New York: McGraw-Hill Book Co.; 2005: 2152-62.
22. Varzi HN, Esmailzadeh S, Morovvati H, Avizeh R, Shahriari A, Givi ME. Effect of silymarin and vitamin E on gentamicin-induced nephrotoxicity in dogs. J Vet Pharmacol Ther. 2007;30(5):477-81.