

Research Article

Neuroprotective Effect of Silymarin by Modulation of Endogenous Biomarkers in Streptozotocin Induced Painful Diabetic Neuropathy

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Abstract: Aim of the present study is to investigate the effect of silymarin (SM), a potent antioxidant and anti-inflammatory compound on experimentally-induced Diabetic Neuropathy (DN) in male Wistar rats. Diabetes was induced by single streptozotocin (STZ) injection in rats. Pain-related behavior tests were performed including tail flick, paw-pressure analgesia and Rota-rod performance. Silymarin treatment was started after 21st day of diabetes induction and continued for 6 consecutive weeks. In serum fasting glucose, insulin, tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6) and interleukin-1 β (IL-1 β) levels were estimated and in sciatic nerve, thiobarbituric acid reactive substances (TBARS), reduced glutathione (GSH), Superoxide Dismutase (SOD), catalase (CAT), glutathione-s-transferase (GST), glutathione-reductase (GR) and glutathione peroxidase (GSH-Px) activities were measured. Diabetic rats developed neuropathy which was apparent from decreased tail-flick latency and paw-withdrawal latency. This was escorted by decreased motor coordination as assessed by performance on Rota-rod treadmill. Treatment with SM ameliorated the hyperalgesia, analgesia and improved motor coordination. STZ significantly increased TBARS and decreased GSH levels in sciatic nerve where silymarin treatment significantly protected those changes. Enzymatic activities such as SOD, CAT, GST, GSH-Px and GR were significantly inhibited in sciatic nerve of diabetic rats. The SM treatment significantly ameliorated decrease in antioxidant defense. Our results clearly demonstrate protective effect of SM is mediated through attenuation of oxidative stress and suggest therapeutic potential of SM in attenuation of diabetic neuropathy.

Keywords: Cytokines, diabetes, neuropathy, oxidative stress, silymarin

INTRODUCTION

Neuropathic pain is a form of chronic pain induced by damage or abnormal function of central or peripheral nervous system (Abdi *et al.*, 2004; Woolf, 2004). It is usually results in sensory abnormalities such as burning sensations, hyperalgesia, allodynia and dysesthesia, leading to alteration in patient's quality of life by an emotional well-being (Galer *et al.*, 2000). Diabetic neuropathy is associated with metabolic syndromes like diabetes mellitus. It effect of about 15-25% in type-1 and 30-40% in type-2 diabetic patients, causing disabilities and a high mortality rate (Callaghan *et al.*, 2012). Furthermore, it is a challenge in clinical practice because of its severity, chronicity and resistance to some classical analgesics (Gilron *et al.*, 2006). The pathophysiological mechanisms of DN include a complex network of unified vascular (Cameron and Cotter, 1999); metabolic (Stevens *et al.*, 2000) and neurotrophic (Calcutt, 2004) defects, which end with electrophysiological discrepancies, abnormal sensory perception and progressive damage and loss of unmyelinated and myelinated nerve fibers (Sima *et al.*, 2000). Oxidative stress can be also one of the caustic

mechanisms associated with DN and evidence about its possible role in the development of diabetic complications is now available (Ceriello, 2006).

Oxidative stress is believed to be a biochemical trigger for sciatic nerve dysfunction and reduced endoneurial blood flow in diabetic rats (Figuroa-Romero *et al.*, 2008; Zhrebetskaya *et al.*, 2009). In this regards, the potential sources of Reactive Oxygen Species (ROS) including endothelial NAD(P)H oxidase, xanthine oxidase, nitric oxide synthase and mitochondrial respiratory chain inefficiency are more notable (Cameron and Cotter, 1999). Furthermore, diabetes is linked with reduced activity of GST, GSH-Px, GR, Cu-Zn superoxide dismutase and lower levels of glutathione (Yu *et al.*, 2006; Arora *et al.*, 2008; Cui *et al.*, 2008). Opposite to this, diabetes causes increase in the lipid peroxidation products such as MDA or conjugated dienes in sciatic nerves (Cunha *et al.*, 2008). Enhanced oxidative stress consecutively activates nuclear factor kappa B (NF- κ B), which up-regulates genes such as cytokines, adhesion molecules, endothelin-1 and tissue factor (Bierhaus *et al.*, 1998).

Silymarin, an extract from the seeds of the milk thistle plant, *Silybummarianum*, has been used for

centuries against liver diseases. Silymarin is a mixture of seven flavonolignans:

- Silybin-A
- Silybin-B
- Isosilybin-A
- Isosilybin-B
- Silychristin
- Silydianin
- Isosilychristin and one flavonoid named taxifolin

Experimentally evidenced that silymarin has anti-oxidant, immunomodulatory, anti-fibrotic, anti-proliferative and anti-viral activities although its mechanism of action is incompletely understood till date (Agarwal *et al.*, 2006; Polyak *et al.*, 2007; Jacobs *et al.*, 2002). Its clinical efficacy even in chronic liver disease has not yet been demonstrated (Saller *et al.*, 2001) as results have been inconsistent. Problems with the studies have included insufficient power, use of varying doses and the use of different non-standardized preparations of silymarin, making it difficult to compare results among studies. Silymarin is known to prevent lipid peroxidation (Velussi *et al.*, 1997), inhibit low-density lipoprotein oxidation (Sobolová *et al.*, 2006) and scavenge Reactive Oxygen Species (ROS) (Dehmlow *et al.*, 1996). It can also increase antioxidative enzyme levels and limit lipid peroxidation (Soto *et al.*, 2003) and enhance Superoxide Dismutase (SOD) activity (Baluchnejadmojarad *et al.*, 2010). It also proposed as a nutritional supplement for liver health (Wellington and Jarvis, 2001) and for lowering some risk factors of atherosclerosis including very low- and high-density lipoprotein-cholesterol (Imanek *et al.*, 2001). In addition, hypoglycemic effect of chronic silymarin treatment in Type 2 diabetic patients has been reported (Huseini *et al.*, 2006). Silymarin is a safe herbal product, since no health hazards or side effects are known in conjunction with the proper administration of designed therapeutic dosages (Montvale, 2000). Thus, we designed this study to investigate the potent effects of silymarin supplementation on diabetic-induced changes in pro-inflammatory cytokines, and oxidative stress in sciatic nerve.

MATERIALS AND METHODS

Animals: Male Wistar albino rats, roughly the same age of 3 months, weighing 250-280 g were received from the Experimental Animal Care Center (King Saud University, Riyadh, Saudi Arabia). They were maintained under controlled conditions of temperature (22±1°C), humidity (50-55%), and light (12 h light/dark cycles) and were provided with Purina chow (Grain Silos & Flour Mills Organization, Riyadh, Saudi Arabia) and drinking water *ad libitum*. All procedures

including euthanasia procedure were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, Institute for Laboratory Animal Research (NIH Publications No. 80-23; 1996) and the Ethical Guidelines of the Experimental Animal Care Center (College of Pharmacy, King Saud University, Riyadh, Saudi Arabia).

Diabetes induction: Diabetes was induced in overnight fasted rats by a single intraperitoneal injection of streptozotocin (SIGMA Chemicals, USA) at a dose of 65 mg/kg body weight freshly dissolved in 0.1 mol/L citrate buffer, pH 4.5. Control rats as vehicle received equal volume of citrate buffer. The animals with fasting blood glucose values more than 250 mg/dl after 72 h of STZ injection were considered diabetic and included in the study.

Experimental design: Normal healthy rats were divided in five groups (six rats in each group):

- Control (vehicle)
- SM (60 mg/kg/day, orally) treated to normal rats (SM 60), and the STZ-induced diabetic rats were randomly divided as
- Diabetic (STZ)
- SM (30mg/kg/day) treated to diabetic rats (SM 30+STZ)
- SM (60 mg/kg/day) treated to diabetic rats (SM 60+STZ)

Vehicle and drug treatment were started three weeks after the diabetes induction and continued for six consecutive weeks. Behavioral assessments were undertaken before and after treatments.

Mechanical hyperalgesia (Randall and Selitto method): Mean right and left paw pressure thresholds were determined using the paw pressure analgesia meter (MK-20D Analgesymeter, Muromachi KIKAI CO. Ltd., Japan). Pressure increased at a linear rate of 10 mm Hg with a cut-off at 500 mm Hg to avoid tissue injury. Pressure was applied to the center of the hindpaw. When the animals displayed pain by withdrawal of the paw, the applied paw pressure was recorded by an analgesia meter and expressed as mmHg. Three tests separated by at least 10 min were performed for each rat, and mean of value is used.

Tail flick test: The method described by Sugimoto *et al.* (2008) used with slight modifications. Acute nociception was induced by using a tail flick apparatus (Tail Flick model DS 20 Sorrel Apex, France). Briefly, each rat placed in a restrainer and the tail flick latency was determined by focusing the intensity controlled beam of light on the distal last 2 cm of the

animal's tail and recording the time taken to remove the tail from the noxious thermal stimulus. For each animal, 2 to 3 recordings were made at an interval of 15 min; the mean value was used for statistical analysis.

Rota rod treadmill test: Treadmill test was performed by using Rota-Rod Treadmill for rats and mice (Model MK-670, Muromachi Kikai Co, Ltd., Tokyo, Japan) to evaluate motor coordination of the animals (Cartmell *et al.*, 1991). Animals were initially trained to maintain themselves on the rotating rod for more than 2 min. A day before treatment starts and at end of the treatment, the rats were placed on rotating rod for two trails each. Animals were scored for their latency to fall (in seconds) in each trial.

Sample collections: At end of the treatment and behavioral assessments, animals were fasted overnight, under deep anesthesia, blood samples were collected through cardiac puncture and then they sacrificed, and sciatic nerves were rapidly removed and dipped in liquid nitrogen for a minute and kept in deep freezer at -80°C till analysis. Blood samples were centrifuged at 3,000 rpm for 10 min and serum samples were stored at -20°C till analysis.

Serum parameters: Serum fasting glucose, AST, ALT, APL, TP, CRP and Albumin levels were estimated by using commercially available kits (RANDOX Laboratories Ltd., UK) and insulin levels were measured by insulin enzyme immunoassay (ELISA) kit (DRG, Germany). Serum pro-inflammatory cytokines including TNF- α , IL-6 and I β concentrations were assayed by an enzyme-linked immunosorbent assay kit (ShangHaiSenXiong Science and Technology Company, China). The levels were estimated by following the instruction provided by the manufacturer.

Tissue parameters: Sciatic nerves were homogenized in 50 mM phosphate buffered saline (pH 7.4) by using a glass homogenizer (Omni International, Kennesaw, GA, USA). Around a milliliter homogenate was centrifuged at 1000 g for 10 min at 4 °C to separate nuclei and unbroken cells. The pellet was discarded and a portion of supernatant was again centrifuged at 12000 g for 20 min to obtain post-mitochondrial supernatant. In homogenate, MDA and GSH levels were estimated. In post-mitochondrial supernatant, SOD, CAT, GST, GSH-Px and GR activities were measured.

Estimation of TBARS levels: A TBARS assay kit (ZeptoMetrix) was used to measure the lipid peroxidation products, malondialdehyde (MDA)

equivalents. One hundred microliters of homogenate was mixed with 2.5 ml reaction buffer (provided by the kit) and heated at 95 °C for 60 min. After the mixture had cooled, the absorbance of the supernatant was measured at 532 nm using a spectrophotometer. The lipid peroxidation products are expressed in terms of nmoles MDA/mg protein using molar extinction coefficient of MDA-thiobarbituric chromophore (1.56×10^5 M/cm).

Estimations of GSH levels: The concentration of GSH was measured using the method described by Sedlak and Lindsay (1968). Homogenate was mixed with 0.2 M Tris buffer, pH 8.2 and 0.1 mL of 0.01 M Ellman's reagent, [5,5'-dithiobis-(2-nitro-benzoic acid)] (DTNB). Each sample tube was centrifuged at 3000 g at room temperature for 15 min. The absorbance of the clear supernatants was measured using spectrophotometer at 412 nm in one centimeter quartz cells.

Estimations of SOD activity: The activity of SOD in sciatic nerve was estimated using the method described by Kono (1978) with the aid of nitroblue tetrazolium as the indicator. Superoxide anions are generated by the oxidation of hydroxylamine hydrochloride. The reduction of nitrobluetetrazolium to blue formazon mediated by superoxide anions was measured 560 nm under aerobic conditions. Addition of superoxide dismutase inhibits the reduction of nitroblue tetrazolium and the extent of inhibition is taken as a measure of enzyme activity. The SOD activity was expressed as units/mg protein.

Estimation of CAT activity: The CAT activity was measured by the method of Aebi (1984) using hydrogen peroxide as substrate in post-mitochondrial supernatant. The hydrogen peroxide decomposition by catalase was monitored spectrophotometrically (LKB-Pharmacia, Mark II, Ireland) by following the decrease in absorbance at 240 nm. The activity of enzyme was expressed as units of decomposed/min/mg proteins by using molar extinction coefficient of H₂O₂ (71/M/cm).

Estimations of GST activity: The GST activity in sciatic nerve was measured by the method of Habig *et al.* (1974). The reaction mixture consisted of 0.067 mM GSH, 0.067 mM 1-chloro-2, 4-dinitrobenzene (CDNB), 0.1 M phosphate buffer (pH 6.0) and 0.1 ml of post-mitochondrial supernatant in a total volume of 3 ml. Absorbance was read at 340 nm for 10 min every 30 sec by an optical plate reader and the enzyme activity was calculated as mMol CDNB conjugate formed min⁻¹/mg protein using a molar extinction coefficient of 9.6×10^3 M/cm.

Estimations of GSH-Px activity: Glutathione peroxidase activity was modified from the method of Flohe and Gunzler (1984). For the enzyme reaction, 0.2 mL of the post-mitochondrial supernatant was placed into a tube and mixed with 0.4 mL reduced glutathione and the mixture was put into an ice bath for 30 min. Then the mixture was centrifuged for 10 min at 3000 rpm, 0.48 mL of the supernatant was placed into a cuvette, and 2.2 mL of 0.32 MNa_2HPO_4 and 0.32 ml of 1.0 mMol/L DTNB were added for color development. The absorbance at wavelength 412 nm was measured on spectrophotometer (LKB-Pharmacia, Mark II, Ireland) after 5 min. The enzyme activity was calculated as nMol/mg protein.

Estimations of GR activity: Glutathione reductase activity was measured in the post-mitochondrial supernatant by the method of Carlberg and Mannervik (1985). GSSG is reduced to GSH by NADPH in the presence of GR. Enzyme activity was measured by following the decrease in absorbance (oxidation of NADPH) for 3 min spectrophotometrically at 340 nm. The activity of enzyme was expressed as nmoles NADPH oxidized/min/mg protein, using molar extinction coefficient of NADPH ($6.22 \cdot 10^6/M/cm$).

Histopathological screening of sciatic nerve: A part of sciatic nerve was fixed in 10% neutral buffered formalin, embedded in paraffin wax, sectioned at 3 μm , stained with Hematoxylin and Eosin (H & E) stain and placed in slides for under light microscopic examination.

Statistical analysis: Data were expressed as means \pm SD. Statistical analysis was carried out using one-way ANOVA followed by newman-keulspost test. P value of ≤ 0.05 was considered statistically significant. All statistics tests were conducted using Graph Pad Prism (version 5) software.

RESULTS

Mean final body weights were significantly decreased in diabetic rats compared to control group. Body weights of the diabetic rats supplemented with SM (30 and 60 mg/kg/day) were found significant increase when compared to untreated diabetic rats respectively (Fig. 1A). Serum fasting glucose levels significantly increased while insulin levels were decreased in STZ-induced diabetic rats. Treatments with both the doses of SM to diabetic rats for 6 consecutive weeks showed significant decrease in fasting glucose and increase in insulin levels when compared to untreated diabetic rats respectively (Fig. 1B and 1C).

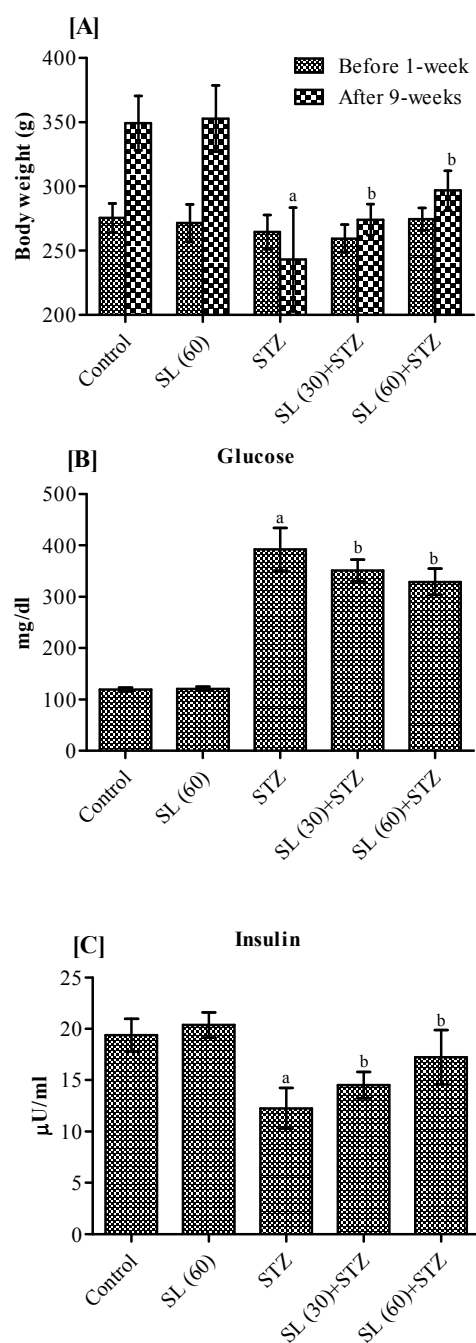


Fig. 1: Effect of silymarin (SM) on body weight, serum glucose and insulin levels of diabetic and non-diabetic animals; One-way ANOVA and Student-Newman-Keuls multiple comparisons test was applied. 'a' Significantly different from control group ($p < 0.05$) and 'b' Significantly different from STZ group ($p < 0.05$). Values are expressed as Mean \pm SD ($n = 6$).

Liver enzymes AST and ALT levels are significantly increased in serum of diabetic rats as

Table 1: Effect of silymarin (SM) on serum liver enzymes and different protein levels of normal and diabetic rats

Treatments (mg/kg/day)	AST (U/L)	ALT (U/L)	Total Protein (mg/dL)	CRP (mg/dL)	Albumin (mg/L)
Control	40.00±3.59	23.36±2.81	9.84±1.20	2.80±0.29	40.23±6.25
SM (60)	37.45±5.04	22.54±3.54	10.20±2.30	2.74±0.54	42.15±5.01
STZ	104.83±11.88 ^a	68.66±14.87 ^a	6.74±0.45 ^a	10.90±1.57 ^a	25.12±5.95 ^a
SM (30)+STZ	74.35±17.24 ^b	56.54±9.29 ^b	7.56±0.61	8.97±1.06 ^b	32.15±3.54 ^b
SM (60)+STZ	57.48±24.87 ^b	42.74±12.74 ^b	8.54±0.57 ^b	7.46±1.15 ^b	37.46±3.68 ^b

One-way ANOVA and Student-Newman-Keuls multiple comparisons test was applied; 'a' Significantly different from control group (p<0.05) and 'b' Significantly different from STZ group (p<0.05); values are expressed as Mean±SD (n = 6)

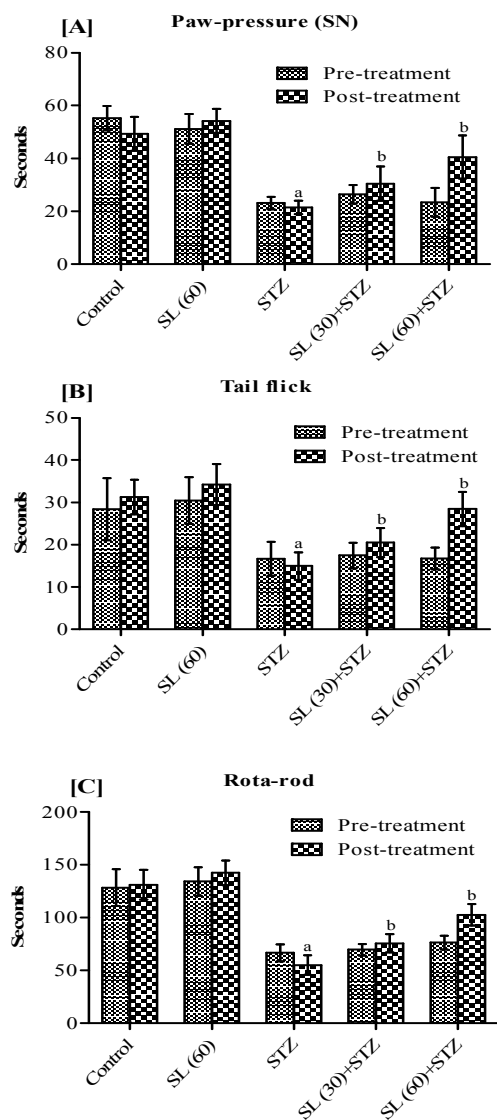


Fig. 2: Effect of silymarin (SM) on pain threshold in paw pressure analgesia, tail flick and Rota-rod treadmill performance of diabetic and non-diabetic animals; One-way ANOVA and Student-Newman-Keuls multiple comparisons test was applied. 'a' Significantly different from control group (p<0.05) and 'b' Significantly different from STZ group (p<0.05). Values are expressed as Mean ± SD (n = 6).

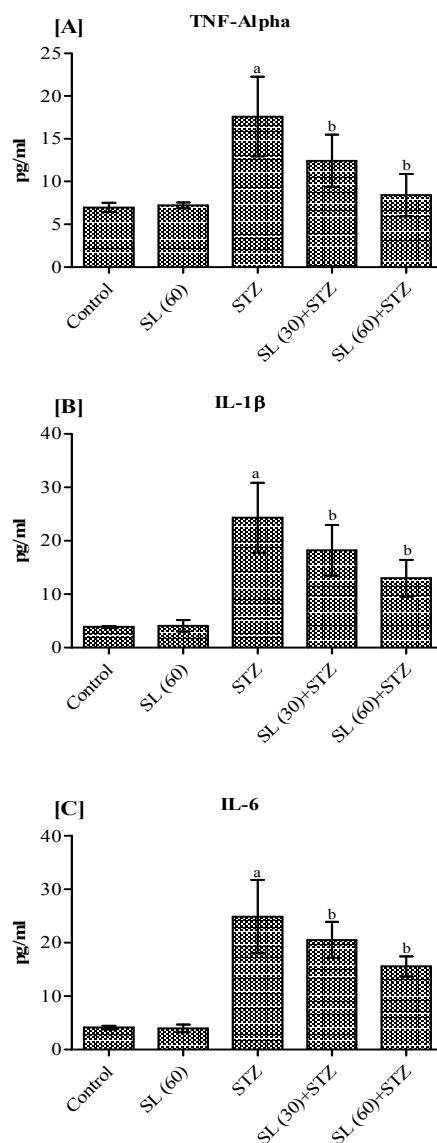


Fig. 3: Effect of silymarin (SM) on serum TNF-α, IL-1β and IL-6 levels of diabetic and non-diabetic rats; One-way ANOVA and Student-Newman-Keuls multiple comparisons test was applied. 'a' Significantly different from control group (p<0.05) and 'b' Significantly different from STZ group (p<0.05). Values are expressed as Mean ± SD (n = 6).

compared to control animals and produced marked decrease in groups supplemented with SM by both the doses. Total protein and albumin levels were significantly decreased in diabetic rats while CRP levels increased as compared to control group. Six weeks SM treatments to diabetic rats, TP and albumin levels significantly increased and CRP levels decreased when compared to untreated diabetic rats (Table 1).

In paw pressure analgesia test, vehicle diabetic rats significantly decreased the Paw Withdraw Latency (PWL) compared to non diabetic animals. The diabetic group of animals treated with SM (30 and 60 mg/kg/day) for 6 weeks significantly increased the PWL time (s) compared the untreated diabetic rats (Fig. 2A). A significant decrease in tail flick latency was also observed in diabetic rats compared to control group and this decrease was markedly eliminated by SM supplementation (Fig. 2B). Rota-rod treadmill performance of diabetic and non-diabetic animals, before and after treatment with two doses of SM is shown in Fig. 2C. The running performance on treadmill was significantly decreased in diabetic animals compared to control rats, after SM treatments to diabetic rats significantly enhanced the performance.

Serum pro-inflammatory markers including TNF- α , IL-1 β and IL6 levels were markedly increased in diabetics as compared to control rats. Treatments with SM by following two doses for six consecutive weeks to diabetic rats significantly decreased the increased levels of TNF- α and interleukins (IL-1 β and IL6), respectively (Fig. 2A, B and C).

TBARS levels significantly increased in sciatic nerve of diabetic animals while GSH levels decreased as compared to control animals. Treatments with SM, by taking two doses to diabetic rats significantly decreased the elevated TBARS levels and increased the inhibited levels of GSH as compared to untreated diabetic rats (Fig. 3A, B). In sciatic nerve, SOD and CAT activities were markedly inhibited in diabetic rats compared to control animals. These activities were significantly enhanced in SM treated diabetic rats when compared to untreated diabetic rats (Fig. 4C, D). Furthermore, GST, GSH-Px and GR activities were also significantly decreased in sciatic nerve of diabetic rats compared to control animals. Six consecutive weeks treatment with SM by taking two doses (30 and 60 mg/kg/day) to diabetic rats produced marked

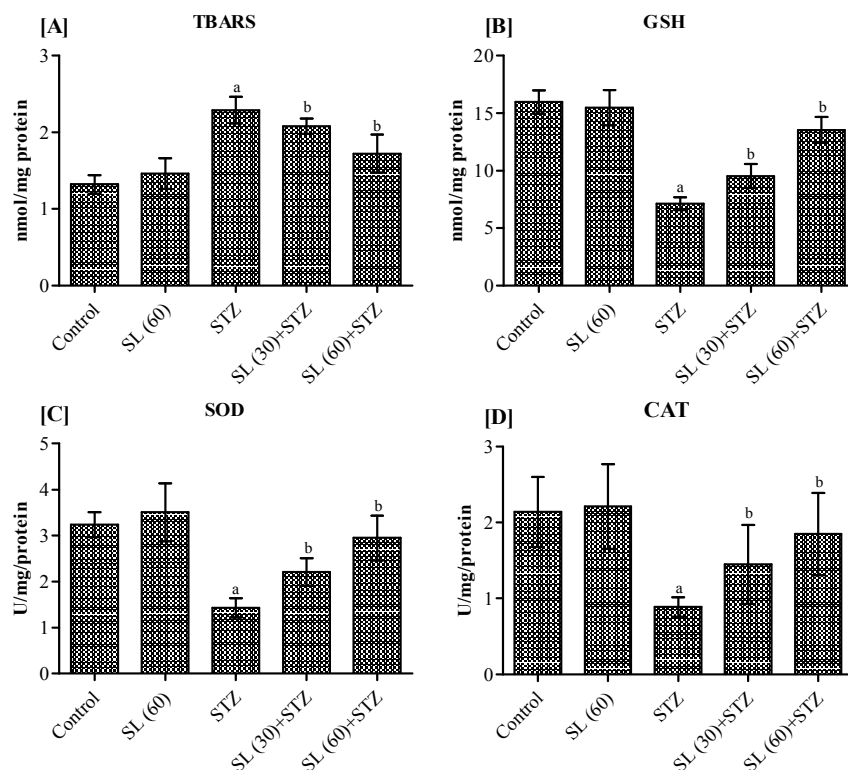


Fig. 4: Effect of silymarin (SM) on TBARS, GSH, SOD and CAT activities in sciatic nerve of diabetic and non-diabetic rats; One-way ANOVA and Student-Newman-Keuls multiple comparisons test was applied. 'a' Significantly different from control group ($p < 0.05$) and 'b' Significantly different from STZ group ($p < 0.05$). Values are expressed as Mean \pm SD (n = 6)

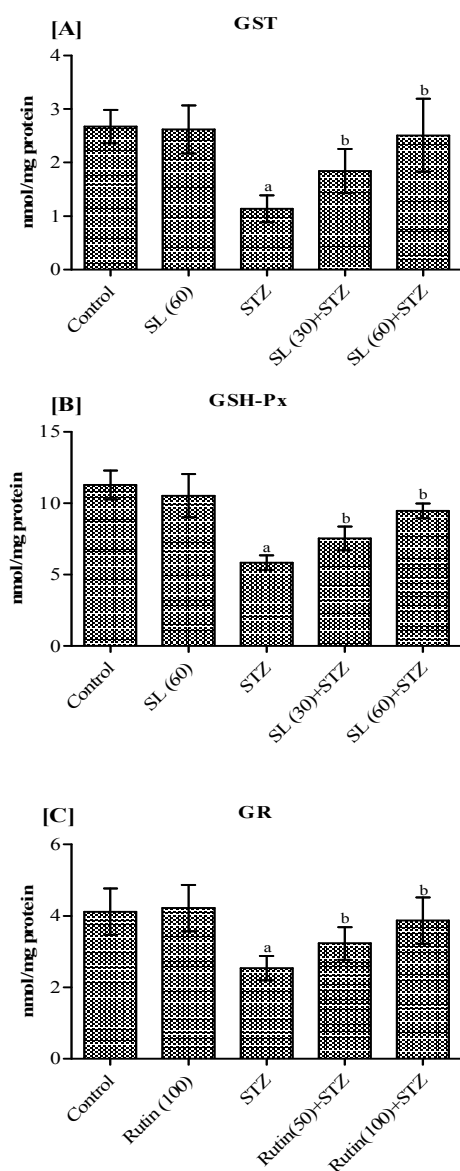


Fig. 5: Effect of silymarin (SM) on GST, GST-Px and GR activities in sciatic nerve of diabetic and non-diabetic rats; One-way ANOVA and Student-Newman-Keuls multiple comparisons test was applied. 'a' Significantly different from control group ($p < 0.05$) and 'b' Significantly different from STZ group ($p < 0.05$). Values are expressed as Mean \pm SD (n = 6)

increase in these activities while compared to untreated diabetic rats (Fig. 5A, B and C).

DISCUSSION

The present results revealed development of diabetic neuropathy in experimentally-induced diabetic

rats which was steady with earlier reports demonstrating a similar reduction in hyperglycemia-induced thermal hyperalgesia and this was accompanied by decreased motor coordination as assessed by performance on Rota-rod treadmill in STZ-induced diabetic rats (Kamboj *et al.*, 2010).

Several studies have shown an association between hyperglycemia and decreased body weight of diabetic animals (Zafar and Naqvi, 2010; Okon *et al.*, 2012). Similar results have seen in the present study, body weights of diabetic animals were significantly decreased. Silymarin treatments for 6 consecutive weeks produced antidiabetic effect showing inhibition in glucose and increase in insulin levels. These results are in agreement with earlier reports showed reduction in glucose levels in SM supplemented diabetic rats (Baluchnejadmojarad *et al.*, 2010; Ashkavand *et al.*, 2012). In a clinical study, reported that SM ingestion by diabetic patients decreased the hyperglycemia and also helped to increase the body weights (Hajiaghamohammadi *et al.*, 2012).

Neuropathic pain is one of the most common complications of diabetes mellitus. Along the disease course, almost 50% of the diabetic patients develop neuropathy with symptoms including spontaneous pain, allodynia and hyperalgesia (Apfel *et al.*, 2001). STZ-induced diabetic animals are used to model chronic neuropathic pain with hyperalgesia and allodynia that reflect symptoms observed in diabetics (Gul *et al.*, 2000; Kamei *et al.*, 2001). In present study, diabetic rats, the tail withdrawal latency was significantly shorter than that observed in control animals, indicating development of thermal hyperalgesia. This was accompanied by decreased motor coordination as assessed by performance on Rota-rod treadmill. This is in line with the observations that STZ-induced diabetic animals show thermal hyperalgesia when the tail is exposed to noxious stimuli (Ohsawa and Kamei, 1999; Kamboj *et al.*, 2010). Most of the phenolic compounds are known to have anti-inflammatory, analgesic and also have antinociceptive properties (Kamboj *et al.*, 2010; Ramirez *et al.*, 2010; Lee *et al.*, 2006). This may be because of SM treatment to diabetic rats for 6 consecutive weeks showed significant improvement in tail withdrawal latency rate while compared to untreated diabetic animals in present study. Earlier reports documented that, SM has anti-inflammatory and analgesic properties (Ashkavand *et al.*, 2012; Gharagozloo *et al.*, 2010). Inflammatory cytokines such as TNF- α and other inflammatory marker including IL-1 β and IL-6 are known to stimulate the acute phase reaction (Locksley *et al.*, 2001). The promoter polymorphism in the TNF gene has been implicated in the regulation of TNF- α production and has been

associated with a wide spectrum of inflammatory and infectious diseases and has been reported in diabetic states to be a consequence of hyperglycemia (Brownlee, 2005; Navarro-Gonzalez and Mora-Fernandez, 2008). In present study, serum proinflammatory markers including TNF- α , IL-6 and IL-1 β are significantly increased in STZ-induced diabetic rats. SM treatment to the diabetic rats significantly reduced such markers in present study. This may be because of SM showed antioxidant and anti-inflammatory properties in earlier studies (Nichols and Katiyar, 2010; Nabavi *et al.*, 2012).

Chronic hyperglycemia induces oxidative stress by the autoxidation of monosaccharides (Bonfont-Rousselot, 2002), which leads to production of superoxide and hydroxyl radicals. It is well known that pain transmission requires production of reactive oxygen species (Viggiano *et al.*, 2005). We observed a significantly higher level of lipid peroxidation marker MDA in sciatic nerve of diabetic animals. Glutathione, a potent endogenous antioxidant is a first line of defense against free radicals. In present study, GSH levels were significantly lowered in the sciatic nerve of diabetic animals. These observations are in agreement with the previous findings showing reduction in GSH levels in diabetes (Kuzumoto *et al.*, 2006; Arora *et al.*, 2008). Intracellular GSH levels have been observed to decrease in brain (Kamboj *et al.*, 2010) and sciatic nerve (Kuzumoto *et al.*, 2006) of diabetic animals. SM treatment significantly reduced lipid peroxidation and regenerated intracellular GSH content in the sciatic nerve; this is probably because of its free radical scavenging activity or endogenous synthesis of GSH by SM (Raza *et al.*, 2011).

The results from the present study are in agreement with earlier studies wherein decreased SOD activity was observed in nerves isolated from diabetic rats (Cui *et al.*, 2008). SOD and CAT are major antioxidant enzymes involved in protection from oxidative stress and offers protection from highly reactive superoxide anions ($O_2^{\cdot-}$) and converts them to H_2O_2 (Halliwell, 1991). Hyperglycemia caused reduction in the activity of SOD in sciatic nerve of diabetic animals. Reduction in SOD activity in hyperglycemia might involve non-enzymatic glycosylation (Arai *et al.*, 1987). The results from the present study are in agreement with earlier studies wherein decreased SOD activity was observed in nerves isolated from diabetic rats (Cui *et al.*, 2008). Increased SOD activity after SM administration to the diabetic animals is in accordance with reported restoration of SOD activity by SM in serum (Cecen *et al.*, 2011) and liver (Jain *et al.*, 2011). CAT is responsible for the catalytic decomposition of H_2O_2 to O_2 and H_2O . The decreased CAT activity in diabetes

might reduce protection against free radicals. It is clear that the simultaneous reduction in the activity of both SOD and CAT makes the sciatic nerve more vulnerable to hyperglycemia-induced oxidative stress. Reports are available wherein SM has been shown to bring about improvement in the CAT activity during diabetic-induced nephrotoxicity in rats (Soto *et al.*, 2010). The results obtained emphasize that SM protects the sciatic nerve from hyperglycemia induced damage by restoring the activity of both these enzymes. Glutathione reductase is an important enzyme involved in maintaining high GSH/GSSG ratios (Carlberg and Mannervik, 1985). Present data showed a significant decrease in the activity of GR in sciatic nerve of diabetic animals. The results obtained from the earlier studies also showed depressed GR activity in sciatic nerve (Kamboj *et al.*, 2010), brain and other organs of diabetic animals (Ulusu *et al.*, 2003). The reversal of GR activity by SM treatment might result in increasing intracellular GSH levels. Recently, Ahmad (2012) and his colleagues reported that, SM markedly protected the PQ-induced hepatotoxicity by reducing oxidative stress, inflammation and by modulating xenobiotic metabolizing machinery. In present studies, the activity of GSH-Px was found to be significantly depressed in sciatic nerve of diabetic rats. This decrease in GSH-Px activity was reversed by the SM treatment. Glutathione reductase is responsible for the regeneration of GSH, whereas GSH-Px and GST work together with GSH in the decomposition of H_2O_2 or other organic hydroperoxides. A reduction observed in sciatic nerve GR, GSH-Px and GST activity in diabetic rats might be reflection of decreased protein thiols observed in the study as -GSH groups play a critical role in enzyme catalysis (Mak *et al.*, 1996). Silymarin treatment ameliorates decrease in the activity of these enzymes which might be mediated by GSH regeneration.

In conclusion, results obtained from the present study revealed that SM ameliorates hyperglycemia-induced thermal hyperalgesia, improves the neuropathic pain by reducing oxidative stress in the nerve of diabetic rats by virtue of its antioxidative property. Morphological assessments also show that the damage caused by streptozotocin to the sciatic nerve was also markedly reduced by the administration of SM. Finally these findings suggest that SM treatment might be beneficial in chronic diabetics exhibiting neuropathy.

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