

## Chronic oral silybum marianum aqueous extract attenuates streptozotocin-diabetic neuropathy

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### Abstract

**Background:** Oxidative stress is implicated as a final common pathway in the development of diabetic neuropathy and pharmacological interventions targeted at inhibiting free radical production have shown beneficial effects. Since Silybum Marianum (SM) is a plant rich in phenolics which their role against oxidative stress and inflammation have been shown in prior studies, we tried to determine the protective effect of aqueous extract of SM in streptozotocin (STZ)-induced diabetic neuropathy in rats.

**Methods:** Male wistar rats (n =40) were randomly divided into 4 groups, i.e. Vehicle-treated control (VC), Extract-treated control (EC), Vehicle-treated diabetic (VD), and Extract-treated diabetic(ED) groups. Animals in VC, EC, VD and ED groups received sterile saline or saline plus SM extract (200mg/kg) orally for 8 weeks. Diabetes was induced by a single intraperitoneal injection of STZ (60 mg/kg).

**Results:** We found significant reduction in motor nerve conduction velocity, tail flick latency in hot immersion test and hyperalgesia in both phases of formalin test of diabetic rats compared to age matched non-diabetic rats. SM treatment significantly improved the nerve conduction velocity and nociception in diabetic rats. The changes in lipid peroxidation status and anti-oxidant enzyme (Superoxide dismutase) levels observed in diabetic rats were significantly restored by SM treatment.

**Conclusion:** This study provides experimental evidence for protective effect of SM extract on nerve function and oxidative stress in animal model of diabetic neuropathy.

**Key words:** Silybum marianum, Nerve function, Diabetic neuropathy, Oxidative stress, Nociception

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## Introduction

Diabetes mellitus is a global health problem in developing as well as developed countries [1]. Uncontrolled chronic hyperglycemia in diabetic patients despite appropriate therapeutic management, leads to several complications including retinopathy, nephropathy, autonomic dysfunctions and neuropathy. Diabetic neuropathy is the most common complication affecting more than 50% of diabetic patients. Diabetes-induced deficits in motor and sensory nerve conduction velocities and other manifestations of peripheral diabetic neuropathy (PDN) have been well correlated with chronic hyperglycemia. Hyperglycemia has been reported to result in increased polyol pathway activity, oxidative stress, advanced glycation end product (AGE) formation, nerve hypoxia/ischemia, increased activation of protein kinase C and impaired nerve growth factor support. Oxidative stress resulting from enhanced reactive oxygen (superoxide radical, hydrogen peroxide and hydroxyl radical) and nitrogen species (peroxynitrite) and/or a defect in antioxidant defenses has been implicated in the pathogenesis of experimental diabetic neuropathy [2]. Antioxidant enzyme defense system (Superoxide dismutase, catalase and glutathione peroxidase) is also attenuated in peripheral nerves of diabetic animals indicating the vital role of oxidative stress in diabetic neuropathy [3]. Oxidative stress also activates down stream pathways such as poly (ADP-ribose) polymerase (PARP) and mitogen-activated protein kinases (MAPK) [4]. Long-term oxidative stress can mediate apoptosis of neurons and Schwann cells leading to nerve damage [5]. Above-mentioned studies suggest that oxidative stress may play a role in the etiology and pathogenesis of chronic complications of diabetes, and in the development of diabetic neuropathy. A large body of literature indicates that widespread plant phenolics—flavonoids, flavonolignans and phenolic acids—act at the molecular level as antioxidants [6, 7] and possess health-promoting properties [8]. *Silybum*

*marianum* L. (Asteraceae) is a plant rich in phenolics that contains flavonolignans (silibinin, also called silybin, and its diastereoisomers isosilybin, silydianin and silychristin), flavonoid taxifolin and other minor compounds [9, 10]. Silymarin, a standardized extract from the seeds of *S. marianum*, contains mainly silibinin and a minority of the other phenolics. The pharmacological profile and particularly, the cytoprotective function of silymarin is well documented. Nowadays, silymarin is used in supportive therapy namely of human chronic liver diseases [11]. The known modes of action of silymarin or silybin include both direct antioxidant activity mediated through scavenging of free radicals, and modulation of antioxidant and inflammatory enzymes [12], inhibition of mitogenic and cell survival signaling or modulations of cell-cycle regulators [13]. The main goal of the present study was to determine the neuroprotective effectiveness of *Silybum marianum* extract on nerve functions, nociception and oxidative stress in streptozotocin (STZ)-induced diabetic neuropathy model in rats.

## Methods

### Animals

Male albino Wistar rats (the Pasteur institute of Iran, Tehran), weighing 250-270 g (9-10 weeks old) were housed in an air-conditioned colony room on a light/dark cycle at  $21 \pm 3^\circ\text{C}$  and supplied with standard pellet diet and tap water ad libitum. Procedures involving animals and their care were conducted in conformity with the standard guidelines set out for the care and use of laboratory animals. All animals were acclimatized for minimum period of 1 week prior to the beginning of study. The animals were randomly divided into four experimental groups; i.e. vehicle-control (VC, n=9), extract-treated control (EC, n=9), saline-treated diabetic (VD, n=9) and extract-treated diabetic (ED, n=9). For formalin test two groups, sodium salicylate (SS)-treated control and diabetic groups were used as positive control for formalin

test. In this respect, VC and VD groups, received orally 0.5 ml of sterile saline (once daily for 8-weeks) and EC and ED groups were fed with aqueous extract of *Silybum marianum* using a gavage needle at a dose of 200 mg/kg once daily for a period of 8 weeks or after diabetes induction. SS (200 mg/kg, i.p.) was administered 1 h before conducting the formalin test.

Diabetes was induced by a single intraperitoneal injection of STZ (60 mg/kg) dissolved in cold 0.9% saline immediately before use. Serum glucose level and body weight were monitored at the beginning and the end of the experiment. Diabetes was confirmed after 48h of STZ injection by determining plasma glucose levels using GOD/POD kit (Zistchimie, Tehran, Iran). Rats with plasma glucose levels of >250 mg/dl were included for further studies.

#### **Preparation of *Silybum marianum* extract**

Fresh leaves of *Silybum marianum* were separated, cleaned and dried at room temperature under shade. Thereafter, 150 g of dried leaves was grounded and the obtained powder was mixed with 1000 ml of distilled boiling water for a period of 15 min under continuous stirring. The obtained mixture was filtered funnel, and the obtained liquid was vacuum dried until a concentrated residue (24%, w/w) was obtained. This stock extract was maintained at -20°C until being used. Lower concentrations of the extract were prepared by dilution of the stock with cold and sterile 0.9% saline solution.

#### **Experimental procedure**

There is some evidence that diabetic neuropathy develop within 6-weeks after the induction of diabetes (Kumar et al., 2005). In this study, development of diabetic neuropathy was ensured before starting the treatment by measuring motor nerve conduction velocity (MNCV) and hyperalgesia after 6-weeks of diabetes induction. All parameters (MNCV, hyperalgesia, lipid peroxidation and antioxidant enzyme activity level) were

evaluated at the completion of treatment (8-weeks). STZ was obtained from Pharmacia and Upjohn (USA). All other chemicals were purchased from Merck (Germany). STZ was freshly dissolved in 0.9% saline solution.

#### **MNCV measurement**

At the completion of the 8-weeks feeding protocol, animals were anaesthetized by i.p. injection of xylazine (20 mg/kg) and ketamine (100 mg/kg). Stimulation and recording were performed on the exposed sciatic nerve trunk using platinum electrodes. Apertures were carefully made through the overlying skin and muscle so that the sciatic nerve in the right leg was exposed at three sites. The two proximal sites, the first located near the sciatic notch and the second set to be approximately 15 mm distal, were used for stimulating, and the third site at the distal end of the tibial nerve, located above the foot, was used for recording. The stimulating and recording electrodes were placed directly under the sciatic nerve. The nerve was bathed in paraffin and maintained at 37°C under radiant heat. The whole animal body temperature was maintained using a small animal thermal operating table. The sciatic nerve of each rat was stimulated by monophasic voltage pulses delivered directly to the nerve at 20% above threshold for the myelinated A-fibers for each animal. The compound action potentials were fed through protek pc-based storage oscilloscope into related computer software for capture and analysis (Hungchong, Korea). Action potentials from both stimulating sites were averaged six to eight times. Following the MNCV recordings, sutures were placed around the sciatic nerve at the sites of the stimulating electrodes and the actual distance between them on the excised nerve was measured using vernier calipers. Conduction velocity was determined from the difference in latencies of the action potentials and the nerve length separating the two cathodal stimulation points [14].

**Thermal hyperalgesia: hot immersion test**

The tail of each rat was submerged in 29 °C water for 30 min before testing began. Thereafter, the whole tail of each rat was submerged in 49 °C water. The time taken for the rat to show a characteristic tail flick response was recorded with a stopwatch. The test was repeated three times for each rat, with at least one minute between each measurement, and the average of the three measurements was recorded as the withdrawal latency for each rat. The tail was removed from the water if the rat had not reacted after 30 s [15].

**Formalin test**

For assessment of pain, formalin test was used according to the previously described method [16]. Briefly, each animal was acclimatized to the observation box before any testing began. Then, it was given a subcutaneous injection of 50 µl of 2.5% formalin into the plantar surface of one hind paw. It was then immediately placed in a Plexiglas box. Observations continued for the next 60 min. A nociceptive score was determined for 5 min blocks by measuring the amount of time spent in each of the four behavioral categories: 0, the position and posture of the injected hind paw is indistinguishable from the contralateral paw; 1, the injected paw has little or no weight placed on it; 2, the injected paw is elevated and is not in contact with any surface; 3, the injected paw is licked, bitten, or shaken. Then, a weighted nociceptive score, ranging from 0 to 3 was calculated by multiplying the time spent in each category by the category weight, summing these products and dividing by the total time for each 5 min block of time. The first 10 min post-injection was considered as the early phase, and the time interval 15–60 as the late phase.

**Determination of MDA concentration**

After a 6-h incubation of sciatic nerve, it was blotted dry and weighed, then made into 5% tissue homogenate in ice-cold 0.9% NaCl solution. A supernatant was obtained from tissue homogenate by centrifugation

(1000×g, 4 °C, 10 min). The MDA concentration (thiobarbituric acid reactive substances, TBARS) in the supernatant was measured. Briefly, 1.0 mL of 20% trichloroacetic acid and 1.0 mL of 1% TBARS reagent were added to 100 µL supernatant, then mixed and incubated at 100 °C for 80 min. After cooling on ice, samples were centrifuged at 1000×g for 20 min and the absorbance of the supernatant was read at 532 nm. TBARS results were expressed as MDA equivalents using tetraethoxypropane as standard [17].

**Assay of SOD activity in sciatic nerve**

The supernatant of tissue homogenate of the sciatic nerve was obtained as described earlier. A competitive inhibition assay was performed by using xanthine/xanthine oxidase reaction-generated superoxide radicals to reduce nitro blue tetrazolium (NBT) quantitatively to blue formazan. Conversion of superoxide radicals to hydrogen peroxide by superoxide dismutase inhibited dye formation and served as a measure of superoxide dismutase activity. Briefly, the supernatant of 0.5 mL with xanthine 50 µmol/l and xanthine oxidase 2.5 µmol/L in potassium phosphate buffer 50 mmol/L (pH 7.8, 37 °C) were incubated for 40 min and NBT was added. Blue formazan was then monitored spectrophotometrically at 550 nm. The amount of protein that inhibited NBT reduction to 50% maximum was defined as 1 nitrite unit (NU) of SOD activity [17].

**Statistical analysis**

All values were given as mean ± SEM. Statistical analysis was carried out using student's paired *t*-test and one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Statistical P-value less than 0.05 was considered as significant.

**Results**

Body weight and serum glucose level have been shown in Table 1. No marked alteration in body weight or food or water

intake was observed following 8-weeks administration of silybum marianum extract (200 mg/kg/day) in EC group compared to VC group. After 8 weeks, the weight of vehicle-treated diabetic rats was found to be significantly decreased compared to

control rats ( $P=0.03$ ). Untreated diabetic rats had also an elevated serum glucose level over those of control rats ( $P<0.0001$ ). Treatment of diabetic rats with aqueous extract of silybum marianum, significantly lowered the serum glucose ( $P<0.0001$ ).

**Table 1: The effect of eight week treatment of silybum marianum extract on body weight and serum glucose level**

Parameters	Vehicle+control VC	Extract+control EC	Vehicle+diabetic VD	Extract+diabetic ED
Body wt.(g)	282.33±3.38	279.92±6.75	238.33±6*	259.7±9.01
Serum glucose (mg/dl)	119.4±6.6	139.7±8.39	355±12.1†	107.42±5.82‡

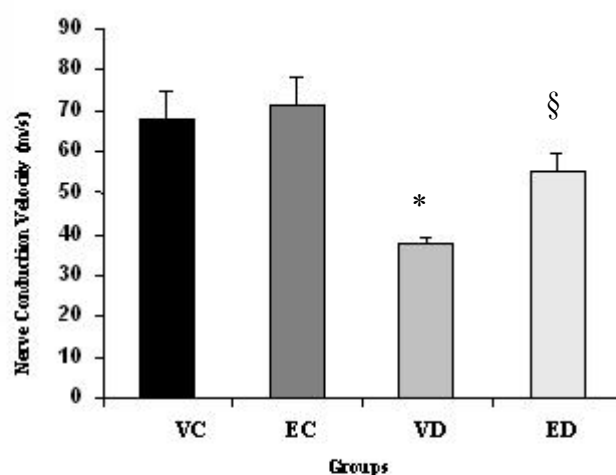
Data are represented in Mean±SEM, \* $P=0.003$  vs VC group; †  $P<0.0001$  vs VC group; ‡ $P<0.0001$  vs VD group

### Motor nerve conduction velocity (MNCV)

A significant decrease in MNCV was observed after 8 weeks of diabetes induction ( $P<0.01$ ). Eight weeks feeding with silybum marianum extract (200mg/kg/day) significantly reversed MNCV deficits in diabetic rats ( $P=0.03$ ) But there were no significant changes in MNCV in EC group compared to VC group (Fig. 1).

### Thermal hyperalgesia

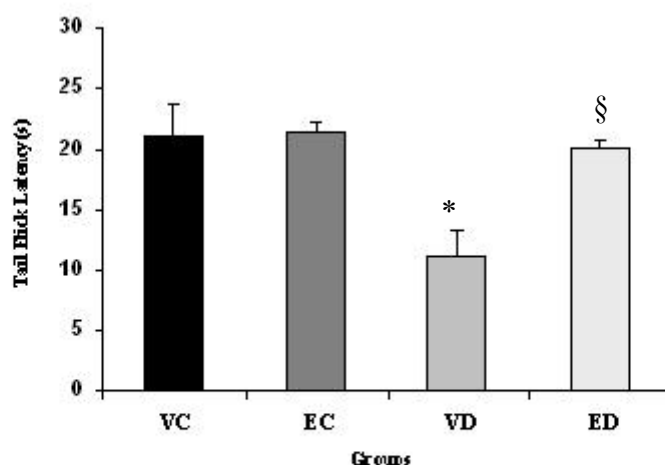
A significant decrease in tail flick latency was observed after 8 weeks of diabetes induction in hot immersion test ( $P=0.0002$ ). This deficit in tail flick response latency was significantly reversed at eighth week treatment with extract of silybum marianum ( $P=0.02$ ). Extract treatment of control group caused no considerable change in tail flick response latency compared to VC group (Fig. 2).



**Figure 1. The effect of 8-weeks treatment with silybum marianum extract on motor nerve conduction velocity.**

All data represent mean±SEM (n=9). \*  $P<0.01$  compared with VC group; §  $P=0.03$  compared with VD group





**Figure 2.** The effect of 8-weeks treatment with silybum marianum extract on hot immersion test.

All data represent mean  $\pm$  SEM (n=9). \*P=0.0002 compared with VC group; § P=0.03 comparing to VD group

### Formalin test

Hind limb formalin injection produced a marked biphasic response in the rats of all groups. Hyperalgesia was significantly ( $P = 0.03-0.006$ ) greater in untreated diabetic than in control rats in both phases of the test. Treatment of control and diabetic rats with sodium salicylate (200 mg/kg, i.p.) caused a significant reduction ( $P < 0.01$ ) in nociceptive score only in the second phase of the formalin test. In addition, diabetic animals receiving silybum marianum extract showed a less intensive nociceptive behavior in both phase of the formalin test ( $P=0.006$ ) (Fig. 3).

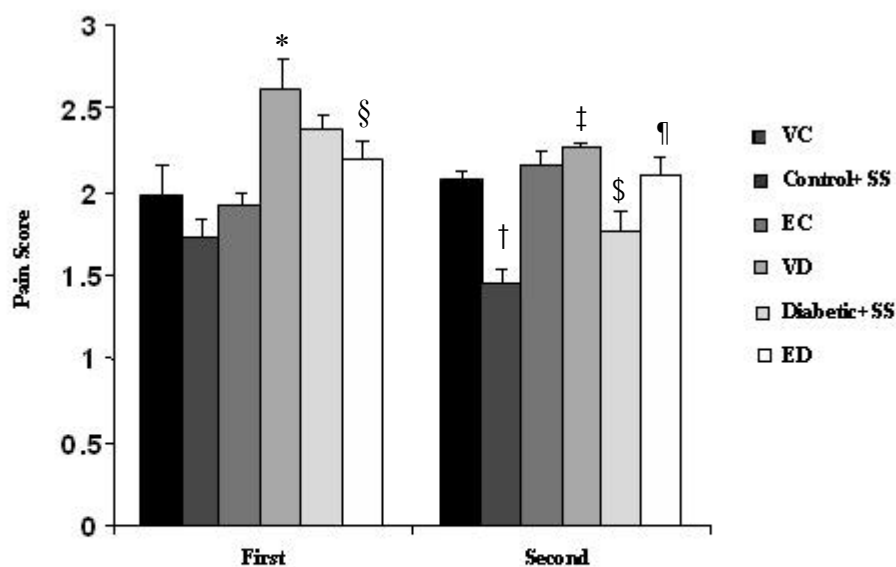
### Lipid peroxidation

MDA level in eighth week diabetic rats was significantly ( $P < 0.001$ ) increased as compared to the age-matched control rats ( $3.56 \pm 0.46$  vs.  $10.12 \pm 0.79$  nM/ml). Eight

weeks treatment with extract of silybum marianum inhibited this increase in MDA levels in treated diabetic rats ( $P < 0.01$ ). There was no significant change on MDA level in EC group compare to VC group (Fig.4).

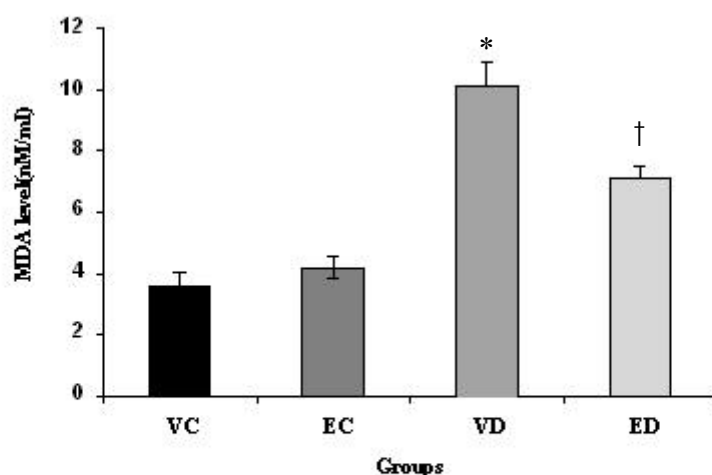
### SOD activity

At the eighth week, SOD activity of diabetic rats was significantly decreased ( $94.75 \pm 2.72$  U/ml) as compared to the age-matched control rats ( $154 \pm 23.58$  U/ml). Eight weeks treatment with extract of silybum marianum significantly inhibited decline in SOD activity of treated diabetic rats as compared to vehicle-treated rats ( $P < 0.01$ ). Extract treatment of control group caused no considerable change on SOD activity compared to VC group (Fig. 5).



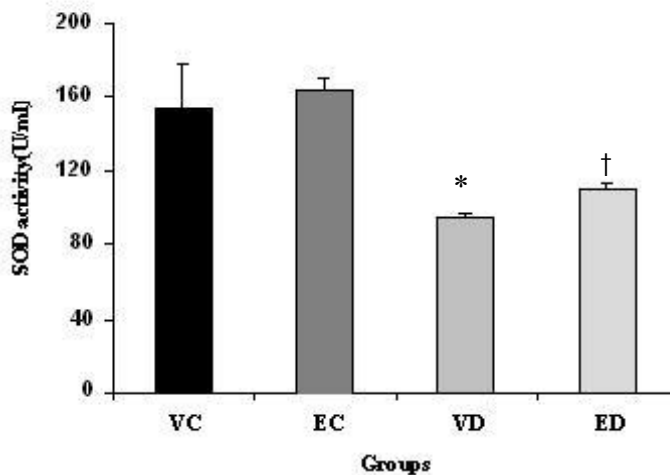
**Figure 3.** The effect of 8-weeks treatment with silybum marianum extract (200 mg/kg) and sodium salicylate (SS, 200 mg/kg) on nociceptive scores in the first (early) and second (late) phases of the formalin test.

All data represent mean±SEM (n=9). \*P=0.03 compared with VC group; §P=0.03 compared with VD group; †P=0.0003 compared with VC group; ‡P=0.006 compared with VC group; §P=0.01 compared with VC group; ¶P=0.03 compared with VD group



**Figure 4.** The effect of silybum marianum extract on serum MDA level.

data represent mean±SEM (n=9). \*P<0.001 compared with VC group; †P=0.02 compared with VD group



**Figure 5. The effect of 8 weeks treatment with silybum marianum extract on serum SOD activity.**  
All data represent mean $\pm$ SEM (n=9). \* P<0.001 compared with VC group; † P=0.02 compared with VD group

## Discussion

Development of diabetic neuropathy was evident in 8-weeks induced diabetic rats from reduction in MNCV with hyperalgesia. We observed about 42% deficit in MNCV after 8-weeks of diabetes induction. These results are consistent with the previous reports, wherein similar reductions of MNCV in STZ-induced diabetic rats were reported [18].

Diabetic neuropathy is a heterogeneous group of disorders and with widely varying pathology [2]. Several interactive pathogenetic mechanisms have been identified and correlated with elevated blood glucose levels.

Various pathways like activation of polyol pathway, activation of protein kinase C, oxidative stress, formation of advanced glycation end (AGE) product, MAPK activation and PARP activation have been reported to play an important role in diabetic neuropathy. Oxidative stress is one common link between all these pathways and causes vascular impairment leading to endoneurial hypoxia. Decrease in nerve blood flow and resulting endoneurial hypoxia may lead to reduced motor nerve

conduction velocity [19]. Impaired endothelium dependent vasodilatation has been demonstrated in various vascular beds in diabetic patients and diabetic animals [20]. Free radicals such as superoxides and hydroxyl radicals cause vascular endothelial damage and reduced nitric oxide mediated vasodilatation [21]. Studies have provided evidence that superoxides and peroxynitrite impairs endothelium dependent vascular relaxation of epineural arterioles of the sciatic nerve from diabetic rats [22]. Hence, from the above results it is convincing to assume that the amelioration of oxidative stress using potent antioxidants can be beneficial in diabetic neuropathy.

In this study, we evaluated the effect of protective properties of silybum marianum extract on the development of diabetic neuropathy. The ability of SM extract to protect a cell membrane against xenobiotic injury is attributed principally to its antioxidant potential to eliminate reactive oxygen species (ROS) [23, 24]. It can be associated with free radical scavenging [24], chain-breaking activity [25] and a reduction in ROS production [26]. It is possible that the silybum marianum



treatment schedule reversed motor nerve conduction velocity through improvement of nerve blood flow. In addition to vascular mechanisms, non-vascular mechanisms have also been reported to cause nerve conduction deficits [27]. The PARP inhibitors known to inhibit energy depletion due to oxidative stress, restore nerve energy status and prevent reduction in conduction deficits [4]. Antioxidants such as  $\alpha$ -Lipoic acid, vitamin E, vitamin C, and N-acetyl-L-cysteine increase energy metabolism, reduce advanced glycation end product formation [28] and inflammation.  $\alpha$ -Lipoic acid also increases energy metabolism and myo-inositol levels in nerves isolated from diabetic animals [29]. N-acetyl-L-cysteine and  $\alpha$ -tocopherol are reported to reduce pro-inflammatory cytokines (IL, TNF- $\alpha$ ), chemokines and C-reactive proteins in diabetic rats [30]. Silymarin is reported to improve energy metabolism and reduce inflammation via inhibition COX-2, 5-lipoxygenase activity and reduction NF- $\kappa$ B and leukotriene levels, although not in the diabetic context [31]. Silymarin is also shown to reduce the activity of polyol pathway by inactivation of aldose reductase enzyme. Also, in diabetic rats, we observed a significant increase in MDA levels and reduction in SOD activity. Kishi et al. have reported that changes in antioxidant enzyme activity may be related to duration of diabetes or due to post-translational modifications [32]. In our study, lipid peroxidation (MDA levels) was significantly reduced and SOD activity was significantly improved following treatment with silybum marianum. Similar to silybum marianum,  $\alpha$ -Lipoic acid is also reported to improve levels of these antioxidant enzymes and restore deficits in diabetic neuropathy [33].

Neuropathic pain is most common symptom associated with diabetic neuropathy, thus we evaluated the nociceptive response in diabetic rats. Nociception was observed in diabetic rats, which is in accordance with several other

reports [34]. In the present study, we observed reduction in tail flick latencies in hot immersion test and greater hyperalgesia in untreated diabetic than in control rats in both phases of the formalin test.

Several Mechanisms, such as direct functional toxicity of hyperglycemia in the peripheral nervous system [35], an increased activity of primary afferent fibers leading to an increased excitatory tone within the spinal cord, increased release of glutamate and activation of the NMDA receptors, reduced activity of both opioidergic and GABAergic inhibitory systems [36], decreased activity of nNOS-cGMP system in neurons of dorsal root ganglion [37], altered sensitivity of the dopaminergic receptors and altered responsiveness of the dopaminergic system, possibly through the enhancement and/or deactivation of the endogenous Met-enkephalinergic system [38], and alterations in L-type  $\text{Ca}^{2+}$  channels [39] tissue injury due to ischemia, sensitization of peripheral receptors and ectopic activity in sprouting fibers and alterations in dorsal root ganglia cells, increased cyclooxygenase-2 (COX-2) protein and COX mediated PGE2 release could be involved in the modulation of nociception in diabetic rats. In the present study, we observed a significant improvement in tail flick response latency for hot immersion performance and analgesic effect in both phases of the formalin test in diabetic rats upon Silybum marianum extract treatment. On the other hand, sodium salicylate significantly reduced the nociceptive score only in the second phase of the formalin test in control and diabetic rats. It has been known that central acting drugs like narcotics inhibit both phases of the formalin test equally [40], while peripheral acting drugs like aspirin only inhibit the late phase [41]. Therefore, the effect of Silybum marianum extract in this study could be mediated possibly through a central and/or a peripheral mechanism. Of possible mechanisms that could partly explain the

beneficial analgesic properties of Silybum marianum extract may be attributed to its hypoglycemic [42], antioxidant [43] and anti-inflammatory effects.

In conclusion, silybum marianum extract revealed significant protective effects on diabetic neuropathy as evident from improvement in MNCV and reduction in nociception. Protective effects of silybum

marianum extract may be attributed to reduction in oxidative stress (reduction in MDA and increase in SOD levels).

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