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# Improvement of inflammatory and toxic stress biomarkers by silymarin in a murine model of type one diabetes mellitus

**Research Article**

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**Abstract:** Type 1 diabetes mellitus (T1DM) is characterized by an impairment of the insulin-secreting beta cells with an immunologic base. Inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β and free radicals are believed to play key roles in destruction of pancreatic β cells. The present study was designed to investigate the effect of *Silybum marianum* seed extract (silymarin), a combination of several flavonolignans with immunomodulatory, anti-oxidant, and anti-inflammatory potential on streptozotocin (STZ)-induced T1DM in mouse. Experimental T1DM was induced in male albino mice by IV injection of multiple-low-doses of STZ for 5 days. Seventy-two male mice in separate groups received various doses of silymarin (20, 40, and 80 mg/kg) concomitant or after induction of diabetes for 21 days. Blood glucose and pancreatic biomarkers of inflammation and toxic stress (IL-1β, TNF-α, myeloperoxidase, lipid peroxidation, protein oxidation, thiol molecules, and total antioxidant capacity) were determined. Silymarin treatment reduced levels of inflammatory cytokines such as TNF-α and IL-1β and oxidative stress mediators like myeloperoxidase activity, lipid peroxidation, carbonyl and thiol content of pancreatic tissue in an almost dose dependent manner. No marked difference between the prevention of T1DM and the reversion of this disease by silymarin was found. Use of silymarin seems to be helpful in T1DM when used as pretreatment or treatment. Benefit of silymarin in human T1DM remains to be elucidated by clinical trials.

**Keywords:** *Silymarin • Diabetes • Antioxidants • Streptozotocin • Oxidative stress • Inflammatory cytokines*



## 1. Introduction

Diabetes mellitus is characterized by hyperglycemia and some metabolic dysfunctions [1]. Type one diabetes mellitus (T1DM) as an autoimmune disease is defined as a condition in which insulin-producing β-cells (IPβCs) in pancreatic islets are destroyed through a T-cell mediated process. Although the exact trigger for initiation of T1DM is unclear, several mechanisms have been proposed. T1DM is associated with a massive sequestration of immune cells into the pancreas leading to insulitis. Accumulated immune cells in these lesions result in an immense release of cytokines and activation of other immune cells triggering a cascade of destruction. Activated T-cells not only devastate IPβCs cytotoxically, but also are able to produce a large amount of cytotoxic components including various proinflammatory cytokines and reactive oxygen species (ROS) leading to toxicity and cell death. Furthermore, effector T cells can promote production of autoantibodies and autoimmune process by activating B cells [2].

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Hyperglycemia is the main complication of diabetes. Investigations show that glucose is a proinflammatory mediator, which enhances intracellular levels of nuclear factor-kappa B (NF-κB), the main mediator of tumor necrosis factor (TNF)-α. It has been illustrated that NF-κB is responsible for over-expression of innate immune mediators [3]. Activation of NF-κB causes destruction and dysfunction of IPβCs [4]. Moreover, glucose itself promotes proinflammatory cytokines and inflammatory matrix metalloproteinase (MMP)-2. Insulin resistance, which occurs through overexpression of c-jun N-terminal kinase (JNK), a mediator of stress and inflammation, is also a consequence of hyperglycemia [5].

One major mechanism in pathogenesis of T1DM is toxic stress. Referring to oxidative and nitrosative stress, toxic stress is a result of an imbalance between production of ROS (which are by-products of glucose auto-oxidation) and radical-scavenging systems. The detrimental activity of these molecules not only damages IPβCs but also is responsible for many micro- and macrovascular complications of diabetes [6-8]. Insulin-therapy is the main consideration in management of T1DM.

For many years, there has been a huge interest in using herbal extracts in order to control the initiation or perpetuation of diabetes [9,10]. One of the most remarkable herbal extracts in this regard is silymarin. Silymarin, which is isolated from seeds of *Silybum marianum* (milk thistle), is a phenolic compound [11,12] whose effectiveness in attenuating Type 2 diabetes has been previously confirmed in human and animal models [13,14]. Silymarin is shown to have anti-hyperglycemic effect through inhibiting hepatic glucose-6-phosphatase and gluconeogenesis [13]. Parallel to this research, a recent investigation on an STZ-induced model of diabetes has exhibited that silymarin can increase hepatocytes membrane permeability to glucose [15]. It is mainly recommended for the treatment of hepatic disorders, since it is shown to accelerate protein biosynthesis and cell-regeneration in hepatic cells alongside showing anti-lipid peroxidation traits [16]. It is demonstrated that silymarin not only possesses anti-oxidant and free radical scavenging properties but also is able to restore depleted levels of glutathione, upregulate expression of superoxide dismutase and enhance activity of catalase [12,17]. Finally, research demonstrates that this flavonoid possesses a phosphodiesterase (PDE) inhibitory effect [18,19]. Accordingly, it is able to enhance levels of cyclic AMP, which ameliorates the harmful consequences of oxidative stress on cell functions [20-22].

Another conspicuous characteristic of silymarin is having anti-inflammatory and immunomodulatory traits. There is evidence that silymarin regulates inflammatory mediators like TNF-α, interleukin (IL)-1β, IL-6 [23], IL-1 receptor antagonist and nitric oxide [24]; down-regulates leukotriene and prostaglandin synthesis, which are potent neutrophil chemoattractants [23,25,26] through inhibition of cyclooxygenase-II; stabilizes mast cells; lessens the cytotoxic activity and proliferation of $CD8^+$ lymphocytes and decreases neutrophil sequestration to the site of inflammation [23]. Silymarin inhibits NF-κB signaling pathway, which is the main pathway in TNF-α destructive effects [27].

Recent study indicated the potential of silymarin in reduction of toxic stress and inflammatory biomarkers in experimental inflammatory bowel disease [17], which has a similar immunological pathology to diabetes [28].

The notable anti-hyperglycemic, immunomodulatory, anti-oxidative and anti-inflammatory properties of silymarin led us to investigate the possible benefit of silymarin in a murine model of T1DM by evaluating the biomarkers of inflammation and toxic stress.

## 2. Experimental Procedures

### 2.1 Chemicals

2,4,6-Trinitrobenzene sulphonic acid (TNBS), thiobarbituric acid (TBA), trichloroacetic acid (TCA), n-butanol, hexadecyl trimethyl ammonium bromide (HETAB), 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), hydrochloric acid (HCL), malondialdehyde (MDA), ethylene diamine tetra acetic acid (EDTA), 2,4-dinitrophenylhydrazine (DNPH), O-dianisidine hydrochloride, hydrogen peroxide, Coomassie Brilliant Blue G-250, sodium hydroxide (NaOH), acetic acid, sodium acetate, bovine serum albumin (BSA), ferric chloride ($FeCl_3$-$6H_2O$), sodium sulphate ($Na_2SO_4$), sulphuric acid ($H_2SO_4$), phosphoric acid ($H_3PO_4$), potassium dihydrogen phosphate ($KH_2PO_4$), potassium hydrogen diphosphate ($K_2HPO_4$), hydrogen peroxide ($H_2O_2$), sodium carbonate ($Na_2CO_3$), Na-K-tartarate, cupric sulphate ($CuSO_4$-$5H_2O$), guanidine hydrochloride, trifluoroacetic acid (TFA), ethanol, ethyl acetate, from Merck (Tehran), silymarin from Goldaru (Esfahan), dexamethasone from Daru-Pakhsh (Tehran), mouse specific TNF-α and IL-1β ELISA kits from Nimapouyesh (Tehran), Accu-Chek Active glucose meter from Roche (Germany) were used in this study.

### 2.2 Animals

Male albino mice from animal house of the School of Pharmacy, TUMS were used. Animals were kept under standard circumstances of temperature (23±1ºC), relative humidity (55±10%), and 12/12 hours light/

dark cycle, and fed with a standard pellet diet and water *ad libitum*. Animals were maintained in standard polypropylene cages with a wire mesh top. All ethical themes of the studies on animals were considered carefully and the experimental protocol was approved by the IRB.

## 2.3 Experimental design

Eight groups of nine male mice were considered in this study. Diabetes was induced by multiple low dose (40 mg/kg/day) administration of streptozotocin (STZ), for 5 consecutive days [29]. Six groups were considered as silymarin-treated (Sil) groups, in which silymarin was administered in three doses of 20 (Sil-20), 40 (Sil-40) and 80 (Sil-80) mg/kg. Two other groups were controls, one which received STZ and the second a sham group in which diabetes was not induced. Animals were considered diabetic where non-fasting blood glucose levels were more than 200 mg/dl. Tail vein blood samples were collected in days 1, 7, 14 and 21 following the first injection of STZ for determination of fed blood glucose (Fed-BG). The Fed-BG was measured using a blood glucose meter (Accu-Chek Active).

Administration of silymarin was started after induction of diabetes from day 5 post administration of STZ and continued for 21 days as Sil-1 series. In Sil-2 series animals received silymarin simultaneous with STZ started from day 1 and continued for 21 days. Silymarin was administered by gavage in a volume of 0.01 ml/kg. Control and sham groups received normal saline in the same volume. On the 22$^{th}$ day, animals were anaesthetized using sodium pentobarbital at 45 mg/kg [28]; the abdomen was dissected and the pancreas removed. At the end of the procedure, an overdose of ether inhalation was used to sacrifice all mice.

## 2.4 Pancreatic sample preparation

The samples were rinsed gently using normal saline, weighed and homogenized in 10 volumes ice cold 50 mM potassium phosphate buffer (pH 7.4). 100 µl of each sample was taken for total anti-oxidant assay and stored at -80ºC until analysis. The remaining homogenates were sonicated and then centrifuged for 30 min at 3500 g. The plates were separated and the supernatants were transferred into several microtubes for separate biochemical assays and all were maintained at -80ºC until analyses [28].

## 2.5 IL-1β and TNF-α in pancreatic tissue

An enzyme-linked immunosorbaent assay mouse specific ELISA kit was used for quantitative detection of TNF-α and IL-1β levels in pancreatic homogenate. According to this procedure the intensity of final colored product is proportional to the concentration of cytokine in the sample. The absorbance was measured in 450 and 620 nm respectively as the primary wavelength and the reference wavelength. Data was expressed as pg/mg protein of tissue.

## 2.6 Lipid peroxidation in pancreatic tissue

Thiobarbituric acid-reactive substance (TBARS) assay was used to measure lipid peroxidation level in pancreatic homogenate. Results were reported as µM/g of tissue. Details have been described previously [30].

## 2.7 Protein oxidation in pancreatic tissue

The carbonyl molecules (CM) of tissue as a marker of protein oxidation was measured using DNPH as a reagent which reacts with CM producing hydrazones which can be measured by absorbance at 370 nm spectrophotometerically [31]. Data was reported as nM/mg protein of tissue.

## 2.8 Total anti-oxidant capacity (TAC) of pancreatic tissue

Ferric reducing anti-oxidant power (FRAP) assay was used to evaluate TAC in sample homogenates. Data was expressed as mM ferric ions reduced to ferrous form *per* g of tissue as described previously [32].

## 2.9 Total thiol molecules (TTM) in pancreatic tissue

TTM was measured using DTNB as a reagent. Reaction of DTNB with TTM leads to production of a yellow complex measured spectrophotometrically by absorbance at 412 nm. Results were expressed as µM/mg protein of tissue [33].

## 2.10 Neutrophil infiltration in pancreatic tissue

Inflammation leads to a massive sequestration of activated immune cells, especially neutrophils, to the site of inflammation. Myeloperoxidase (MPO) is an oxidative enzyme released from neutrophils during the course of inflammation. Its activity and levels has been used in order to evaluate neutrophil infiltration to the injured tissue previously. Data was reported as mU/mg protein of tissue [34].

## 2.11 Total protein (TP) of pancreatic tissue

According to Bradford method, following binding to proteins, the maximum absorbance of the colored reagent Coomassie Brilliant Blue changes from 465 nm to 595 nm and the latter is measured spectrophotometrically [35]. Data was expressed as mg/ml of homogenized pancreatic tissue.

### 2.12 Statistical analysis

The data were analyzed by one-way ANOVA followed by Newman Keul's *post hoc* test for multiple comparisons to make sure the variances of data are distributed properly. Results are reported as mean ± standard error of the mean (SEM) and a P value less than 0.05 was considered significant.

## 3. Results

### 3.1 Plasma glucose levels

Fed-BG levels were measured in days 1, 7, 14 and 21 subsequent to the first administration of STZ. The Fed-BG concentration was not altered significantly following administration of STZ in day 1 as compared to sham group. Treatment of animals with silymarin did not change this result in day 1 in either group (P>0.05). Furthermore, the results of Fed-BG measurement in day 7 showed that administration of silymarin did not improve hyperglycemia (P>0.05). Fed-BG levels in day 14 demonstrated that hyperglycemia improved noticeably in Sil-40-1 and Sil-80-1 groups compared to diabetic rats (P<0.01) while no significant difference was observed between these two groups (P= 0.96). Moreover administration of silymarin normalized Fed-BG in Sil-40-2 and Sil-80-2 groups (P<0.01) when compared to control group whereas there was no significant difference between these two groups (P= 0.96). There was no considerable difference between Sil-40-1 and Sil-40-2 (P= 0.99) groups or Sil-80-1 and Sil-80-2 (P= 0.18) groups in terms of Fed-BG levels. Measuring Fed-BG at day 21, animals became normoglycemic when compared to control group (P<0.01). In addition, no significant difference was observed between silymarin-treated groups in terms of Fed-BG concentration (P>0.05) (Figure 1 and 2).

### 3.2 Pancreatic TNF-α level

Inflammation resulted in a significant increase in TNF-α level in diabetic rats in comparison with the sham group (P<0.01). TNF-α was considerably decreased in Sil-20-1, Sil-40-1 and Sil-80-1 groups (P<0.01). Its level in Sil-20-1 was approximately near the level gained in Sil-40-1 group (P=0.98). Additionally, among these three groups, TNF-α level was significantly lower in Sil-80-1 group when compared to others (P<0.01).

Administration of silymarin caused a significant decrease in TNF-α level of Sil-20-2, Sil-40-2, and Sil-80-2 groups (P<0.01). TNF-α levels were significantly

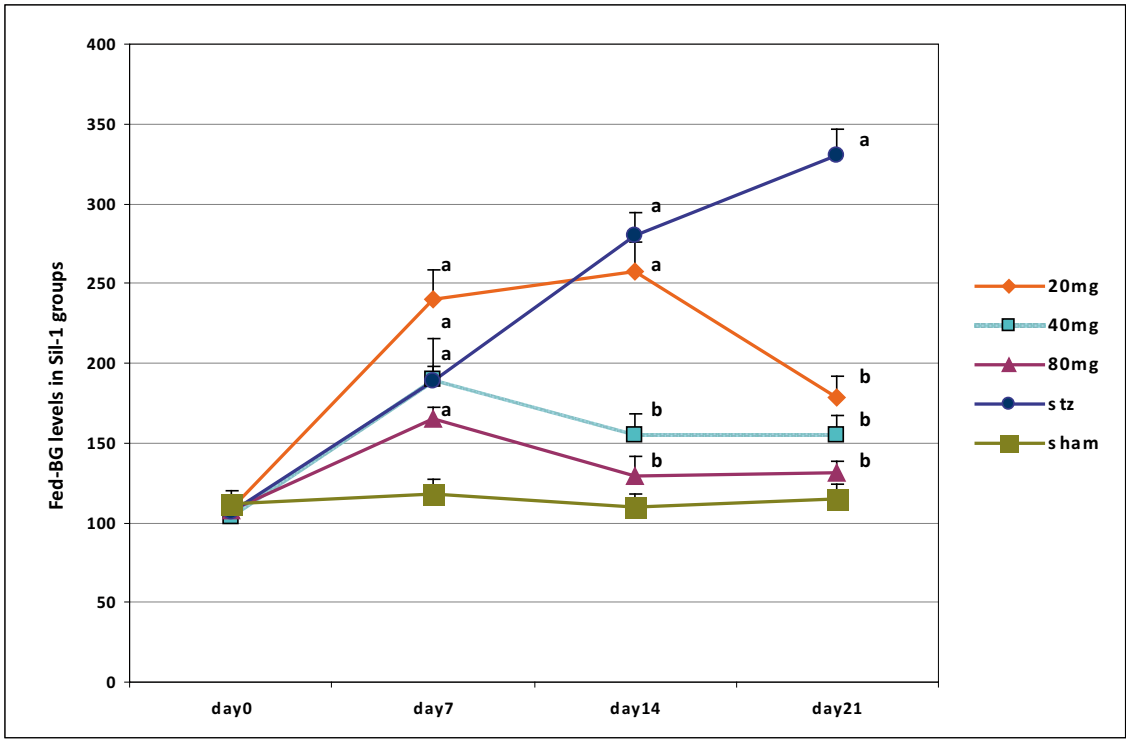


**Figure 1.** Fed blood glucose in serum of Sil-1 groups of rats. Values are mean±SEM. [a]Significantly different from sham group at P<0.01. [b]Significantly different from control group at P<0.01.

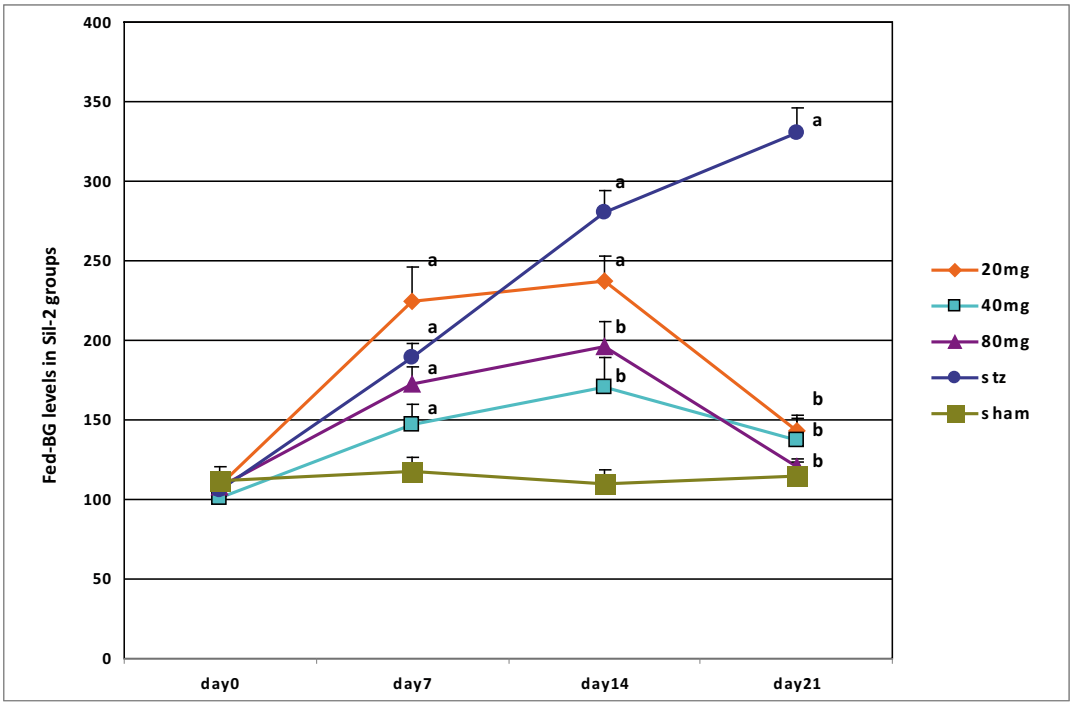


**Figure 2.** Fed blood glucose in serum of Sil-2 groups of rats. Values are mean±SEM. [a]Significantly different from sham group at P<0.01. [b]Significantly different from control group at P<0.01.

lower in Sil-40-2 and Sil-80-2 groups when compared to Sil-20-2 group (P<0.01), while no considerable difference was observed between Sil-40-2 and Sil-80-2 groups (P=1.00).

There was no considerable difference between Sil-20-1 and Sil-20-2 groups (P=0.98) and also between Sil-80-1 and Sil-80-2 groups (P=1.00). However, TNF-α level was significantly lower in Sil-40-2 group when compared to Sil-40-1 group (P<0.01) (Figure 3).

## 3.3 Pancreatic IL-1β levels

IL-1β level was significantly increased in diabetic rats compared to normal rats (P<0.01). IL-1β level was significantly lowered in Sil-20-1, Sil-40-1 and Sil-80-1 groups (P<0.01). Although its level was significantly higher in Sil-20-1 when compared to two other groups (P<0.01), no significant difference was observed between Sil-40-1 and Sil-80-1 in terms of IL-1β level (P=0.61).

IL-1β was considerably lower in Sil-20-2, Sil-40-2 and Sil-80-2 when compared to control group, although there was no significant difference in terms of IL-1β level between these three groups (P>0.05).

IL-1β level was significantly lower in Sil-20-2 group in comparison with Sil-20-1 group (P<0.01). However, there was no significant difference between Sil-40-1 and Sil-40-2 groups (P=0.83), and also Sil-80-1 and Sil-80-2 groups (P=0.96) (Figure 4).

## 3.4 Pancreatic lipid peroxidation as TBARS

The oxidative stress condition led to a significant increase in TBARS levels in control group as compared to sham group (P<0.01). Even though TBARS value was not improved significantly in Sil-20-1 group (P=0.71), it was considerably decreased in Sil-40-1 and Sil-80-1 groups in comparison to diabetic rats (P<0.01). There was no significant difference between Sil-20-1 and Sil-40-1 groups (P=0.18) and also Sil-40-1 and Sil-80-1 groups (P=0.95). However, a significant decrease in TBARS value was seen in Sil-80-1 group as compared to Sil-20-1 group (P<0.01).

Although administration of silymarin caused a significant decrease in Sil-40-2 and Sil-80-2 groups in terms of TBARS value (P<0.01), its value was not diminished considerably in Sil-20-2 group when compared to diabetic rats (P=0.94).

No significant difference was evident in Sil-20-1, Sil-40-1, and Sil-80-1 groups when compared to Sil-20-2 (P=1.00), Sil-40-2 (P=0.9), and Sil-80-2 (P=1.00) groups, orderly (Figure 5).

## 3.5 Pancreatic protein oxidation as CM

The circumstance of toxic stress caused a significant rise in CM of diabetic rats compared to sham group (P<0.01). There was a significant difference in terms of CM between all silymarin-treated groups and

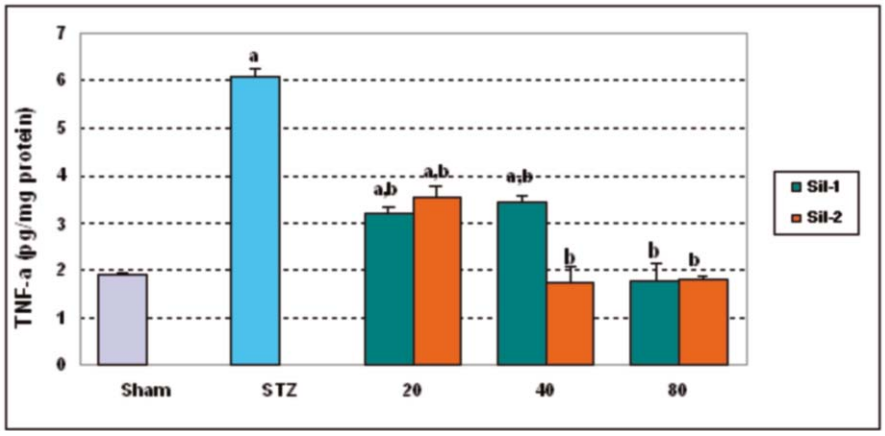


**Figure 3.** Tumor necrosis factor-alpha (TNF-α) level in pancreatic tissue. Values are mean±SEM. [a]Significantly different from sham group at P<0.05. [b]Significantly different from control group at P<0.05. Administration of silymarin was started after induction of diabetes from day 5 post administration of STZ and continued for 21 as Sil-1 series. In Sil-2 series, animals received silymarin simultaneous with STZ started from day 1 and continued for 21 days.

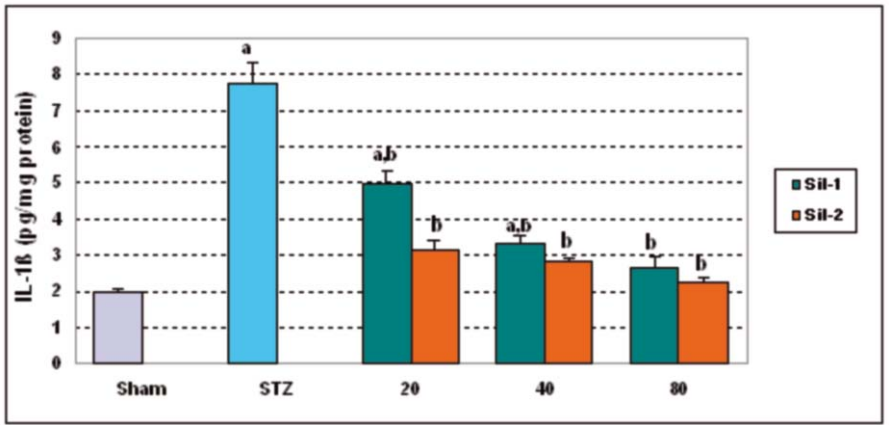


**Figure 4.** Interleukin-1β (IL-1β) level in pancreatic tissue. Values are mean±SEM. [a]Significantly different from sham group at P<0.05. [b]Significantly different from control group at P<0.05. Administration of silymarin was started after induction of diabetes from day 5 post administration of STZ and continued for 21 as Sil-1 series. In Sil-2 series, animals received silymarin simultaneous with STZ started from day 1 and continued for 21 days.

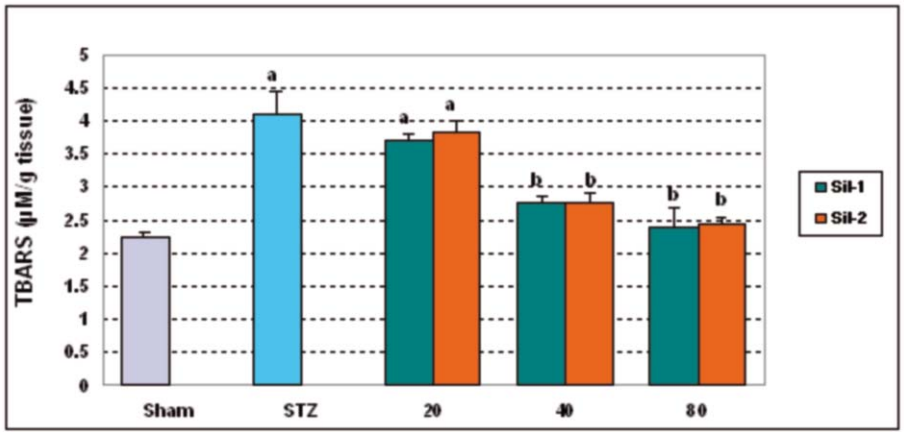


**Figure 5.** Lipid peroxidation as TBARS in pancreatic tissue. Values are mean±SEM. [a]Significantly different from sham group at P<0.05. [b]Significantly different from control group at P<0.05. Administration of silymarin was started after induction of diabetes from day 5 post administration of STZ and continued for 21 as Sil-1 series. In Sil-2 series, animals received silymarin simultaneous with STZ started from day 1 and continued for 21 days.

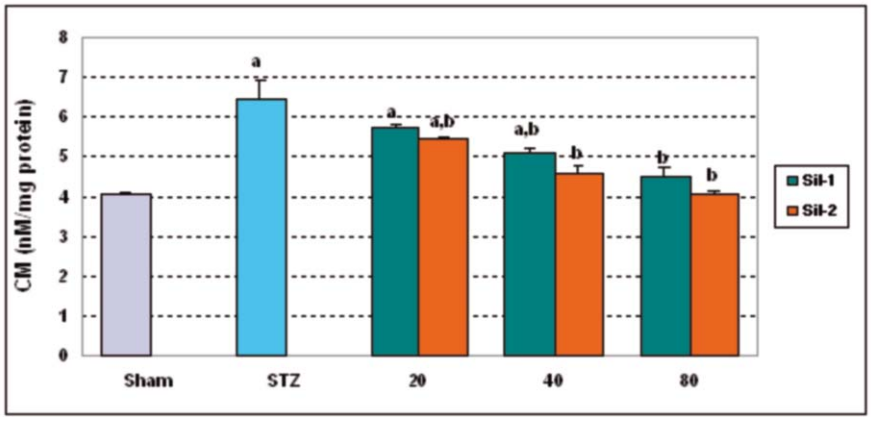


**Figure 6.** Protein oxidation as carbonyl content in pancreatic tissue. Values are mean±SEM. [a]Significantly different from sham group at P<0.05. [b]Significantly different from control group at P<0.05. Administration of silymarin was started after induction of diabetes from day 5 post administration of STZ and continued for 21 as Sil-1 series. In Sil-2 series, animals received silymarin simultaneous with STZ started from day 1 and continued for 21 days.

control group (P<0.05) except Sil-20-1, where CM was statistically near that of the control group (P=0.62).

CM was considerably higher in Sil-20-1 group in comparison to Sil-80-1 group (P<0.01), however no significant difference was observed between Sil-20-1 and Sil-40-1 groups (P=0.38) and also Sil-40-1 and Sil-80-1 groups (P=0.12).

Administration of silymarin improved CM in Sil-20-2, Sil-40-2, and Sil-80-2 considerably in a dose-dependent manner (P<0.05).

There was no statistical difference in terms of CM between Sil-20-1, Sil-40-1, and Sil-80-1 groups and those of Sil-20-2 (P=0.8), Sil-40-2 (P=0.63), and Sil-80-2 (P=0.39) groups respectively (Figure 6).

## 3.6 Pancreatic TAP as FRAP value

Induction of diabetes caused a significant decrease in FRAP value in the control group compared to the sham group (P<0.01). Treatment of animals with silymarin significantly increased FRAP in Sil-40-1 and Sil-80-1 (P<0.01) groups, however there was no significant increase in FRAP value for the Sil-20-1 group compared to control. Also, there was no significant difference between Sil-40-1 and Sil-80-1 groups (P=0.32).

While FRAP value was not significantly improved in Sil-20-2 group (P=1.00), it was significantly increased in Sil-40-2 (P<0.01) and Sil-80-2 (P<0.01) when compared to control group. There was not a significant difference between Sil-40-2 and Sil-80-2 in terms of FRAP value (P=0.82).

The FRAP values gained in Sil-20-1, Sil-40-1, and Sil-80-1 groups were approximately near the values gained by Sil-20-2 (P=0.99), Sil-40-2 (P=0.65), and Sil-80-2 (P=1.00), respectively (Figure 7).

## 3.7 Pancreatic TTM

A significant decrease in TTM was seen in diabetic rats when compared to normal rats of sham group (P<0.01). Albeit TTM was noticeably increased in Sil-40-1 and Sil-80-1 groups (P<0.01), there was no significant difference in terms of TTM with Sil-20-1 group (P=0.45) when compared to control group. While, its level was significantly higher in Sil-80-1 in comparison with Sil-20-1 group, TTM in Sil-40-1 reached near values gained in Sil-20-1 (P=0.64) and Sil-80-1 (P=0.25) groups statistically.

Administration of silymarin caused a significant reduction in TTM in all Sil-20-2, Sil-40-2, and Sil-80-2 groups when compared to control group (P<0.05). TTM of Sil-80-2 was noticeably higher than Sil-20-2 (P<0.01). Nevertheless, its value in Sil-40-2 group was not different statistically from Sil-20-2 (P=0.06) and Sil-80-2 (P=0.96) groups.

Administration of silymarin in Sil-20-1, Sil-40-1 and Sil-80-1 groups led to results similar to those for Sil-20-2 (P=0.88), Sil-40-2 (P=0.1) and Sil-80-2 (P=0.85) groups, respectively (Figure 8).

## 3.8 Pancreatic neutrophil infiltration as MPO activity

MPO activity was increased significantly in pancreatic tissue of the control group when compared with the sham group (P<0.01). The MPO activity was lessened considerably in Sil-20-1, Sil-40-1, and Sil-80-1 groups as compared to control group (P<0.01). While there was not a significant difference between Sil-20-1 and Sil-40-1 in terms of MPO activity (P=0.6), its value was significantly lower in Sil-80-1 when compared to group Sil-20-1 (P<0.01).

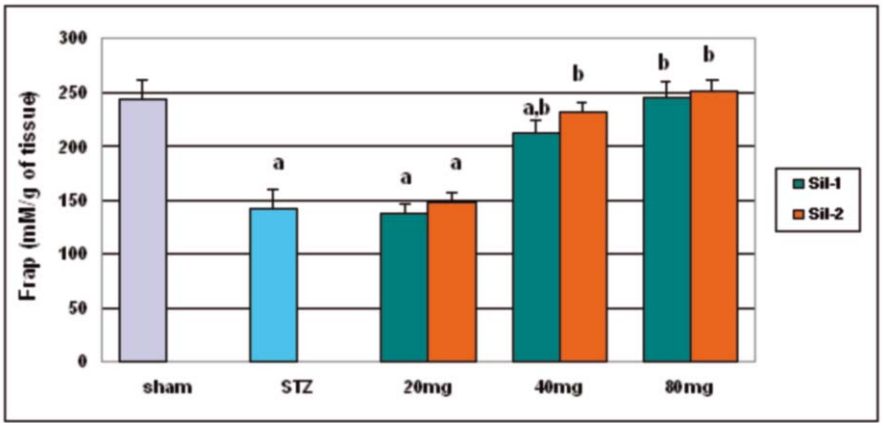


**Figure 7.** Total anti-oxidant capacity as FRAP in pancreatic tissue. Values are mean±SEM. [a]Significantly different from sham group at P<0.05. [b]Significantly different from control group at P<0.05. Administration of silymarin was started after induction of diabetes from day 5 post administration of STZ and continued for 21 as Sil-1 series. In Sil-2 series, animals received silymarin simultaneous with STZ started from day 1 and continued for 21days.

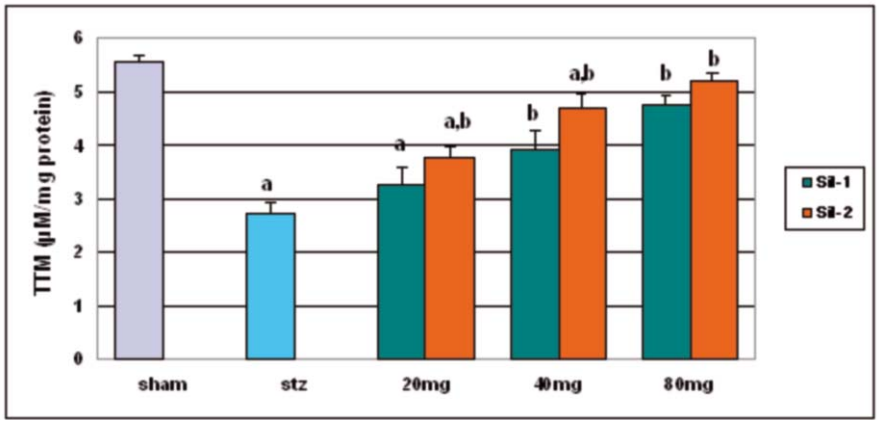


**Figure 8.** Total thiol molecules in pancreatic tissue. Values are mean±SEM. [a]Significantly different from sham group at P<0.05. [b]Significantly different from control group at P<0.05. Administration of silymarin was started after induction of diabetes from day 5 post administration of STZ and continued for 21 as Sil-1 series. In Sil-2 series, animals received silymarin simultaneous with STZ started from day 1 and continued for 21 days.

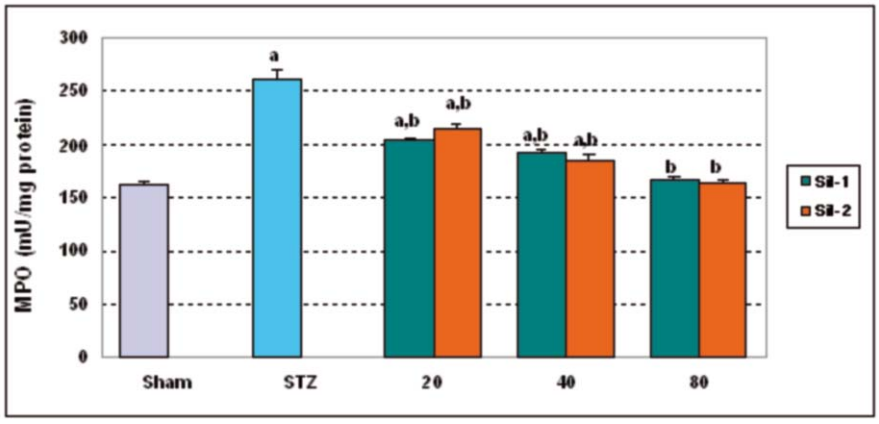


**Figure 9.** Neutrophil infiltration as MPO activity in pancreatic tissue. Values are mean±SEM. [a]Significantly different from sham group at P<0.01. [b]Significantly different from control group at P<0.01. Administration of silymarin was started after induction of diabetes from day 5 post administration of STZ and continued for 21 as Sil-1 series. In Sil-2 series, animals received silymarin simultaneous with STZ started from day 1 and continued for 21 days.

Administration of silymarin decreased the MPO activity in three groups of Sil-20-2, Sil-40-2, and Sil-80-2 in comparison with control group ($P<0.01$), whereas its value was the lowest in Sil-80-2 group ($P<0.05$). In addition a significant difference was observed between Sil-20 and Sil-40 groups ($P<0.01$).

Administration of silymarin in Sil-20-1, Sil-40-1 and Silymarin-80-1 groups resulted in values similar to those for the Sil-20-2 ($P=0.88$), Sil-40-2 ($P=0.93$) and Sil-80-2 ($p=1.00$) groups, respectively (Figure 9).

## 4. Discussion

The results of the present study demonstrate that silymarin, the extract of *Silybum marianum* (milk thistle) seeds, has an overall beneficial effect in an experimental model of autoimmune diabetes induced by multiple-low-dose injection of STZ. Silymarin markedly improved biomarkers of inflammation and toxic stress alongside hyperglycemia, the main complication of diabetes. The present study elucidates that silymarin serves its protective effects in an almost dose dependent manner. Moreover, no marked difference between the prevention of T1DM and the reversion of this disease by silymarin was found. Therefore, it is surprising to conclude that same mechanisms are involved in preventive and remissive effects of the silymarin in T1DM. In support of the present findings, remissive effects of silymarin have also been reported for an alloxan-induced model of diabetes [36-38]. Silymarin at a dose of 200 mg/kg not only increased the level of anti-oxidant enzymes and showed anti-oxidant properties but also enhanced insulin levels and recovered pancreatic function [36-38]. As mentioned before, in another similar study study, Vengerovskii *et al*. showed beneficial effects of silymarin on single-dose-STZ-induced diabetes [15]. In that study, silymarin was used at a dose of 70 mg/kg suspended in starch gel and was administered for 14 days. Also, levels of serum glucose, total cholesterol, lipid peroxidation in liver homogenates and function of hepatocyte mitochondria were assessed. According to their results, silymarin can successfully reverse hyperglycemia and hypercholesterolemia while improving lipid peroxidation and liver bioenergetics.

A number of mechanisms have been suggested for initiation and perpetuation of T1DM and its complications. Amongst these, toxic stress especially oxidative stress is believed to play a key role. Oxidative stress in T1DM may be due to glucose auto-oxidation resulting in generation of free radicals. On the other hand, it may be a result of cellular imbalances such as impairment in activity of anti-oxidant enzymes, anti-oxidant depletion or overproduction of pro-oxidant components [39]. As shown in the present results, in T1DM there is extra free radical damage as confirmed by changes in cellular lipid and protein oxidation. There is also an imbalance between oxidant and anti-oxidant status of pancreas as confirmed by reduced TTM and TAC. It has been shown that ROS are released from various infiltrated immune cells into pancreatic islets. These non-specific inflammatory mediators can lead to IPβC destruction by damaging their plasma membrane [40]. Using animal models, it has been demonstrated that several anti-oxidant compounds can successfully reduce oxidative stress, prevent destruction of IPβCs and alleviate diabetic complications [40,41].

Proinflammatory cytokines are produced in tremendous levels during the inflammatory process [5,42]. These cytokines, such as IL-1β and TNF-α, are believed to have destructive effects on pancreatic β-cells in both human and rodent T1DM. It seems that activated macrophages and T cells are the major sources of these cytokines. One mechanism underlying their destructive effects is triggering detrimental pathways in IPβCs like activating synthesis of toxic components. Moreover, IL-1 and TNF-α can lessen glucose-stimulated insulin secretion resulting in insulin resistance [43,44]. It is believed that some cytodestructive effects of proinflammatory cytokines may be due to ROS. In other words, it seems that ROS are intermediates of cytokine-induced IPβC damage. Consequently, IL-1 in the company of TNF-α can induce ROS generation, lipid peroxidation and generation of toxic aldehydes [40].

This study demonstrates that silymarin is able to reduce pancreatic content of proinflammatory cytokines like IL-1β and TNF-α, probably through inhibition of their production or release. These findings are comparable with previous evidence on anti-inflammatory effects of silymarin [17]. According to Matsuda *et al.* (2005), silymarin is able to save IPβCs against cytokines toxicity [45]. Regarding the wide variety in action of these cytokines, this inhibition can result in reduced release or production of other inflammatory and toxic stress mediators.

One major group of toxic stress mediators are free radicals, especially ROS [8]. The results of this study show that silymarin is able to enhance TAC of pancreatic tissue, which was decreased noticeably following induction of injury. This property is possibly due to its ability to scavenge free radicals alongside enhancing the activity of anti-oxidant enzymes [12]. It should be mentioned that alternations in activity of anti-oxidant enzymes have been reported previously in STZ-induced T1DM [41]. Parallel to this property, this investigation reveals that silymarin is able to enhance

TTM in pancreatic tissue. TTM is a biomarker of thiol-related anti-oxidants, specially glutathione content [46], a critical anti-oxidant enzyme which acts as a direct free radical scavenger. It has been shown that STZ-induced diabetes leads to a significant reduction in glutathione content of pancreatic tissue [41]. The effects seen herein support the mentioned anti-oxidant traits of silymarin.

Since silymarin is able to inhibit the generation and activity of free radicals, it is expected that silymarin can also improve the consequences of toxic stress. One of the main complications of toxic stress is oxidation of critical molecules like lipids and proteins [47]. Lipid peroxidation was evaluated by TBARS assay in the present study. It has been previously demonstrated that TBARS level was noticeably increased following induction of diabetes using STZ [41]. In TBARS assay the level of toxic aldehydes like MDA, which are by-products of this phenomenon, is measured. Results of TBARS assay in this study show that silymarin is able to diminish lipid peroxidation which validates the previous claims on this property of silymarin.

On the other hand, our results show that silymarin is able to reduce protein oxidation, evaluated by measuring CM of pancreatic tissue. The cleavage of amino acid backbones of lysine, arginine, histidine, proline, glutamic acid, and threonine occurs as a result of toxic stress. This can lead to detrimental consequences especially cell dysfunction. In oxidized proteins, new carbonyl groups and protein hydroperoxides are formed and protein thiols may be a target for ROS. The relationship between protein oxidation and inflammatory conditions has been illustrated previously [48].

The inflammatory and toxic stress mediators are important chemotactic molecules which attract immune cells to the site of injury. Neutrophils are a group of immune cells whose infiltration to the site of damage during inflammation leads to harmful consequences especially release of further toxic mediators. MPO is a protective enzyme, located in granules of neutrophils, and its activity has been used as a biomarker of neutrophil sequestration previously [28]. According to our results, silymarin can decrease neutrophil migration to the injured area which not only lessens damage but also has additive beneficial effects by preventing further release of chemotactic molecules.

In addition, as mentioned earlier, silymarin as a PDE inhibitor is able to increase cAMP and cGMP levels [18,19]. Good evidence indicates that cyclic nucleotides are able to act against oxidative stress [49,50] that may explain anti-toxic stress potential of silymarin in the present model of T1DM. Regarding existing literature, silymarin has enough potential to be tested for improvement of pancreatic islet transplantation procedures [51].

Collectively, the results of the present study confirm the reports of anti-oxidative and anti-inflammatory traits of silymarin of previous investigations. Many mechanisms like free radical scavenging, up-regulating expression of anti-oxidant enzymes, PDE inhibition, obstructing NF-κB activities, impeding synthesis or activity of inflammatory mediators and ultimately diminishing immune cell infiltration to the site of injury are possibly responsible for the herein observed effects of silymarin [52]. Regarding the low toxicity and rare side effects of silymarin [11], it is hoped to see silymarin in therapy of human T1DM in near future. However, further animal and clinical researches are required to prove this thesis.

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