

Comparison of the Antioxidant and Anti-Inflammatory Effects of Silymarin and Nano-Silymarin in Hyperlipidemic Rats

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ABSTRACT

Research Paper

Background and Objective: For centuries, medicinal plants have been used in the treatment of many diseases and hyperlipidemia. The present study was conducted to compare the antioxidant and anti-inflammatory effects of silymarin and nano-silymarin in hyperlipidemic rats receiving a high-fat diet (HFD).

Methods: In this experimental study, 35 male rats were divided into 5 groups of 7, including control, negative control (HFD), HFD and atorvastatin (20 mg/kg) (positive control), HFD and silymarin (100 mg/kg), and HFD and nano-silymarin (100 mg/kg). Receiving a high-fat diet for 12 weeks induced oxidative stress. Drugs were administered through gavage feeding for 12 weeks. Finally, blood samples were taken to measure lipid profile, tumor necrosis factor alpha (TNF- α), interleukin 17, malondialdehyde (MDA), and superoxide dismutase (SOD).

Findings: Nano-silymarin was able to significantly reduce triglyceride levels by 2.93 units compared to the negative control ($p<0.001$), but the reduction of cholesterol and LDL-C by nano-silymarin was not significant. TNF- α in the negative control group was significantly higher by 31 units compared to the control ($p=0.006$). TNF- α and interleukin 17 levels were not significantly reduced in the groups receiving atorvastatin, silymarin and nano-silymarin compared to the negative control group. MDA levels in the negative control group increased by 0.8 units compared to the control group ($p=0.001$), but in the silymarin, nano-silymarin and atorvastatin groups, they showed a significant decrease by 0.8, 1 and 0.9 units compared to the negative control group, respectively ($p<0.001$).

Conclusion: According to the results of this study, the use of nano-silymarin compared to silymarin in rats receiving a high-fat diet led to a relative reduction in lipid profiles, but there was no difference in terms of antioxidant and anti-inflammatory properties.

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Introduction

Hyperlipidemia is caused by an increase in lipids or lipoproteins in the blood. Based on the type of lipid elevation, hyperlipidemia is divided into hypertriglyceridemia, hypercholesterolemia, or a combination of both (1). Eating disorders, obesity, genetic diseases, or other diseases such as diabetes can all lead to an increase in blood lipids (2). The risk of developing cardiovascular diseases is twice as high in patients with hyperlipidemia. According to clinical studies, the risk of non-ischemic heart failure is increased in hyperlipidemia, while lowering serum lipids can reverse cardiac dysfunction. In addition to the fact that cardiac function is indirectly affected by hyperlipidemia through atherosclerosis, it also has a direct effect on systolic function and electrophysiological response of the heart. The heart is directly affected by hyperlipidemia, but the underlying mechanism is not fully understood (3).

Inflammation plays a key role in the development and progression of atherosclerosis and, consequently, cardiovascular diseases. The role of the immune system and subsequent systemic inflammation is now well established. Multiple lines of evidence, from experimental models and histopathological evaluation of tissues to systemic biomarkers and epidemiological or clinical associations, have shown that inflammation is one of the fundamental mechanisms in the instability of atherogenic plaques leading to clinical events. Several inflammatory markers have been associated with lipid levels and the course of atherosclerosis. The best known of these are IL-6 (interleukin 6) and interleukin 1 β , C-reactive protein, TNF- α (tumor necrosis factor alpha), pentraxin-3, serum amyloid A, monocyte chemoattractant protein-1, and chemokine 16 (4). Through stimulation of oxygen free radicals, hyperlipidemia is not only directly but also indirectly effective in accelerating the formation of atherosclerotic plaques in the development of coronary heart disease (5).

Oxidative stress is a tissue injury caused by excessive production of oxidizing compounds or the ineffectiveness of antioxidant defense mechanisms in eliminating them. Oxidative compounds can be produced naturally in the body, but inappropriate activation of oxidative stress can cause vascular damage and atherosclerosis. In the presence of oxidative stress, molecules are easily oxidized, and LDL-C (low-density lipoprotein) and oxidized molecules initiate and exacerbate vascular inflammation, atherosclerotic plaque formation, and atherosclerosis (6). The progression of atherosclerotic cardiovascular disease is often influenced by inflammation, oxidative stress, and dyslipidemia (7).

Sage, scientifically known as *Salvia officinalis*, is a plant that plays a major role in improving metabolic disorders (8). The extract of this plant contains flavonoids, the complex of which is called silymarin, which constitutes the main component of the essential oil of this plant (9). Silymarin has significant antioxidant properties and reduces free radicals, inhibits lipid peroxidation, and increases the activity of the enzyme superoxide dismutase (SOD) (10). In addition, it has anti-inflammatory effects and increases protein synthesis by liver cells when the liver parenchyma is damaged (11). It also has significant effects on kidney damage (12). Poor water solubility is a major challenge in the development of silymarin formulations (13). Silymarin has numerous biological properties, but its poor water solubility hinders its bioavailability and therapeutic efficacy (14-16). Liposomes, nanoemulsions, micelles, nanocrystals, and polymeric nanoparticles can be used to increase solubility and target desired cells with minimal damage to normal cells.

In another study conducted by Nouri et al., the antioxidant effect of silymarin against diclofenac-induced renal injury was investigated. The results showed that the activity of antioxidant enzymes in the silymarin-treated group was significantly increased compared to the injured group, and the levels of malondialdehyde, urea, creatinine, and inflammatory factor gene expression were significantly reduced (17). Laboratory studies have shown that silymarin significantly reduces cholesterol absorption, thereby reducing blood

cholesterol and LDL-C levels and increasing HDL-C levels (18). Considering the association between oxidative stress and inflammatory factors with increased blood lipids, this study was conducted to determine and compare the effects of nanosilymarin, silymarin, and atorvastatin on lipid profiles, oxidative stress, and inflammation in rats receiving high-fat diet (HFD).

Methods

After approval by the Ethics Committee of Gonabad University of Medical Sciences with the code IR.GMU.REC.1400.070, this experimental study was conducted on 35 adult male Wistar rats, weighing 270 to 330 g, which were kept in the Laboratory Animal Center of Gonabad University of Medical Sciences. The animals were adapted to the environment by being kept for two weeks at an average temperature of $23\pm2^{\circ}\text{C}$. Ethical principles of working with animals were observed during the 12-h-light/12-h-dark cycle with free access to food and water. In this study, sampling was done using convenience method and animals were randomly assigned to different groups.

The resource equalization method was used to calculate the sample size (19). In this method, the value of E, which is the degree of freedom used in the "variable" effect test in analysis of variance (ANOVA), was calculated based on the following formula:

$$\text{E} = \text{Total number of test units} - \text{Total number of groups}$$

First, we considered the number of samples in each group to be 6. The number of groups was also 5 groups and the value of E was calculated: $E = (5 \times 6) - 5 = 25$

To calculate the probable mortality, the probable mortality percentage was multiplied by the calculated number of animals for each group, and the following formula determined the number of animals required for each group:

$$\text{Number of animals required for each group} = \text{Number of animals calculated} + (\text{Percentage of probable mortality} \times \text{Number of animals calculated})$$

Based on this, the number of animals required for each group was determined to be 7 rats, taking into account the probable mortality, and since we had 5 groups, the total number of animals required for this study was determined to be 35 rats.

Rats were randomly divided into 5 groups of 7: Group 1 (control group) (C): fed with standard diet + 1 ml normal saline daily by gavage for 12 weeks, Group 2 (negative control group) (C-): fed with HFD without treatment+ 1 ml normal saline daily by gavage for 12 weeks, Group 3 (positive control group) (C+): fed with HFD + 20 mg/kg atorvastatin daily by gavage for 12 weeks (20), Group 4 (silymarin group) (S): fed with HFD + 100 mg/kg silymarin daily by gavage for 12 weeks (21), Group 5 (nano-silymarin group) (NS): fed with HFD + 100 mg/kg nano-silymarin daily by gavage for 12 weeks (21).

In order to create HFD to induce hyperlipidemia, oxidative stress, and inflammation, a diet including 45% standard rodent food plus 49.5% sheep tail fat, 0.2% cholesterol powder, and 0.5% wheat flour was used for 12 weeks and was made available to the animals ad libitum (22, 23). The method of food preparation was as follows: first, the necessary ingredients were weighed according to the percentages in the formula, and the standard food plates were ground into powder, and then melted sheep tail oil (containing 87.8% fat), cholesterol, and wheat flour were added to this powder. After homogenization of this mixture, the resulting dough was re-formed into balls and stored at room temperature until it was dry. Atorvastatin was purchased from Actoverco Pharmaceutical Company.

Nano-silymarin was prepared as a colloidal solution in amber glass by Exir Nano Sina Co. The dynamic light scattering (DLS) report is shown in Figure 1. Using DLS, the hydrodynamic diameter of nanoparticles in the solution can be measured and their aggregation status can be determined. The nano-silymarin solution

was stored at room temperature and away from light. A 10% concentration of the solution was used, which was diluted with 5% dextrose serum at a ratio of 1: 100 mg/kg for daily use via gavage for each animal. Rats were re-weighed after 12 weeks and anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) by intraperitoneal (IP) injection after 12 hours of fasting. After abdominal incision, blood samples were taken from the apex of the heart.

To prepare serum, blood samples were centrifuged at 4000 rpm for 15 min at 4°C. Then, serum samples were stored in a freezer at -20°C until analysis. Serum lipid profile (cholesterol, triglyceride, HDL-C), oxidative stress markers (MDA, SOD) were measured using standard kits (Pars Azmon, IR). Friedewald Equation was also used to determine the amount of LDL-C in serum (24).

The ELISA method was used to measure cytokines (Carmania Parsgen Co.). The principles of ELISA are the same as the basic principles of immunology, namely the specific binding of antigen and antibody. In the Sandwich ELISA method, to identify specific antigens (TNF- α and IL-17), a specific antibody was attached to the bottom of the container and to prevent non-specific binding, the rest of the container was covered with bovine serum albumin. By adding the liquid containing the antigen to be measured, the antigen was bound to the antibody at the bottom of the container. After washing, the primary antibody was added, which recognized another epitope of the antigen and, together with the antibody at the bottom of the container, took the antigen in between (Sandwich). After washing, the secondary antibody was added, to which the specific HRP enzyme was bound, and this secondary antibody bound to the primary antibody. After washing, the enzyme substrate was added, which was converted by the enzyme into a product that creates a different color from the substrate. Quantitative measurement was performed based on this color change.

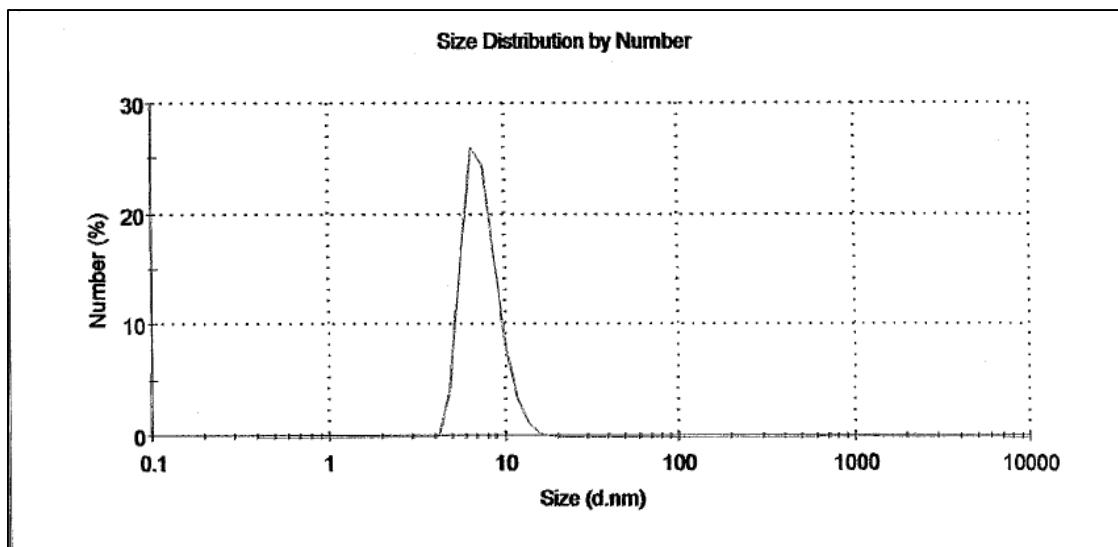


Figure 1. DLS report for nano-silymarin sample

After confirming normality based on Kolmogorov-Smirnov test, one-way analysis of variance statistical test was used to compare variables between different groups, and Tukey's post hoc test was used for paired comparisons between groups. Central (mean) and dispersion (standard deviation) indices were used to describe quantitative variables, and $p < 0.05$ was considered significant.

Results

The negative control group showed a significant increase in serum cholesterol levels compared to the control group ($p<0.001$). There was a significant decrease in cholesterol levels in the positive control group compared to the control group ($p=0.025$). Serum cholesterol levels were significantly higher in the groups receiving silymarin and nano-silymarin compared to the control group ($p<0.001$). The groups receiving silymarin and nano-silymarin showed a decrease in cholesterol levels compared to the negative control group, but it was not significant (Table 1).

Table 1. Serum lipid profile levels in the study groups (n=7)

Lipid profile	Cholesterol	Triglycerides	HDL-C	LDL-C
Group	Mean±SD	Mean±SD	Mean±SD	Mean±SD
Control	62.28±7.04 ^a	61.87±30.34 ^a	35±34.5 ^a	14.9±94.6 ^a
Negative control	122.14±89.20 ^b	226.71±63.48 ^b	28.71±42.2 ^a	48.08±92.19 ^b
Positive control	94±73.9 ^c	98.66±45.03 ^c	33.83±99.6 ^a	40.43±48.5 ^a
Silymarin	115.45±57.23 ^a	169.16±37.41 ^d	28.33±56.6 ^a	53.28±66.18 ^a
Nano-Silymarin	113.16±23.21 ^a	133.5±44.41 ^c	34.38±58.8 ^a	52.08±91.15 ^a
p-value	<0.05	<0.05	>0.05	<0.05

a: No significant difference between groups, b: Significant difference with the control group, c: Significant difference with the negative control group, and d: Significant difference with the positive control group.

According to the results, the negative control group showed a significant increase in triglyceride levels compared to the control group ($p<0.001$). A significant decrease in triglyceride levels was observed in the positive control group compared to the control group ($p<0.001$). The group receiving nano-silymarin had a significant decrease in mean triglycerides, which was evident when comparing the negative control group with the group receiving nano-silymarin ($p=0.004$). The positive control group showed a significant decrease in mean triglycerides compared to the silymarin group ($p=0.049$) (Table 1). There was no difference in HDL-C levels between the groups. As the results showed, the negative control group had a significant increase in serum LDL-C levels compared to the control group ($p=0.002$). The positive control group showed a slight decrease in LDL-C in the comparison, which was not significant (Table 1).

Among different groups, only the mean TNF- α in the negative control group was significantly higher than the control group ($p=0.006$). The groups receiving atorvastatin, silymarin, and nano-silymarin showed a decrease in the levels of TNF- α compared to the negative control group, but it was not significant (Figure 2).

There was no significant difference between the negative control group and the control group in terms of IL-17. There was no significant difference between the treatment groups and the negative control group in terms of serum IL-17 levels. The mean IL-17 in the groups receiving silymarin ($p=0.001$) and nano-silymarin ($p=0.018$) was significantly lower than the control group. A significant decrease in serum IL-17 levels was observed between the silymarin group and the negative control ($p=0.020$). Moreover, the use of silymarin significantly reduced serum IL-17 levels compared to atorvastatin ($p=0.020$) (Figure 3).

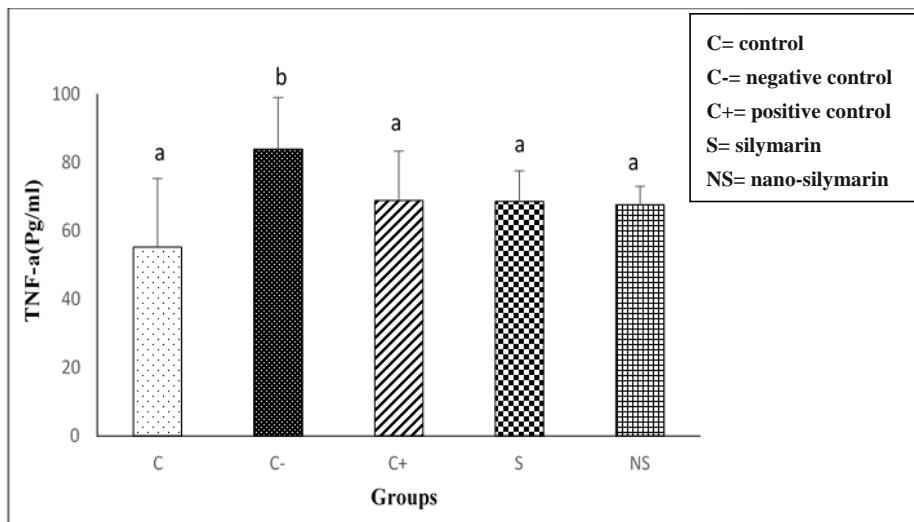


Figure 2. Comparison of mean serum TNF- α levels in the study groups (n=7)

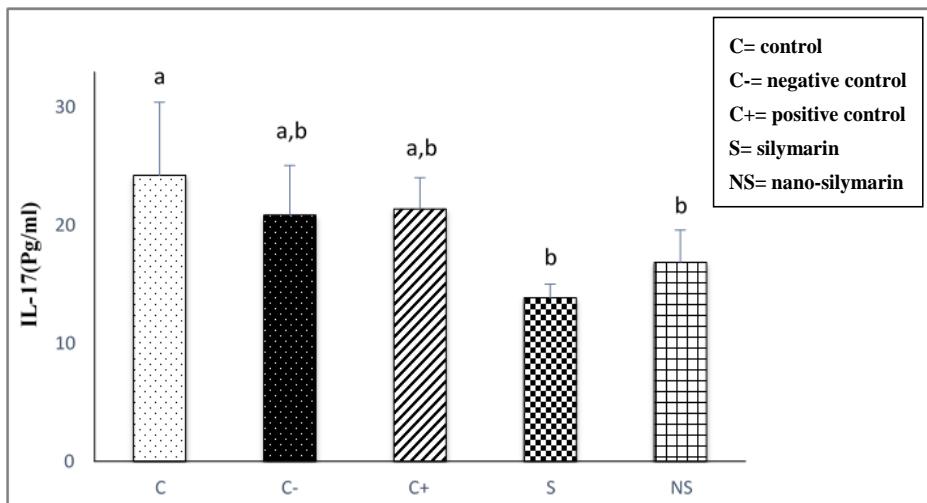


Figure 3. Comparison of mean serum IL-17 levels in the study groups (n=7)

The MDA level in the negative control group was significantly increased compared to the control ($p=0.001$). The comparison of the MDA factor level in the silymarin, nano-silymarin and atorvastatin groups showed a significant decrease compared to the negative control group ($p<0.001$). However, no significant difference was observed between the groups receiving silymarin, nano-silymarin and atorvastatin in terms of changes in MDA levels (Figure 4).

As a result of the Tukey post-hoc test, no significant difference was observed between the SOD levels in the control group and the negative control. The groups receiving atorvastatin and silymarin showed a decrease in the SOD levels compared to the negative control group, but it was not significant. The comparison of the SOD levels in the nano-silymarin group showed a significant increase compared to the positive control group ($p=0.039$). There was no significant difference between the other groups (Figure 5).

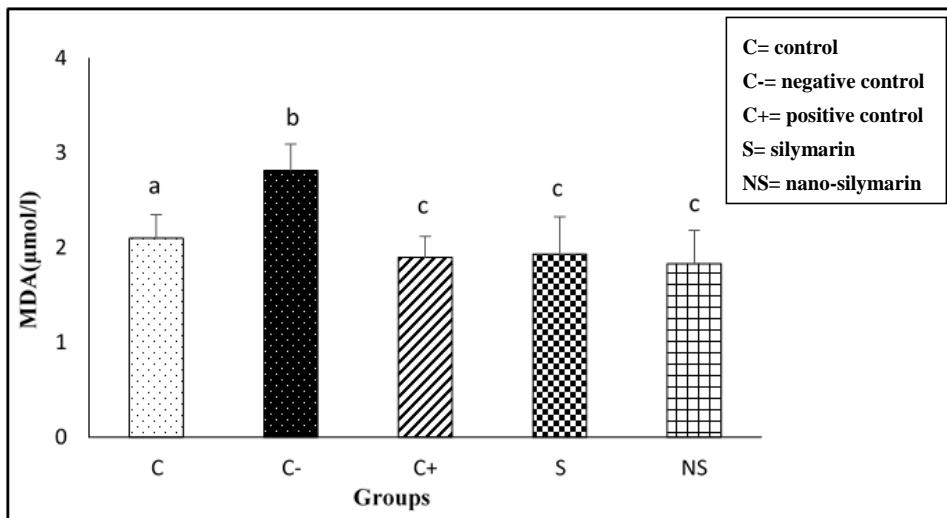


Figure 4. Comparison of mean serum MDA levels in the study groups (n=7)

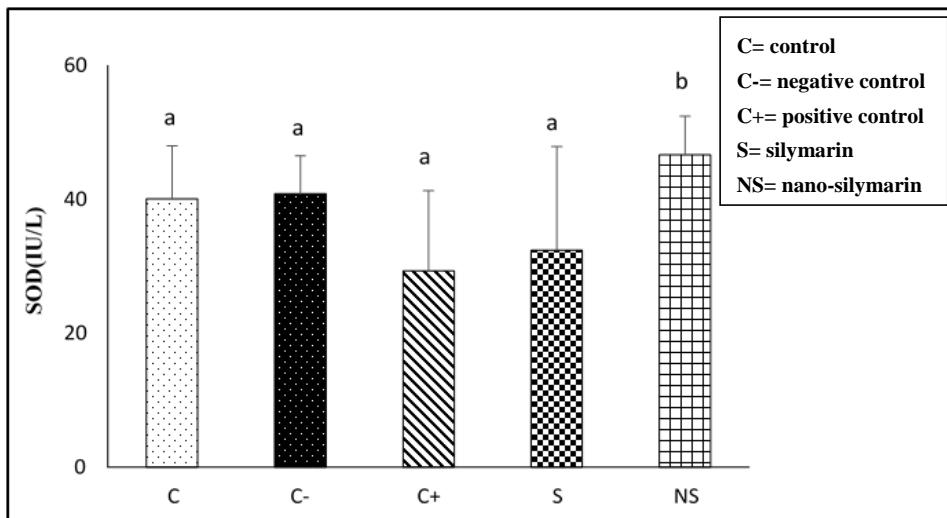


Figure 5. Comparison of mean serum SOD levels in the study groups (n=7)

Discussion

This study showed that nano-silymarin significantly reduced blood triglyceride levels compared to the negative control group, while the reduction in total cholesterol and LDL-C was not significant compared to the negative control group. The level of MDA in the negative control group was significantly increased compared to the control group, and this marker showed a significant decrease in the treatment groups compared to the negative control group. Regarding inflammatory cytokines, only TNF- α was significantly increased in the negative control group compared to the control group.

High-fat diet (HFD) causes oxidative stress and inflammation. Hyperlipidemia is considered a clear risk factor for cardiovascular diseases (25). The results of this study showed that high-fat diet consumption was able to significantly increase serum TG, cholesterol, and LDL-C and also reduce HDL-C. Silymarin consumption along with high-fat diet decreased cholesterol and TG, but this decrease was not significant.

Nano-silymarin consumption significantly decreased TG compared to the negative control group, but the decrease in cholesterol in this group was not significant. The cholesterol and TG-lowering effect of nano-silymarin was greater than that of silymarin. This study shows that nanoparticle technology has been able to improve the therapeutic effects of silymarin. A study by Mohebbati et al. showed the increasing effects of vitamin C on cholesterol levels in Adriamycin-induced hyperlipidemic rats. Therefore, antioxidant agents can also have adverse effects on cholesterol levels (26). In support of our findings, El-Kot et al. reported that there was no significant difference in triglyceride levels between silymarin-treated rats and controls. However, LDL-C levels were higher in silymarin-treated rats compared to controls (27). In the study by Mohammadi et al., it was found that there was no significant difference between the levels of lipid profile and blood glucose in the two groups before and after the experiment, and therefore the findings of this study were in line with the findings of our study (28). In another study conducted by Isfahani et al., it was found that lipid profile parameters in the group treated with nanomicelles were significantly reduced compared to the group treated with silymarin extract (29). In our study, nano-silymarin was also able to cause a greater reduction in TG and cholesterol compared to silymarin.

Atorvastatin and silymarin were used to increase the hypolipidemic effect, reduce adiposity, and improve glucose metabolism, and increase antioxidant and anti-inflammatory effects (30). The present study showed that a significant increase in the inflammatory factor TNF- α was observed in the negative control group compared to the control group. The effectiveness of nano-silymarin, silymarin, and atorvastatin on TNF- α in rats receiving a high-fat diet was different. Although the mean inflammatory factor TNF- α in the three groups of rats receiving silymarin, nano-silymarin, and atorvastatin was lower than the negative control group, there was no significant difference between these groups and the negative control group. There was also no significant difference between the three groups of nano-silymarin, silymarin, and atorvastatin in terms of changes in the level of the inflammatory factor TNF- α .

In the present study, no significant association was observed between silymarin administration (100 mg/kg daily) and TNF- α , which may be due to differences in the diet used and the dose of silymarin administered. In a study conducted by Nouri et al. to determine the nephroprotective effect of silymarin against diclofenac-induced renal injury and oxidative stress, it was shown that silymarin significantly reduced TNF- α compared to the other groups (17). In a study conducted by Amin et al. to investigate the anti-inflammatory and anti-hyperlipidemic effects of silymarin in mice and albino mice, serum TNF- α levels were significantly reduced two hours after silymarin administration (31). In the present study, the effect of silymarin on TNF- α levels in hyperlipidemic rats was investigated, and the daily dose of silymarin was 100 mg/kg instead of 150 mg. Silymarin consumption reduced TNF- α levels compared to the negative control group, but this was not significant. The reason for this difference could be the difference in the last time the rats were treated in the present study (one day before) and the aforementioned study (2 hours before) and the dose of the drug used.

Talaei et al. studied the effect of atorvastatin on inflammatory markers in patients with type 2 diabetes. Their results showed that compared to the control group, TNF- α levels were significantly reduced in the intervention group. The researchers stated that atorvastatin, in addition to its beneficial therapeutic effects on lipid profiles, can also inhibit inflammatory processes (32). In the present study, TNF- α levels were also lower in the atorvastatin group (positive control) compared to the control group, but this was not statistically significant. The differences in results may be due to differences in the study samples (experimental study vs. clinical trial) or sample size (35 rats vs. 88 humans) or the dose of atorvastatin used (20 mg daily vs. 40 mg daily). In our study, TNF- α was significantly increased in the negative control group, while IL-17 did not change in this group. It is likely that induced hyperlipidemia cannot affect Th 17 to produce IL-17 (33).

Silymarin can also reduce TNF- α and IL-17 due to its anti-inflammatory properties. Silymarin has significant antioxidant properties, reducing free radicals and inhibiting lipid peroxidation and increasing superoxide dismutase activity in red blood cells. In addition, it induces anti-inflammatory effects through the inhibition of nuclear factor kB (NF-kB). When the liver parenchyma is damaged, it resists the depletion of glutathione stores and increases protein synthesis by liver cells (11). In previous studies, it has been observed that silymarin not only combines with harmful free radicals but also suppresses pro-inflammatory responses caused by increased TNF- α levels (34).

In a study by Balouchi et al. on serum levels of the cytokines IL-10, IL-17, and IL-23 in patients with beta-thalassemia major, a significant decrease was observed in serum IL-10 levels in patients treated with silymarin compared to baseline IL-10 levels. However, no significant difference was observed between baseline cytokine levels compared to final levels in the placebo group. In the present study, the use of silymarin caused a decrease in IL-17 levels, which could be due to the difference in the daily dose of silymarin (100 mg/kg/day compared to 420 mg/day) and the difference in the selected population (rats versus humans) (35).

In this study, the level of MDA in the negative control group was significantly increased compared to the control group, and in the groups treated with silymarin, nano-silymarin and atorvastatin, this marker showed a significant decrease compared to the negative control group. In line with these results, in a study conducted by Nouri et al. in rats treated with silymarin, it was found that the serum level of MDA was significantly reduced compared to the control group (17). According to the results obtained in this study, nano-silymarin was not superior to silymarin in reducing inflammation and oxidative stress. There are probably several reasons for this situation. Changes in pH and temperature can affect the aggregation of nanoparticles and lead to the relative ineffectiveness of nano-silymarin. In hyperlipidemia, blood acidity increases due to the increase in fatty acids in the blood, and this change in blood pH can probably affect the aggregation of nanoparticles.

According to the results of this study, the use of nano-silymarin compared to silymarin in rats treated with a high-fat diet resulted in a relative reduction in lipid profiles, while there was no difference in antioxidant and anti-inflammatory properties. Changes in pH and temperature can affect the aggregation of nanoparticles and possibly cause the relative inefficiency of nano-silymarin.

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