

Research Article

The Role of Silymarin in Mitigating Inflammation and Cognitive Impairment Induced by Ovariectomy in Wistar Rats

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Background. Silymarin, a polyphenolic flavonoid found in milk thistle, has been used to treat liver and brain injuries in humans and animals. The study aims to investigate the protective effects of silymarin on spatial and passive avoidance memory, oxidative stress, and inflammatory factors in the brain and liver tissues of ovariectomized (OVX) Wistar rats. *Methods.* The study involved 30 female Wistar rats divided into control, sham, and three silymarin-treated groups. After ovariectomy, rats underwent CT scan, and some of them were administered silymarin via gavage for 2 months. Memory and learning were assessed using Morris water maze and shuttle box tests. Brain and liver tissues were analyzed for inflammatory factors (IL-1 β , TNF α , and IL-6) and oxidative stress markers (CAT, SOD, and MDA) after sacrifice. *Results.* Silymarin improved spatial memory and fear learning compared to the sham group ($P \le 0.05$ to $P \le 0.001$). It also significantly reduced IL-1 β , TNF- α , and IL-6 levels in the cortex, hippocampus, and liver ($P \le 0.05$ to $P \le 0.001$) and increased CAT and SOD while decreasing MDA levels ($P \le 0.05$ to $P \le 0.001$) compared to control and sham groups. *Conclusion.* Long-term administration of silymarin extract can improve learning and memory, reverse cognitive impairment caused by ovariectomy, and reduce oxidative stress and inflammatory factors induced by ovariectomy in the liver and brain of Wistar rats. This is due to the reduction in MDA levels and an increase in CAT activity, although silymarin has some effect on SOD at high doses.

1. Introduction

Estrogen is primarily produced in the ovaries, and after menopause, when their production stops, estrogen levels in the bloodstream decrease drastically [1]. This decrease in estrogen levels is significant because it can lead to an increased risk of chronic kidney disease, which is more frequently observed after menopause. Estrogen normally helps to reduce renal superoxide production and protect the kidneys from oxidative damage. However, with an 80% reduction in estrogen levels during menopause, older women become more vulnerable to this risk [2, 3]. The effects of menopause on oxidative stress and inflammation are particularly important in the field of osteoporosis because all these factors contribute to bone loss [1]. The reported prevalence of osteoporosis in women worldwide was 23.1%, while it was found to be 11.7% in men worldwide [4]. Estrogens, which can cross the blood–brain barrier (BBB) and are also endogenously produced in the brain from cholesterol, have been shown to affect various aspects of mitochondrial function. Specifically, they modulate ATP and reactive oxygen species (ROS) production, as well as antioxidant defense, mitochondrial membrane potential, and calcium levels [5, 6].

Sex steroids, such as estrogen, can modulate behavior, cognition, and memory. Brain regions involved in memory and executive function, such as the basal forebrain, hippocampus, and prefrontal cortex, have widely distributed estrogen receptors (ERs). Estradiol, a form of estrogen, has a crucial trophic effect on memory and executive functions in the basal forebrain and hippocampus. Estrogens also mediate neurotransmitter interactions in the prefrontal cortex, which is related to executive functions. Additionally, the hippocampus and prefrontal cortex are responsible for spatial working memory [7, 8]. This hormone has been shown to increase the expression of several antioxidant enzymes, including peroxiredoxin 5, glutaredoxin, and manganese superoxide dismutase (MnSOD) [6]. Additionally, 17β -estradiol (E2) plays a protective role on the BBB by inhibiting the activation of NF- κ B, which leads to the transcription of certain cytokines such as TNF- α , chemokine ligand 2 (CCL2), and IL-6 [9]. Estrogen deficiency has been correlated with higher levels of ROS production [1], which can lead to several undesirable symptoms, including impairment of learning and memory [10].

Specifically, rats that underwent ovariectomy showed a significant increase in hydrogen peroxide (H_2O_2) production in liver mitochondria and peroxisomes, as well as a decrease in antioxidant enzyme activity [1]. On the other hand, estrogen can protect against the progression of chronic liver diseases by reducing inflammation, improving mitochondrial function, and lowering oxidative stress levels [11].

ROS regulate several transcription factors, including NF- κ B, hypoxia-inducible factor 1 subunit alpha (HIF-1 α), nuclear factor erythroid 2-related factor 2 (Nrf2), and activator protein-1 (AP-1), which can also regulate the response to oxidative stress. Recently, estrogen-related receptors have been shown to have a direct role in controlling ROS homeostasis [12]. Additionally, an array of cognitive changes, such as reduced processing speed, impaired verbal memory, and diminished spatial memory, have been observed during the perimenopausal period [13, 14]. Therefore, as mentioned above, methods can be adopted to reduce the inflammatory and oxidative effects caused by reduced estrogen levels after menopause. These methods can play an effective role in promoting overall health [15].

Silymarin is a compound from milk thistle that has six flavonoid isomers, with silybin being the most active [16]. It has liver protective, antioxidant, anti-inflammatory, and anticancer properties. Certain amounts of silymarin can lower the secretion of IL-1 β and TNF- α , inhibit phosphorylation of p65 NF- κ B and p38 MAPK, and block NF- κ B activation by decreasing p65 subunit phosphorylation [17, 18].

Recent studies have shown that many natural products, such as silymarin, can alter cellular metabolism and signal transduction pathways through enzymes such as AMPK and mTOR. These pathways have a direct impact on cellular inflammatory status, such as the NF- κ B pathway, and immune system function [19–21].

Inflammation is a necessary response to various stimuli and involves the migration of immune cells aided by cytokines, chemokines, and acute-phase proteins. However, if inflammation persists, it can lead to tissue damage and chronic inflammation, which is linked to many diseases [22]. An imbalance of natural antioxidants leads to the production of free radicals from various sources, contributing to the onset of inflammatory-related ailments [23].

Silymarin inhibits pro-inflammatory signaling pathways, including NF- κ B and the expression of numerous proinflammatory cytokines and chemokines such as IL-1, TNF- α , and IL-6 [19, 24]. It also has good pharmacological activity in the nervous system, especially for the treatment of Alzheimer's disease, and it can control the production of β -amyloid (A β) by inhibiting the precursor of $A\beta$ and inhibiting the polymerization of A β . Therefore, silymarin can treat Alzheimer's disease by inhibiting the formation of $A\beta$, preventing oxidative stress, and reducing neuroinflammation [25]. Eliminating free radicals, increasing cellular glutathione levels, and improving SOD activity are the key mechanisms attributed to silymarin's antioxidant activities. Silymarin inhibits the activation of microglia as well as the production of inflammatory mediators such as TNF- α and nitric oxide (NO), thus reducing damage to brain dopaminergic neurons. It has been documented in some studies that silymarin reduces the level of α -synuclein protein and increases the level of dopamine. This substance exerts its anti-inflammatory activities mainly by inhibiting the activity of microglia. It reduces the production of inflammatory mediators such as TNF- α , IL-1 β , and NO, and also protects brain dopaminergic neurons [26].

Therefore, this research was conducted to examine the potential in vivo inhibitory effects of silymarin, an herbal medicine, in reducing the complications caused by estrogen deficiency in OVX rats, specifically oxidative stress and inflammation. This could be beneficial in alleviating the complications in postmenopausal women.

2. Materials and Methods

2.1. Animals and the Model of Menopause. Thirty-threemonth-old adult female Wistar rats (220–250 g) were purchased from Animal Center, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran. The rats were fed in the standard experimental conditions (room temperature $22 \pm 1^{\circ}$ C) with light/dark cycles of 12 hr each and were allowed free access to standard laboratory diet and water. Before the experiment, the rats were adapted to the experimental environment for 1 week. All experimental procedures were performed on animals. It was carried out in accordance with the animal care guidelines of Mashhad University of Medical Sciences (MUMS). The ethical code is IR.MUMS. AEC.1401.048. Then, the animals were randomly divided into five groups according to Table 1.

2.1.1. Ovariectomy Method. All rats underwent ovariectomy using the methods previously described by Mostafavinia et al. [27] and Fallahnezhad et al. [28]. Briefly, the rats were placed under general anesthesia using injections of 50 mg/kg ketamine hydrochloride (Rotex Medica, Tritteu, Germany) with 5 mg/kg diazepam (Caspian, Rasht, Iran) intraperitone-ally. Two paravertebral skin incisions were performed, and the uterine tubes were located and closed. The ovaries were then removed, and the incisions were ligated. To prevent

TABLE 1: The grouping of Wistar rats in this study (n = 6 in each group).

Groups	Month 0 (commencement of ovariectomy)	Month 3 (start gavage)	Month 5 (end gavage)
Control	Not done	Not done	Not done
Sham	Done	PBS as a daily gavage	Done
Silymarin (50 mg/kg)	Done	Silymarin as a daily gavage	Done
Silymarin (100 mg/kg)	Done	Silymarin as a daily gavage	Done
Silymarin (150 mg/kg)	Done	Silymarin as a daily gavage	Done

The doses are all in milligrams of silymarin per kilogram of body weight in rats.

infection, a ceftriaxone antibiotic (50 mg/kg, intraperitoneally) was administered immediately before surgery and again at 24 and 48 hr after surgery. At 14 weeks postoperatively, the rats were sent for CT scanning to confirm the induction of the menopause model.

2.1.2. Computed Tomography (CT) Scanning Protocol and Measurement of Hounsfield Units (HU) to Confirm the Induction of Menopause Model. The progression of osteoporosis in all rats that had their ovaries removed was assessed using CT scanning techniques through 16-slice spiral CT scanning (Philips Healthcare, Ingenuity, USA). The specific scanning parameters used were as follows: kV = 100, ma = 50, section = 2 mm, FOV = 240 mm or kVp ¹/₄ 120, mAs/slice ¹/₄ 50, pitch number ¹/₄ 0.54, rotation time ¹/₄ 0.75, and reconstruction iDose ¹/₄ 2. The bone density of the tibia in all rats was then quantitatively determined using CT scanning (Toshiba, Aquilion 16, Japan) under general anesthesia. The Hounsfield units of the defects found in the scans were ultimately reported.

2.2. Behavioral Tests. In this study, a set of behavioral experiments were carried out on 75-day-old rats. The Morris water maze (MWM) test was used to assess spatial learning and memory function, while the passive avoidance training (PAT) test, also known as the shuttle-box test, was utilized to evaluate fear learning and memory function. To avoid circadian rhythm artifacts, all measurements were performed at a specific time of day.

2.2.1. Morris Water Maze (MWM) Test. This experiment is utilized to assess the spatial learning and memory performance of animals. The MWM analysis was conducted following the protocol outlined by Vahdati Hassani et al. [29], with some slight modifications. The apparatus consisted of a round pool constructed from black metal sheets, measuring 136 cm \times 60 cm (diameter \times height). The pool's boundaries were divided into four equal quadrants, labeled as north (N), south (S), east (E), and west (W). The pool was filled with water at a depth of 25 cm, maintained at a temperature of 22 \pm 1°C. At the center of the northeast quadrant, a circular platform made of black Plexiglas with a diameter of 13 cm was positioned 2 cm below the water surface. The surrounding walls of the pool featured fixed visual cues that were visible to the rat. The testing chamber was dimly illuminated, and the room temperature was kept constant at $22 \pm 1^{\circ}$ C. A video camera connected to a computer was installed above the apparatus to monitor the rat's location and record the data obtained from each rat [29, 30].

(1) Acquisition Test. During the training trial period of the acquisition test protocol, each rat underwent five tests per block per day for five consecutive days. The rats were given 60 s to swim and locate the hidden escape platform. Once they found the platform, they remained on it for 15 s. If a rat failed to identify the platform within 60 s, it was manually placed on the platform for 15 s. This 15-s rest period served the purpose of familiarizing the rat with the environment. After each test, the rat was dried with a towel, returned to its home cage, and promptly placed back in the colony room. Throughout the 5-day training trial period, various measurements were automatically recorded using a mounted video camera situated on top of the device. These measurements included the escape latency (s) to detect the hidden platform, the escape pathlength or the total swimming distance (cm, traveled distance to the hidden platform, as the basic motor function), and the swim speed (cm/s) [29].

(2) Probe Test. The probe test was conducted on the 81 days after treatment (or 24 hr after the last training trial period). After removing the hidden platform, the rats underwent a single search trial lasting 60 s to evaluate their spatial memory. The starting position for each rat was set in a way that the rat accidentally entered the water while facing the wall, and it was positioned in the center of one of the quadrants without a platform. At the conclusion of each test, the rat was dried with a towel, returned to its home cage, and immediately placed back in the colony room. The total time taken to discover the hidden platform, known as escape latency time, was recorded. This measurement indicated an inverse relationship between the ability to find the hidden platform location and spatial learning and memory. The traveled distance and the swim speed (cm/s) during the probe test were also automatically recorded using videocamera tracking software connected to a computer (Noldus EthoVision XT, Noldus Information Technology, Wageningen, Netherlands) [29]. All experimental tests were conducted between 8:00 a.m. and 4:00 p.m. to minimize potential biases caused by time differences. Each group consisted of six rats.

2.2.2. Passive Avoidance Training (PAT) Test. The PAT test was performed 2 days following the MVM test. PAT assesses two types of avoidance behaviors known as step-down and step-through. The purpose of the PAT test, which is conducted on the shuttle-box apparatus, is to assess fear learning and memory function in rodent models displaying central nervous system disorders induced by excitatory stimuli like foot shocks. For this study, the PAT protocol was slightly modified based on the method described by Hogan et al. [30]

and Taherian et al. [31]. In brief, the experimental setup consists of a shuttle-box apparatus comprising two chambers of equal size: a dark box and a lightbox. These chambers are constructed using transparent acrylic resin epoxy plexiglass sheet panels, measuring 20 cm in length, 20 cm in width, and 30 cm in height. They are connected by a sliding door (guillotine) measuring 7 cm \times 9 cm, which can be raised up to 10 cm. A parallel stainless-steel grid with a diameter of 2.5 mm and intervals of 1 cm is positioned on the floor of the dark chamber. This grid is connected to an electrical shock generator (Borj Sanat Co., Tehran, Iran) and capable of producing electrical signals that are transmitted to the grid. The PAT test comprises three phases: habituation, training (acquisition trial), and retention.

(1) Habituation Phase. In the first step, following a period of 2 days' rest, the rat was gently introduced to the light chamber to habituate to the device. After a brief interval of 10 s, the sliding door was raised. Subsequently, the rat was permitted to enter the dark chamber and remained there (experimental room) for a duration of 10 min. Upon completion of the 10-min period, the rat promptly returned to its designated home cage. Thirty minutes after the first stage of habituation, an identical habituation test was conducted once again. Following the 10-min period, the rat was immediately returned to its home cage. Rats that took more than 120 s to enter the dark box were not included in this study.

(2) Training Phase (Acquisition Trial). This step was carried out 30 min after the second trial of habituation. The procedure is as follows: the rat was gently placed in the light chamber, and then the middle guillotine lid was slowly opened. Due to their innate desire, rats typically enter the dark chamber. Once the rat had completely entered the dark chamber (four paws), the middle guillotine lid was closed (exploration time). After that, a single electric current (intensity: 1 mA; frequency: 50 Hz; duration: 3 s) (Borj Sanat Co., Tehran, Iran) was instantly applied to the steel grids of the dark chamber floor through an electrical generating device to induce shock to the rat's paws. Following the administration of the foot shock, a period of 20 s elapsed before the rat was removed from the apparatus. The rat was then returned to its home cage and promptly placed back in the colony room. Each rat underwent this training process up to three times.

(3) Retention (Retrieval) Test. The memory retention phase, also known as the last step, took place on 24 hr after the training phase (acquisition trial), but this time no electric shock was administered to the rats' paws. The work phases were carried out as follows: once the rat was placed in the lightbox, the door opened after a 10-s delay. The inhibitory avoidance memory was assessed by measuring the stepthrough latency (STL) or time latency, which represents the time interval it took for the rat to move from the lightbox to the dark box. The cut-off time of STL measurement for all rats that remained in the light chamber was 300 s, or the test ended when the rat entered the dark chamber. Each rat individually performed all behavioral steps, following the sequence mentioned above, between 8:00 a.m. and 2:00 p.m. This timeframe was chosen to minimize any potential biases caused by variations in time. There were six rats in each group.

Throughout the trials, a camera positioned directly above the apparatus recorded all the events. The camera was connected to a computer that automatically analyzed all events (Noldus EthoVision XT, Noldus Information Technology, Wageningen, Netherlands). After completing the behavioral tests for each rat, the boxes were cleaned using a damp sponge soaked in hot water and a small amount of 100% ethanol. They were then dried with a clean towel to remove any odors left by the previous animal.

2.3. Drugs and Reagents. Silymarin nanomicelle in capsule form was purchased from Elixir Nano Sina Co. (Iran) and each capsule contains 70 mg of purified silymarin. Rat IL-1 β , TNF- α , and IL-6 primers were purchased from Metabion Co. (Germany). RNA extraction and complementary DNA (cDNA) synthesis kit were purchased from Parstous Co. (Iran). Malon-dialdehyde (MDA), superoxide dismutase (SOD), and catalase (CAT) reagent kits were purchased from Teb Pazhouhan Razi Co. (Iran). Bicinchoninic acid (BCA) protein assay kit was purchased from Bio Basic Co. (Canada).

2.4. Detection of mRNA Expression Levels by Real-Time Polymerase Chain Reaction (qRT-PCR). Sacrifice and sampling of all rats were performed at a specific time of day, which further minimizes the potential for circadian rhythm artifacts. Liver and cortex, as well as hippocampus of the brain tissues, were homogenized for RNA extraction. The RNA extraction method used was the same as described in the Parstous RNA extraction kit (Iran) protocol. The extracted RNA was then converted to complementary DNA (cDNA) using the Parstous cDNA synthesis kit (Iran) according to the manufacturer's instructions. cDNA sequences for IL-1 β , TNF- α , and IL-6 genes were obtained from the NCBI database.

To analyze gene expression, qRT-PCR amplification of target genes was performed using Roche real-time thermal cycler (Mannheim, Germany) with appropriate primers and SYBR Green qPCR Master Mix. The expression levels of the desired genes were normalized using a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as an internal reference gene. The $2^{-\Delta\Delta Ct}$ method was used to determine the relative changes in gene expression.

2.5. Measurement of Oxidative and Antioxidant Parameters in the Brain and Liver. The liver, cortex, and hippocampus tissues were mixed with PBS and processed according to the instructions provided in the relevant kits. The resulting supernatant was used to determine the levels of MDA as well as the activity of SOD and CAT. To measure protein concentration, a BCA protein assay reagent kit was utilized. These values were then standardized based on total protein content in the liver, cortex, and hippocampus tissues. The resulting measurements were presented as μ M MDA/mg protein for MDA levels, μ M protein for SOD activity, and nmol/min/ml for CAT activity.

2.6. Statistical Analysis. Statistical analysis was performed using GraphPad Prism 8. The HU examination was analyzed using Student's *t*-test. The MWM probe trials and PA tests were analyzed using one-way ANOVA. The time spent and distance moved during MWM training were analyzed using two-way ANOVA with repeated measures to assess learning rate differences. Tukey's post hoc test was used for group comparisons when significant differences were observed. Inflammation and oxidative stress levels among different groups were compared using ANOVA followed by Bonferroni's *t*-test. The average results were demonstrated as mean \pm standard deviation (SD). Statistical significance was determined when the *P* value was below 0.05 ($P \le 0.05$).

3. Results

3.1. Results of HU Examination. The analysis showed a significant decrease in tibial bone density in the OVX group (529.4 \pm 38 HU) compared to the control rats (681.2 \pm 23 HU) (P = 0.0001). Therefore, ovariectomy has led to the development of osteoporosis and, consequently, the menopausal model has been induced.

3.2. Evaluating the Impact of Silymarin on Spatial and Fear Learning and Memory Function in the MWM and Shuttle Box Tests after Menopause. Our results indicate that silymarin enhances memory function in OVX rats. In all of the memory assessments performed, such as the MWM and shuttle box tests (Figures 1 and 2), the sham group exhibited a statistically significant difference when compared to the control group. Thus, it can be inferred that ovariectomy is linked to memory impairment.

Based on the data received from the MVM test, the mean escape latency time (s) of sham group has significantly increased compared to the control group during the training days (practice from the first day to the fifth day). However, the prescription of silymarin on the training days (practice from the first to the fifth day) with doses of 100 and 150 mg/kg, as well as with a dose of 50 mg/kg only on the fifth day, has significantly reduced the mean escape latency time (s) compared to sham group (Figure 1(b)). Also, the mean time spent in the target quadrant (s) of sham group has significantly decreased compared to the control group on the test day (probe). However, the prescription of silymarin with doses of 100 and 150 mg/kg on the test day (probe) has significantly increased the mean time spent in the target quadrant (s) compared to sham group (Figure 1(a)). In addition, the traveled distance (cm) during the training days (practice from the first day to the fifth day) of sham group has significantly increased compared to the control group. However, the prescription of silymarin with doses of 100 (on the third to the fifth day) and 150 (on the second to the fifth day) mg/kg has significantly reduced the traveled distance (cm) compared to sham group (Figure 1(d)). Furthermore, the mean traveled distance (cm) in the target quarter on the test day (probe) in sham group has significantly decreased compared to the control group. However, the administration of silymarin with doses of 100 and 150 mg/kg on the test day (probe) has significantly reduced the mean traveled distance in the target quarter (cm) compared to sham group (Figure 1(c)). Another outcome of this study showed that the mean swimming speed of rats (cm/s) in sham group has significantly decreased compared to the control group on the test day (probe) (Figure 1(e)) and the training days (practice from the first day to the fifth day) (Figure 1(f)). However, the administration of silymarin with doses of 100 and 150 mg/kg on the test day (probe) (Figure 1(e)) and also the administration of silymarin with doses of 100 mg/kg on the training days (practice on the fourth and fifth days) and 150 mg/kg on the training days (practice from the second day to the fifth day) (Figure 1(f)) has significantly increased the mean swimming speed of rats (cm/s) compared to sham group.

Based on the data obtained from the shuttle box test, the mean STL (s) in the sham group has significantly decreased compared to the control group. However, silymarin at doses of 100 and 150 mg/kg has significantly increased STL, and fear learning and memory compared to the sham group (Figure 2). Consequently, silymarin can improve spatial and fear learning and memory function in menopausal women.

3.3. Silymarin Efficiently Inhibited the Expression Levels IL-1 β , TNF- α , and IL-6 in the Liver after Menopause. Our results showed that silymarin dose-dependently reduced the levels of mRNA IL-1 β , TNF- α , and IL-6 in the liver of OVX rats (Figure 3). As shown in Figure 3(a), the sham group had a higher expression of IL-1 β compared to the control group, indicating that ovariectomy led to increased mRNA expression of IL-1 β in the liver. In the intervention groups, the expression of IL-1 β mRNA was significantly decreased compared to both the control and sham groups. Administration of silymarin extract at doses of 50, 100, and 150 mg/kg body weight in OVX rats showed statistically significant differences compared to the control and sham groups.

Furthermore, as shown in Figure 3(b), the sham group had higher expression of TNF- α compared to the control group, indicating that ovariectomy led to increased mRNA expression of TNF- α in the liver. In the intervention groups, the expression of TNF- α mRNA was significantly decreased compared to both the control and sham groups. In OVX rats, administration of silymarin extract at a dose of 150 mg/kg body weight showed statistically significant differences compared to the control group. Moreover, the administration of silymarin extract at doses of 50, 100, and 150 mg/kg body weight exhibited significant differences compared to the sham group.

As demonstrated in Figure 3(c), the sham group did not show a significant difference in the expression of IL-6 compared to the control group. In the intervention groups, the expression of IL-6 mRNA was significantly decreased compared to both the control and sham groups. In OVX rats, the administration of silymarin extract at doses of 50, 100, and 150 mg/kg body weight showed statistically significant differences compared to the control and sham groups.

The study showed that the highest reduction in mRNA expression of IL-1 β in the liver of OVX rats was observed at doses of 100 and 150 mg/kg body weight of silymarin. The maximum reduction in mRNA expression of TNF- α and IL-6 in the liver was observed at a dose of 150 mg/kg body weight of silymarin in OVX rats. Therefore, silymarin has anti-inflammatory properties in the liver of menopausal women.

3.4. Silymarin Efficiently Inhibited the Expression Levels IL-1 β , TNF- α , and IL-6 in the Hippocampus and Cortex after Menopause. Our results demonstrated that silymarin reduced the mRNA levels of IL-1 β , TNF- α , and IL-6 in both the hippocampus (Figure 4) and cortex (Figure 5) of OVX rats.



FIGURE 1: MWM behavioral outcomes of five groups based on the time spent in target quadrant (a, b), the travelled distance in target quadrant (c, d), and swim speed (e, f). Data are presented as mean \pm SEM (n=6 in each group). (* $P \le 0.05$; ** $P \le 0.01$; *** $P \le 0.001$) indicated statistical significance compared to the control group. (* $P \le 0.05$; ** $P \le 0.001$) indicated statistical significance compared to the sham group.



FIGURE 2: PAT test outcomes of five groups. Data are presented as mean \pm SEM (n=6 in each group). (*** $P \le 0.001$) indicated statistical significance compared to the control group. (${}^{\#}P \le 0.05$; ${}^{\#}P \le 0.01$) indicated statistical significance compared to the sham group.

Figures 4(a) and 5(a) show that the sham group did not exhibit a significant difference in IL-1 β expression compared to the control group. However, in the intervention groups, there was a significant decrease in IL-1 β mRNA expression compared to both the control and sham groups. In OVX rats, administration of silymarin extract at doses of 50, 100, and 150 mg/kg body weight resulted in statistically significant differences compared to the control and sham groups in both the hippocampus and cortex.

Figures 4(b) and 5(b) show that the sham group did not exhibit a significant difference in TNF- α expression compared to the control group. However, in the intervention groups, TNF- α mRNA expression significantly decreased compared to both the control and sham groups. In OVX rats, administration of silymarin extract at doses of 50, 100, and 150 mg/kg body weight resulted in statistically significant differences compared to the control group in the hippocampus. Furthermore, at a dose of 150 mg/kg body weight, silymarin extract showed statistically significant differences compared to the sham group in the hippocampus. Administration of silymarin extract at doses of 50, 100, and 150 mg/kg body weight showed statistically significant differences compared to the control and sham groups in the cortex.

Figures 4(c) and 5(c) show that the sham group had a higher expression of IL-6 than the control group, indicating that ovariectomy increased the mRNA expression of IL-6 in the hippocampus and cortex. However, in the intervention groups, there was a significant reduction in the expression of IL-6 mRNA compared to both the control and sham groups. In OVX rats, administration of silymarin extract at doses of 50, 100, and 150 mg/kg body weight resulted in statistically significant differences compared to both the sham and control groups in the hippocampus and cortex.

The study showed that silymarin has anti-inflammatory properties in the hippocampus and cortex of menopausal women. In OVX rats, administering a dose of 150 mg/kg body weight of silymarin resulted in the highest reduction in mRNA expression of IL-1 β , TNF- α , and IL-6 in the hippocampus. In the cortex, the maximum reduction in mRNA expression of IL-1 β and TNF- α was observed at doses of 100 and 150 mg/kg body weight of silymarin, while the greatest decrease in mRNA expression of IL-6 was observed at doses of 50, 100, and 150 mg/kg body weight of silymarin. Thus, silymarin has anti-inflammatory properties in both the hippocampus and cortex of menopausal women.

3.5. Evaluating the Impact of Silymarin on MDA Levels, CAT, and SOD Activity in the Liver after Menopause. Our results showed that silymarin reduced MDA levels and increases CAT and SOD activity in the liver of OVX rats (Figure 6). As shown in Figure 6(a), the sham group had higher MDA level than the control group, indicating that ovariectomy led to increased MDA levels in the liver. In the intervention groups, MDA levels were significantly decreased compared to both the control and sham groups. In OVX rats, silymarin extract doses of 100 and 150 mg/kg body weight showed statistically significant differences compared to the control group. Additionally, silymarin extract doses of 50, 100, and 150 mg/kg body weight exhibited significant differences compared to the sham group. As shown in Figure 6(b), the sham group did not show a significant difference in CAT activity compared to the control group. In the intervention groups, CAT activity was significantly increased compared to both the control and sham groups. In OVX rats, administration of silymarin extract at doses of 50, 100, and 150 mg/kg body weight showed statistically significant differences compared to the control and sham groups. According to Figure 6(c), the sham group did not show a significant difference in SOD activity compared to the control group. In the intervention groups, administration of silymarin extract at a dose of 150 mg/kg body weight showed statistically significant differences compared to the control and sham groups.

The study showed that administration of silymarin at a dose of 150 mg/kg body weight resulted in the most notable decrease in MDA levels and increase in SOD activity in the liver. The highest increase in CAT activity in the liver was observed in OVX rats that were administered a dose of 100 mg/kg body weight of silymarin. Therefore, silymarin alleviates oxidative stress in the liver of menopausal women.

3.6. Evaluating the Impact of Silymarin on MDA Levels, CAT, and SOD Activity in the Hippocampus and Cortex after Menopause. The findings revealed that silymarin had a positive impact on OVX rats, specifically reducing MDA levels and increasing CAT and SOD activity in both the hippocampus (Figure 7) and cortex (Figure 8) regions of the brain.

Figures 7(a) and 8(a) show that the sham group did not exhibit a significant difference in MDA levels compared to the control group. However, in the intervention groups, MDA levels significantly decreased compared to both the control and sham groups. Administration of silymarin extract at doses of 50, 100, and 150 mg/kg body weight to OVX rats resulted in statistically significant differences compared to the control and sham groups in the hippocampus. Additionally, administration



FIGURE 3: Silymarin inhibits the expression of IL-1 β , TNF- α , and IL-6 in the liver after menopause. The mRNA expression levels of IL-1 β (a), TNF- α (b), and IL-6 (c) were evaluated by qRT-PCR in samples of liver tissue from five groups of rats. The experiments were conducted in triplicates, and the results are illustrated as the mean \pm SD (n=6 in each group). (* $P \le 0.05$; ** $P \le 0.001$; *** $P \le 0.001$; **** $P \le 0.0001$) indicated statistical significance compared to the control group.(* $P \le 0.05$; ** $P \le 0.0001$) indicated statistical significance compared to the sham group.

of silymarin extract at doses of 100 and 150 mg/kg body weight to OVX rats resulted in statistically significant differences compared to the control and sham groups in the cortex.

Figures 7(b) and 8(b) show that the sham group did not exhibit a significant difference in CAT activity compared to the control group. However, in the intervention groups, CAT activity significantly increased compared to both the control and sham groups. In OVX rats, administration of silymarin extract at doses of 50, 100, and 150 mg/kg body weight resulted in statistically significant differences compared to the control group in the hippocampus. Additionally, administration of silymarin extract at doses of 100 and 150 mg/kg body weight exhibited significant differences compared to the sham group in the hippocampus. Administration of



FIGURE 4: Silymarin inhibits the expression of IL-1 β , TNF- α , and IL-6 in the hippocampus after menopause. The mRNA expression levels of IL-1 β (a), TNF- α (b), and IL-6 (c) were evaluated by qRT-PCR in samples of hippocampus tissue from five groups of rats. The experiments were conducted in triplicates, and the results are illustrated as the mean \pm SD (n = 6 in each group). (** $P \le 0.001$; *** $P \le 0.001$) indicated statistical significance compared to the control group. (* $P \le 0.05$; ** $P \le 0.001$; *** $P \le 0.001$) indicated statistical significance compared to the sham group.

silymarin extract at doses of 100 and 150 mg/kg body weight showed statistically significant differences compared to the control group in the cortex. Additionally, administration of silymarin extract at doses of 50, 100, and 150 mg/kg body weight exhibited significant differences compared to the sham group in the cortex.

Figures 7(c) and 8(c) show that the sham group did not exhibit a significant difference in SOD activity compared to



FIGURE 5: Silymarin inhibits the expression of IL-1 β , TNF- α , and IL-6 in the cortex after menopause. The mRNA expression levels of IL-1 β (a), TNF- α (b), and IL-6 (c) were evaluated by qRT-PCR in samples of cortex tissue from five groups of rats. The experiments were conducted in triplicates, and the results are illustrated as the mean \pm SD (n=6 in each group). (* $P \le 0.05$; ** $P \le 0.001$; **** $P \le 0.001$) indicated statistical significance compared to the control group. (* $^{\#\#}P \le 0.001$; *** $^{\#\#\#}P \le 0.0001$) indicated statistical significance compared to the sham group.

the control group. However, administration of silymarin extract at doses of 100 and 150 mg/kg body weight to OVX rats resulted in statistically significant differences in SOD activity in the hippocampus compared to the control group. Additionally, OVX rats when were administered silymarin extract at doses of 100 and 150 mg/kg body weight exhibited statistically significant differences in SOD activity in the cortex compared with the sham group.

The study showed that administration of silymarin at a dose of 150 mg/kg body weight resulted in the most significant decrease in MDA levels in the hippocampus. Additionally, the highest increase in CAT and SOD activity in the



FIGURE 6: Comparison of MDA concentration (a), CAT activity (b), and SOD activity (c) in the liver tissue of five groups. Data are presented as mean \pm SEM (n=6 in each group). (* $P \le 0.05$; ** $P \le 0.01$) indicated statistical significance compared to the control group. (* $P \le 0.05$; ** $P \le 0.01$; ** $P \le 0.01$; ** $P \le 0.001$) indicated statistical significance compared to the sham group.

hippocampus was observed in OVX rats at doses of 150 mg/ kg for CAT activity and 100 and 150 mg/kg for SOD activity. Similarly, the study also revealed that the most significant decrease in MDA levels in the cortex occurred at a dose of 150 mg/kg body weight of silymarin. Moreover, the greatest increase in CAT and SOD activity in the cortex was observed in OVX rats when silymarin was administered at doses of 100 mg/kg for CAT activity and 100 and 150 mg/kg for SOD activity, based on body weight. Consequently, silymarin can alleviate oxidative stress in the hippocampus and cortex of menopausal women.

4. Discussion

In rats undergoing ovariectomy, the levels of TNF- α , IL-1 β , and IL-6 increase, which are pro-inflammatory cytokines responsible for creating inflammation [32, 33]. During menopause, the decrease in estrogen levels leads to an increase in inflammation. This can result in the development of cerebral edema and disorders in the nervous system caused by intrace-rebral hemorrhage. Estrogen's protective effects extend beyond reducing inflammation and oxidative stress [2, 34]. It also strengthens the BBB by inhibiting NF- κ B [9]. Estrogen reduces



FIGURE 7: Comparison of MDA concentration (a), CAT activity (b), and SOD activity (c) in the hippocampus tissue of five groups. Data are presented as mean \pm SEM (n=6 in each group). (* $P \le 0.05$; ** $P \le 0.01$; *** $P \le 0.001$) indicated statistical significance compared to the control group. (* $P \le 0.05$; ** $P \le 0.001$) indicated statistical significance compared to the sham group.

mitochondrial lipid peroxidation, enhances the expression of the SOD2 gene [35], and regulates the production of ROS in cardiac mitochondria [5, 36, 37].

In the study we conducted, we found several lines of evidence suggesting that silymarin may be considered as an antiinflammatory and antioxidant agent in menopausal women. Silymarin efficiently inhibits the expression of TNF- α , IL-1 β , and IL-6 in liver, hippocampus, and cortex. It can also increase the activity of SOD and CAT, which play a crucial role in protecting cells from oxidative damage caused by ROS and decrease MDA levels in the tissues mentioned. Additionally, it can improve learning and memory and reverse cognitive impairment caused by menopause. After menopause, several changes occur in the body. One of these changes is an increase in the production of H_2O_2 by the liver's mitochondria [6, 38]. At the same time, there is a decrease in the expression of antioxidant enzymes, specifically SOD and GPX. This imbalance between oxidants and antioxidants leads to oxidative stress [1]. Oxidative stress arises when there is an uneven distribution favoring oxidants over antioxidants, resulting in excessive generation of ROS or free radicals that can inflict damage on biological systems [39, 40]. Additionally, the enzyme NADPH oxidase becomes more active, promoting ROS production and worsening oxidative stress [1]. Elevated levels of oxidative stress markers like MDA [41] and GSSG indicate the presence of oxidative



FIGURE 8: Comparison of MDA concentration (a), CAT activity (b), and SOD activity (c) in the cortex tissue of five groups. Data are presented as mean \pm SEM (n = 6 in each group). (* $P \le 0.05$; ** $P \le 0.01$; *** $P \le 0.001$) indicated statistical significance compared to the control group. (* $P \le 0.05$; ** $P \le 0.001$) indicated statistical significance compared to the sham group.

stress [1]. In addition to oxidative stress, menopause also leads to other systemic changes. There is a significant increase in the levels of pro-inflammatory cytokines, specifically IL-6, in the bloodstream. These cytokines play a role in inflammation and can contribute to the development of chronic diseases [42]. In our study, the levels of IL-6 and MDA increased in the sham group, which was used as the menopause model. Therefore, our study is consistent with these earlier studies. The pro-inflammatory effects of estrogen deficiency are carried out through multiple mechanisms. A study by Xu et al. [43] showed that ovariectomy increases significant active NF-kB, pro-IL-1 β , and pro-IL-18 in the hippocampus of mice, leading to neuroinflammation. Furthermore, menopausal women who commonly experience hot flashes also exhibit heightened levels of IL-8 and TNF- α [42]. Therefore, an approach is needed to reduce these inflammations and oxidative stress caused by menopause. In our study, we

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were not given any silymarin extract, had higher levels of IL-1 β and TNF- α , which is not surprising based on previous study.

Recently conducted studies have shown that silybin, the main component of silymarin extracted from milk thistle, has antioxidant and anti-inflammatory effects on liver cells [20, 44], and the anti-inflammatory effects of silymarin are due to its reduction of TNF- α [45]. As our study has shown, silymarin has a protective effect on the liver and reduces TNF- α . These results are not unexpected based on previous researches.

Silybin is known as a cell proliferation inhibitor, apoptosis inducer, and angiogenesis inhibitor and has antitumor effects in cancers such as skin, liver, lung, bladder [20], stomach [46], colon [47], prostate [48], and breast. It can help reduce the expression of pro-inflammatory interleukins and TGF- β in certain types of cancer cells [49]. So, our study results indicate that the reduction of inflammatory factors such as IL-1 β and IL-6 by silymarin is consistent with this study.

Silvbin affects both ER, α and β , but it has opposite effects on these two receptors. Its effect on $ER\alpha$ is through signaling pathways PI3K/AKT/mTOR and RAS/ERK, while its effect on $\text{Er}\beta$ increases the number of apoptotic cells [20]. Additionally, the PI3K/AKT pathway regulates various transcriptional factors such as NF- κ B, leading to diverse cellular responses like cell apoptosis, invasion, and inflammation [50]. Silymarin has been demonstrated to be superior to estrogen doses in enhancing vascular health in menopausal women. This is likely due to its efficacy in treating agerelated endothelial damage by modulating NO signaling and calcium homeostasis in blood vessels [51]. Additionally, silvmarin has been shown to increase the thickness of the luminal epithelium and the endometrium, suggesting that it has uterine growth-promoting effects [52]. These findings suggest that silymarin may be a promising therapeutic agent for both vascular health and reproductive function in menopausal women.

In recent years, silymarin has been increasingly studied for its potential protective effects on the nervous system. Studies have investigated its impact on various neurological disorders, including Alzheimer's, Parkinson's, and cerebral ischemia. In these studies, silymarin was found to reduce oxidative stress and inflammatory cytokines, ultimately leading to improved outcomes in these conditions [26], which all align with confirming the results of our study.

A study by Tao et al. [53] showed that administration of ammonium ferric citrate to OVX rats resulted in decreased levels of SOD2 and total antioxidant capacity and increased levels of MDA in serum. However, treatment with silymarin reduced oxidative stress in the serum of these rats. Therefore, the results of this study are consistent with our study, and silymarin was able to reduce oxidative stress by decreasing MDA and increasing SOD2 levels [53].

The effects of estrogen injection and silymarin on oxidative stress and inflammation after menopause differ, as shown below. In OVX rats, E2 treatment reduces lipid peroxidation [15, 54] and does not significantly affect TNF- α and IL-1 β levels in the plasma [54]. Our study demonstrated that silymarin treatment reduced the levels of TNF- α and IL-1 β in the brain and liver of OVX rats. Therefore, it can be concluded that silymarin is more effective than estradiol in reducing these inflammatory factors after menopause.

Early onset of estrogen replacement therapy (ERT) prevents the ovariectomy-associated increase in mitochondrial H_2O_2 levels and decrease in CAT activity [15]. E2 treatment also reduces MDA levels and increases CAT and SOD levels in the brain cortex and liver of OVX rats [55, 56]. Furthermore, E2 treatment upregulates the expression of MnSOD in the brain [57] and attenuates the decrease in SOD1 in the rostral ventrolateral medulla (RVLM) of OVX rats [58]. In our study, we found that silymarin treatment was able to increase SOD and CAT levels and decrease MDA levels in the brain and liver of OVX rats, like estrogen treatment. However, estrogen injection must be started immediately after ovariectomy to achieve this effect. Otherwise, the effects of estrogen will decrease over time [15].

The hippocampus and prefrontal cortex are involved in spatial working memory [7, 59]. Memory encoding is linked to the anterior hippocampus, while memory retrieval is linked to the posterior hippocampus [60, 61]. Moreover, increased levels of pro-inflammatory cytokines in specific brain regions can cause cognitive deficits [59]. Targeting TNF- α can potentially prevent Alzheimer's and enhance cognition and memory [62, 63], while knocking down IL-1 β in the hippocampus has been shown to significantly alleviate memory deficits and depressive behaviors in mice [64]. Furthermore, chronic peripheral elevation of IL-6 in humans has been linked to cognitive impairments [65]. Based on these studies, it can be concluded that reducing these three factors (IL-6, IL-1 β , and TNF- α) in these regions of brain improves memory and cognition, as observed in our study.

A comparison of the effects of estrogen injection and silymarin on memory after menopause is presented below.

Luo et al. [66] found that 1 month of ERT improved spatial learning capacity and myelin sheaths in OVX rats. Our study found that silymarin injection also improved spatial learning capacity in OVX rats. These findings suggest that estrogen and silymarin injections may be effective in improving memory after menopause.

In general, although ERT has been shown to be effective in some studies, its safety has been questioned by some serious studies, including the Women's Health Initiative Memory Study and the Women's Health Initiative Study of Cognitive Aging. These studies found that ERT was associated with an increased risk of certain health problems, such as breast cancer, stroke, and heart disease [15]. As a result of these concerns, the clinical use of ERT is controversial and even not recommended. Silymarin is a safe and effective alternative to ERT. Silymarin has anti-inflammatory and antioxidant properties that may be beneficial for menopausal women.

Based on these findings, researchers believe that silymarin could be effective in suppressing mechanisms that



FIGURE 9: This graphical abstract illustrates the possible mechanisms of the effect of silymarin on liver and brain tissue during menopause.

cause inflammation and oxidative stress in menopausal women [67]. Silymarin can help to protect cells from damage caused by free radicals. Free radicals are unstable molecules that can damage DNA, proteins, and lipids. They are thought to play a role in the development of chronic diseases, such as Alzheimer's disease and Parkinson's disease [26]. In our study, silymarin increased the activity of antioxidant enzymes, such as SOD and CAT in the brains and livers of rats that underwent ovariectomy. SOD and CAT help to break down free radicals and protect cells from damage [26]. In addition, we observed a reduction in MDA, which is a marker of lipid peroxidation [68]. Therefore, the use of silymarin has reduced lipid peroxidation in the brain and liver of OVX rats. Silymarin can also help to reduce inflammation. Inflammation is a natural immune response that can be beneficial in the short term. However, chronic inflammation is thought to play a role in the development of many diseases, including Alzheimer's disease and Parkinson's disease [2, 23, 34]. In our study, silymarin inhibited the production of pro-inflammatory cytokines, such as IL-6, IL-1 β , and TNF- α , in the brains and livers of rats that underwent ovariectomy. These are pro-inflammatory cytokines that has been linked to cognitive decline [69, 70]. Therefore, it is likely that silymarin improved memory in OVX rats by reducing IL-6, IL-1 β , and TNF- α (Figure 9).

Finally, it is important to note that silymarin has been found to have minimal side effects in adults. While some individuals may experience weight gain, nausea, dry mouth, or excitability, severe allergic reactions are rare [71]. Furthermore, long-term use of silymarin for human diseases has not been associated with toxicity [16]. As such, silymarin represents a promising option for alleviating menopausal symptoms with minimal risk of side effects.

5. Conclusions

This study showed that ovariectomy leads to increased levels of MDA, a marker of oxidative stress, and inflammatory factors such as IL-1 β , TNF- α , and IL-6 in the brain and liver of rats. Silymarin was found to effectively reduce MDA levels, with its maximum antioxidant effect attributed to MDA reduction. Additionally, silymarin can increase CAT and reduce oxidative stress through pathways unrelated to SOD. However, at high doses, silymarin may impact SOD activity (Figure 10). Additionally, silymarin improved spatial memory and fear learning in OVX rats. These findings suggest that silymarin has the potential to be used as a drug to alleviate menopause-induced inflammation in women.



FIGURE 10: A qualitative comparison of some inflammatory and oxidative stress factors in three groups: intervention, sham, and control in the liver and brain of rats in a conducted study.

Data Availability

The data used to support the findings of this study are included within the article.

Ethical Approval

All experimental procedures were performed on animals. It was carried out in accordance with the animal care guidelines of Mashhad University of Medical Sciences (MUMS). The ethical code is IR.MUMS.AEC.1401.048.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

SIH, SF, and FG designed the experiments. RM and HJ performed experiments and collected data. All authors discussed the results and strategy. SIH and SF supervised, directed, and managed the study. RM wrote the first draft of the paper, and all authors approved of the version to be published.

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