Physiology and Pharmacology 29 (2025) 232-239
Short Communication

Neuroprotective effect of silibinin linked with attenuated Interleukin-6 and TNFα in an Aβ$_{1-40}$ Induced Alzheimer's rat model

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ABSTRACT

Introduction: The mechanisms behind Alzheimer's disease (AD) remain largely unclear. Reactive oxygen species and inflammatory cytokines contribute to inflammation and synaptic dysfunction in AD. This study investigates whether silibinin's neuroprotective properties act through regulating oxidative stress and inflammation.

Methods: Forty-eight Wistar rats (230±20g) were divided into four groups. The control group comprised healthy animals, while the lesion group received amyloid beta (Aβ $_{1-40}$). The vehicle group received silibinin solvent post-Aβ$_{1-40}$ injection. Treatment groups received silibinin (50, 100, and 200 mg/kg) after Aβ$_{1-40}$ injection. Following a passive avoidance behavior test, biochemical analysis of superoxide dismutase (SOD), malondialdehyde, Tumor necrosis factor α (TNF -α) and interleukin-6 (IL-6) was conducted.

Results: Administration of 100 mg/kg silibinin significantly reduced serum IL-6 levels compared to the lesion group (P=0.005). All silibinin doses significantly decreased TNF-α levels (P≤0.001). Serum superoxide dismutase (SOD) levels increased significantly in animals treated with 100 and 200 mg/kg silibinin compared to the lesion group (P=0.002, P=0.03). Step-through latency improved in silibinin-treated animals (100 and 200 mg/kg) (P≤0.006).

Conclusion: These results suggest silibinin can enhance cognitive function and offer neuroprotection by inhibiting lipid peroxidation and reducing pro-inflammatory cytokines (TNF-α and IL-6) in AD rat models.

Keywords:
Alzheimer's disease
Neuroinflammation
Silibinin
Oxidative stress

Introduction

Alzheimer's disease (AD) is characterized by the abnormal buildup of amyloid-beta protein outside nerve cells and tau protein inside them. These protein accumulations disrupt neuronal communication, ultimately leading to neuron destruction (Mohammadzadeh et al., 2014).

The exact mechanisms responsible for AD remain

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Received 10 March 2024; Revised from 3 October 2024; Accepted 5 October 2024
Citation: Salari S, Azizi M, Alihosseni T, Bagheri M. Neuroprotective effect of silibinin linked with attenuated Interleukin-6 and TNFα in an Aβ1-40 Induced Alzheimer's rat model. Physiology and Pharmacology 2025; 29: 232-239. http://dx.doi.org/10.61882/phypha.29.3.232

TABLE 1: Timeline of the study

Day 1	Day 1-28	Day 30-32	Day 33
surgery	Treatment with Silibinin	Behavioural test	Blood Collection

Amyloid-beta was injected stereotaxically on day 1, followed by silibinin treatment (50, 100, 200 mg/kg). A passive avoidance test was done on day 30, and blood sampling for biochemical analysis was done on day 33.

poorly understood. Some researchers have identified oxidative stress and mitotic changes as primary causes (Zhang et al., 2012). Oxidative stress results from an imbalance between prooxidants and cellular antioxidant defences, leading to cell damage, apoptosis, and memory loss (Cunnane et al., 2009). The brain, with its high oxygen consumption, is particularly vulnerable to oxidative agents.

Free radicals, reactive nitrogen species (RNS), and reactive oxygen species (ROS) damage cells by oxidizing membrane proteins, lipids, and DNA (Schmitt-Schillig et al., 2005). Lipid peroxidation and the subsequent generation of free radicals have been implicated as factors in various diseases, including Alzheimer's dementia (Purdy et al., 2004).

Milk thistle contains flavonoids, such as silybin and its stereoisomers (isosilybin, silychristin, isosilychristin, and silydianin) (Schulz et al., 2001). Silibinin, which comprises 70-80% of silymarin, has neuroprotective, antioxidant, anti-cancer, and anti-inflammatory effects (Mateen et al., 2010).

Prior studies have shown silibinin's neuroprotective properties in brain damage, diabetes, and memory impairment induced by amyloid β_{25-35} and neurotoxicity induced by MPP+, achieved through the improvement of oxidative stress and the inflammatory cascade (Geed et al., 2014; Hou et al., 2010). Additionally, it has been shown to ameliorate behavioral disorders associated with AD (Jangra et al., 2015).

Notably, milk thistle has shown potential in preventing AD progression through its antioxidant properties. It may improve cognitive symptoms and reduce GFAP levels in the astrocytes of Alzheimer's rat brains (Hadinia et al., 2010). Brahmachari et al. (2006) found that GFAP levels increase in astrocytes exposed to oxidizing agents like nitric oxide (Brahmachari et al., 2006).

Nitrosative stress, linked to aging, contributes to neurological diseases through inflammation, cell death, and oxidative stress (Javier Jimenez-Jimenez et al., 2016; Liu et al., 2011). Furthermore, an increase in MDA along with a decrease in SOD level has been proven in AD (Bagheri et al., 2012; Ghofrani et al., 2015). Thus, we aim to explore whether silibinin's neuroprotective effects are mediated through oxidative stress and inflammation.

Materials and Methods

Forty-eight male Wistar rats (230±20g) were randomly divided into six groups (n=8 each): 1-The control group (n=8); healthy animals. 2-The lesion group; stereotaxically administered $A\beta_{1-40}$ (n=8), 3-The vehicle group; $A\beta_{1-40}$ injection and received silibinin solvent (1ml normal saline via gavage for four weeks; n=8), 4-The treatment groups, $A\beta_{1-40}$ injection rats which received concentrations of 50, 100, and 200 mg/kg of silibinin (n=8; for each dose), 1 ml of the specified silibinin concentration/daily for four weeks. Notably, different doses of silibinin have been selected based on our recent *in vivo* studies (Alihosseini et al., 2023b). The timeline of the study is shown in Table 1.

Surgery

Animals were anesthetized with ketamine/xylazine (40/10 mg/kg). Using Paxinos and Watson's atlas, the coordinates for the hippocampus region were determined (AP=-3.5, ML=±2, DV=2.8), marked, and drilled. $A\beta_{1-40}$ solution (6 µl) was bilaterally injected into the hippocampus over 5 minutes using a Hamilton syringe.

The protocol was approved by the ethics committee of Ilam University of Medical Sciences (IR.MEDILAM.REC.1398.036).

Passive Avoidance Behavior

The passive avoidance behavior test was performed in week 5 (day 30 post-surgery) using a shuttle box. The shuttle box consists of two compartments, one light and one dark, each measuring 40x20x20 cm. The floors of both compartments are equipped with metal rods, spaced one centimeter apart with a diameter of 0.5 cm, which deliver electric shocks to the animal. A control unit reg-

ulates the shock's duration, frequency, and intensity via adjustable screws. A guillotine door (8x7 cm) separates the two chambers, allowing the animal to move freely between them. Each compartment has a sliding door on the roof for introducing or removing the animal. In this test, electric shocks are only administered in the dark compartment. The output screw is used to adjust the current intensity, measured in milliamps. The procedure unfolded as follows: Day 1 (Habituation): The first day was dedicated to familiarizing the animal with the shuttle box, without administering any electrical stimulation. The rat was placed in the light compartment and allowed to move freely into the dark compartment. Day 2 (Initial Latency Measurement): On the second day, the rat was placed in the light compartment, and the guillotine door was raised. As soon as the rat's hind legs entered the dark compartment, the door was closed, and an electric shock (50 Hz, 0.5 mA) was delivered. The time taken for the rat to enter the dark compartment was recorded as the initial latency. The cutoff time for the measurement was set at 300 seconds. Day 3 (Step-Through Latency): The procedure was repeated on the third day, but without the electric shock. The time the rat took to enter the dark compartment was recorded again, representing the animal's avoidance memory, or step-through latency (Bagheri et al., 2011).

Sample Processing

Blood sampling was conducted between 2:00 and 4:00 pm in a non-fasting condition, day 33 post-surgery. Since the animals were decapitated after this step, blood was collected via direct cardiac puncture. The samples were kept at room temperature for two hours before centrifugation at 1000 x g for 20 minutes. The serum was then extracted and stored at -20°C until further analysis.

Biochemical Analysis

Interleukin-6 (IL-6)

The Rat IL-6 Quantikine ELISA Kit (R6000B) was used for quantitative sandwich enzyme immunoassay. Following the manufacturer's protocol, a microplate pre-coated with a monoclonal antibody specific to IL-6 was used. Standards, controls, and samples were pipetted into the wells, where any IL-6 present bound to the immobilized antibody. After washing away unbound substances, an enzyme-linked polyclonal antibody specific to rat IL-6 was added. The enzymatic reaction produced a color change proportional to the quantity of IL-6, which was measured at 450 nm (Duan et al., 2023).

Malondialdehyde (MDA)

To assess lipid peroxidation (MDA) levels, we used the MDA Assay Kit (ab118970) following the manufacturer's protocol. In brief, serum samples and standards were incubated with TBA solution at 95°C for 60 minutes and then cooled for 10 minutes. The resulting product was measured calorimetrically at 532 nm (Park et al., 2017).

Superoxide dismutase (SOD)

The Nasdox™ Superoxide Dismutase Activity Assay Kit was used to measure SOD activity. Serum samples were centrifuged at 10,000 rpm for 15 minutes at 4°C, and the resulting supernatant containing Cu/Zn-SOD was used. After a 5-minute incubation at room temperature in the dark, the optical absorbance was recorded at 405 nm.

TNF-α

The TNF-α ELISA Kit (E0764Ra) was used according to the manufacturer's instructions. The microplate was pre-coated with Rat TNF-α antibodies. Biotinylated TNF-α antibodies were added to bind to TNF-α in the samples. Streptavidin-HRP was then added, which attached to the biotinylated TNF-α antibody. After washing away unbound Streptavidin-HRP, color development proportional to the quantity of TNF-α was stopped by a stop solution, and absorbance was measured at 450 nm (Ahmadi-Naji et al., 2017).

Statistical analysis

The dependent variables evaluated in this study were initial latency (IL), step-through latency (STL), IL-6, TNF-α, MDA, and SOD. Data analysis was conducted using GraphPad Prism software, with significance defined as $p<0.05$. Group comparisons were made using two-way ANOVA followed by Tukey's multiple comparison post-test to assess differences between groups. Data are presented as mean ± SEM.

Result

Passive avoidance behavior

No significant differences in Initial latency (IL) were observed across all experimental groups. However, step-

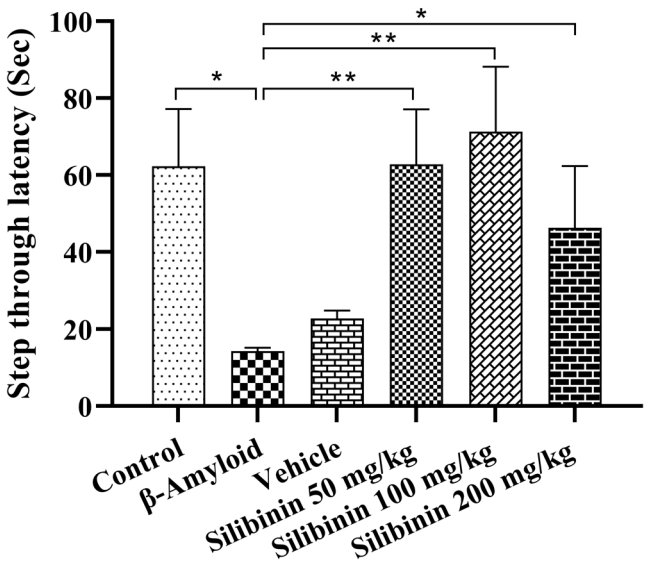

FIGURE 1. Aβ1-40 injected rats showed a significant decrease in step through latency (STL) time vs. the control rats (P=0.009). Aβ1-40-injected rats treated with 50 mg/kg, 100 mg/kg, and 200 mg/kg silibinin showed a significant increase in STL time vs. the lesioned rats (P≤0.006).

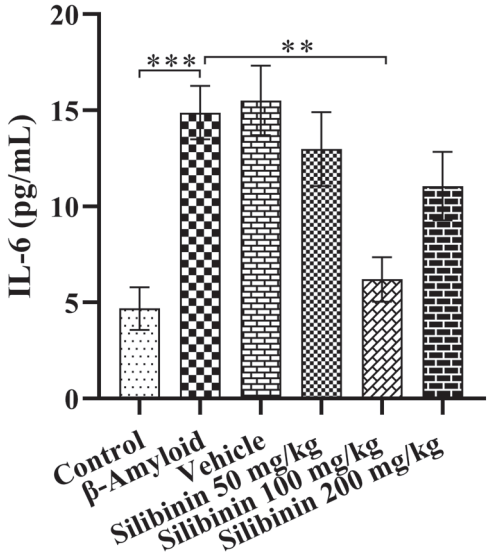

FIGURE 2. The concentration of IL-6 serum levels is shown in all groups. IL-6 significantly increased in Aβ1-40 injected rats vs. the controls (P=0.0007). Lesioned animals that received 100 mg/kg silibinin as a treatment showed a significant decrease in IL-6 serum level vs. the lesioned animals (P=0.005).

through latency (STL), which indicates the animal's ability to retain and recall information, was significantly reduced in lesioned rats (14.2 ± 0.9 seconds) compared to the control group (62 ± 14.9 seconds, P=0.009). In the Alzheimer's groups treated with low, medium, and high doses of silibinin, STL was measured at 62.8 ± 21.5, 71.2 ± 25.8, and 46.2 ± 24.5 seconds, respectively. Statistical analysis revealed significant differences between the treated groups and the untreated lesion group (Figure 1; P ≤ 0.006).

Notably, the vehicle group did not show any statistically significant differences compared to the untreated lesion group, and thus, it will not be discussed further.

IL-6

The IL-6 measurement, as depicted in Figure 2, showed that the IL-6 content was (4.6±1.1) in the control group and (14.8±1.3) in the lesioned group, indicat-

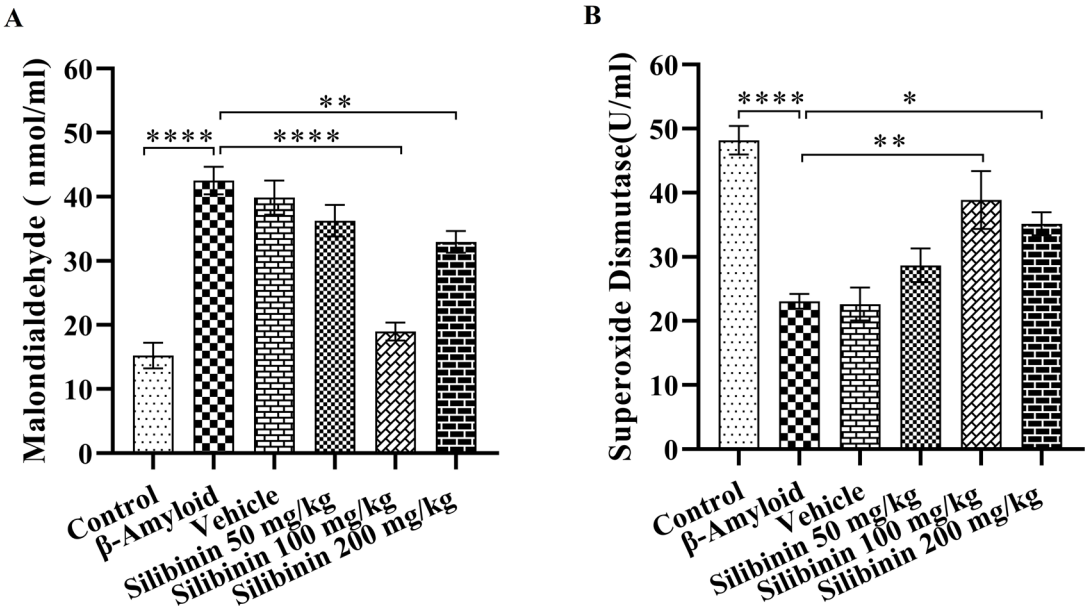

FIGURE 3. A. Serum concentration of malondialdehyde (MDA) and (B) superoxide dismutase (SOD) is shown in all groups. MDA significantly increased in Aβ1-40injected rats vs. the controls (*P*=0.0001). Lesion-treated animals that received 100 mg/kg and 200 mg/kg silibinin showed a significant decrease in MDA concentration vs. lesioned animals (*P*<0.0001, *P*=0.03). B. SOD significantly decreased in Aβ1-40 injected rats vs. the controls (*P*<0.0001). While, animals that received 100mg/kg silibinin after Aβ1-40 injection showed a significant decrease in serum level of SOD vs. the lesioned animals (*P*=0.002, *P*=0.03).

ing a significant increase in the latter (P=0.0007). Furthermore, IL-6 levels were (12.9±1.9) in the lesioned animals treated with 50 mg of silibinin, (6.2±1.1) in those treated with 100 mg of silibinin, and (11±1.7) in those treated with 200 mg of silibinin. Remarkably, only the treatment with 100 mg of silibinin led to a reduction in IL-6 content when compared to the lesioned rats (P=0.005).

MDA

Figure 3A shows the serum level of MDA in all groups. MDA was (15.21±2) in the control group; however, it exhibited a significant increase in the lesioned animals (45.5±2.1, P=0.0001). Additionally, MDA levels were (36.3±2.4) in the group treated with 50 mg of silibinin, (18.9±1.4) in the 100 mg silibinin treatment, and (33±1.7) in the 200 mg silibinin treatment. Interestingly, animals receiving 100 mg and 200 mg of silibinin demonstrated a notable reduction in MDA content when compared to the lesioned animals (P<0.0001, P=0.03).

SOD

The analysis of SOD levels indicated a notable increase in the lesioned animals compared to the control group, with levels of (48.2±2.2) and (23.08±1.1), respectively, resulting in a significant difference (P<0.0001). Moreover, animals treated with 50 mg of silibinin exhibited SOD levels of (28.7±2.6), with no significant improvement, while those treated with 100 mg of silibinin displayed (38.9±4.5), and the 200 mg-treated animals showed (37.2±1.8) SOD content, both demonstrating a significant increase when compared to the lesioned rats (P=0.002, P=0.03), as illustrated in Figure 3B.

TNF-α

The TNF-α measurement is presented in Figure 4. In the control group, TNF-α levels were (17.7±1.7), while in the lesioned group, they were (44.3±2.6), indicating a significant increase in the latter (P<0.0001). Furthermore, TNF-α levels were (30.09±1.6), (19.1±2.1), and (23.17±2.6) in the animals treated with 50 mg, 100 mg, and 200 mg of silibinin, respectively, with significant reductions in all treated rats (P≤0.001).

Discussion

In our current study, we aimed to evaluate the impact of silibinin administered at doses of 50, 100, and 200 mg/kg on the balance between oxidative stress and antioxidant defenses, as well as the inflammatory status in an animal model of AD induced by $A\beta_{1\text{-}40}$. Our findings

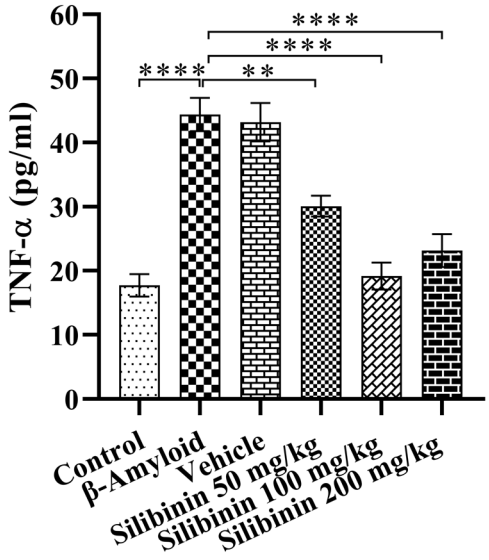

FIGURE 4. Concentration of TNF-α is shown in all groups. Aβ1-40-injected rats showed a significant increase in TNF-α serum level vs. the controls (P<0.0001). Animals that received 50 mg/kg, 100 mg/kg, and 200 mg/kg silibinin as a treatment showed a significant decrease in TNF-α content vs. the lesioned animals (P≤0.001).

showed that treatment with 100 and 200 mg/kg of silibinin yielded more beneficial protective effects in mitigating Aβ neurotoxicity. This was evidenced by a reduction in inflammatory markers such as IL-6 and TNF-α, as well as the lipid peroxidation marker MDA, coupled with an improvement in SOD levels. Additionally, behavioral analysis conducted through the shuttle box test further underscored the neuroprotective characteristics of silibinin, particularly at the higher doses of 100 and 200 mg/kg.

Chronic neuroinflammation plays a significant role in the progression of AD. This inflammation leads to the activation of microglia and astrocytes, which release pro-inflammatory cytokines, ultimately contributing to neurodegeneration (Calsolaro and Edison 2016).

Many research investigations have delved into the impact of silibinin on cognitive function and neuronal health in rat models of AD, yielding results that closely align with our own findings. For instance, Pengsheng Wei's study in 2022 demonstrated that silibinin could mitigate cognitive impairment induced by formaldehyde exposure through the inhibition of oxidative stress (Wei et al., 2022). Similarly, Panwen Liu and colleagues observed improvements in learning and memory in STZ rat models with silibinin treatment (Liu et al., 2020). It is possible that silibinin exerts its beneficial effects on cognitive function by upregulating the insulin signaling pathway and mitigating apoptosis (Liu et al., 2020).

Additionally, research has observed a reduction in neuronal damage, apoptosis, and oxidative stress within the brains of rats treated with silibinin (Bai et al., 2017; Lu et al., 2009). These effects might contribute to the preservation of cognitive function. Furthermore, it has been demonstrated that silibinin inhibits Aβ aggregation and promotes its clearance. Studies involving APP/PS1 transgenic mice, a model for AD, revealed that silibinin administration led to decreased levels of soluble $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$, as well as reduced Aβ deposition in the brain. This effect is attributed to the downregulation of amyloid precursor protein (APP) and β-secretase (BACE1) levels, alongside the upregulation of neprilysin (NEP), an enzyme involved in Aβ degradation (Bai et al., 2019).

Furthermore, inflammation plays a pivotal role in the progression of AD. Silibinin, known for its anti-inflammatory properties, effectively modulated the release of pro-inflammatory cytokines, namely IL-6 and TNF-α (Meng et al., 2022), as our findings showed. This modulation led to an improvement in passive avoidance behavior in the rat models of AD. It is worth noting that while the amelioration of IL-6 was achieved with 100 mg/kg silibinin treatment, all doses of silibinin were effective in reducing serum levels of TNF-α.

Moreover, the antioxidant capacity of silibinin has been demonstrated to some extent in our recent labo-

ratory work (Alihosseini et al., 2023a). Specifically, the administration of 100 and 200 mg/kg of silibinin was shown to enhance the balance in the oxidant-antioxidant status, which was characterized by a decrease in serum levels of MDA and an increase in SOD activity.

These combined anti-inflammatory and antioxidant properties attributed to silibinin may contribute significantly to its overall neuroprotective effects.

In various cognitive assessments, including the Y-maze and Morris water maze tests, silibinin has been reported to improve cognitive deficits in AD models, as we found in passive avoidance behavior. These improvements are linked to its antioxidative and anti-inflammatory actions, which collectively enhance synaptic function and neuroprotection against Aβ-induced damage (Bai et al., 2019; Lu et al., 2009), findings consistent with our current results.

However, it is crucial to acknowledge that while results from various studies are promising, variations in study methodologies, dosages, and the specific rat models employed can lead to differing outcomes. Additionally, the precise mechanisms through which silibinin affects the pathology of Alzheimer's disease require further investigation.

To summarize, emerging findings indicate that silibinin may hold promise as a therapeutic intervention for AD. Specifically, it shows potential for enhancing cognitive function and providing neuroprotection by suppressing lipid peroxidation and pro-inflammatory cytokines in rat models of AD. Nevertheless, there is a need for more extensive and standardized research to confirm these observations and elucidate the precise mechanisms through which silibinin confers its beneficial effects.

Acknowledgement

Our thanks go to Ilam University of Medical Sciences for great support during this project.

Conflict of interest

The authors have no conflict of interest.

Ethic approval

The current protocol was performed after receiving ethical approval from the ethics committee of Ilam University of Medical Sciences, numbered IR.MEDILAM.REC.1398.036.

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