Effects of *Silybum Marianum* on hippocampus GFAP and spatial memory in mice model of Alzheimer's disease

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Abatract: Silybum Marianum has high levels of antioxidant polyphenolic substances and neuro-protective effects on neurodegenerative diseases. The aim of this study was investigation effects of Silybum Marianum on the hippocampus GFAP and spatial memory in mice model of Alzheimer's disease. **Materials & Methods**: Thirty adult male Wistar rats were allocated in three groups: sham group, experimental group, and lesion group which each group was consist of ten rats. The experimental and lesion groups were injected Ibotonic acid in their Nucleus Basalis Magnocellularis (NBM) after fixation in stereotaxic apparatus, whereas the sham group underwent surgical procedure without injection. The experimental group received 200mg/kg of Silybum marianum extract orally, diluted in 1% Arabic gum. The sham group received 1% Arabic gum every day for four weeks also. The lesion group did not receive anything. The behavioral assessment was measured, after treatment, by using of Y maze test on 7thday and 28th in all groups. The ELISA method was used to measure the GFAP level in Hippocampus at the end of behavioral assessment. The collected data was analyzed by the SPSS software using ANOVA and Repeated Measures of Analysis Variance tests. **Results:** The spatial memory in the experimental group compared to the lesion and sham groups were improved significantly on day 7th and 28th ($P < 0.01$ & $P < 0.001$) respectively. The result of ELISA assay showed that level of the GFAP synthesis decreased in the experimental group compared to the lesion and sham groups ($P < 0.001$). **Conclusion:** The Silybum marianum plant has a protective effect on the nerve tissue in a mouse model of Alzheimer's disease by decreasing of the GFAP synthesis and improving behavioral performance.
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Introduction

Alzheimer's disease is characterized by progressive impairment of mental and behavioral abilities which are the most common form of dementia (1). The incidence of Alzheimer's disease is growing and becoming a social problem in many countries (2). Hippocampus damage in adult results in loss of memory (3).Symptoms of Alzheimer's disease begins with memory impairment and leads to severe cognitive disorders, eventually a patient with disability and total dependence that indirectly causes many economic and psychological problems for other family members and community(4). Although there is no treatment for restoration of defects or to stop the progress of this disease, the patients get Medical treatment in order to control its progress. It seems that alternative therapies, especially herbal medicines open a new way to treat Alzheimer's disease (5).

Silybum marianum is an annual or biannual plant of the Asteraceae family. Originally a native of Southern Europe through to Asia, it has been identified in Central and North India, Africa, China, Australia,South America and some parts of North America, South and West of Asia. It is also found in Iranian Gonbad eKavus, Haraz valleys, Dashte Moghan, poshte kooh, Ahvaz, Shoosh, Hamidieh, Ramhormoz, Izeh and Kazeroun as well (6). Its potent extract is used in medicine under the name silymarin (a flavonolignane complex consisting of silibinin A and B/silybin/silymarin I, isosilibinin A and B, silicristin/silymarin II and silidianin (7).For about two thousand years the Silybum marianum has been used for milk stimulation, treatment of irregularities in liver, kidney, spleen, gall stones, jaundice, and menstrual pains and today is used as an effective drug in preventing and healing diseases, liver disorders and liver toxicity caused by fungal toxins. Furthermore, it protects liver cells, against solvents and chemicals. Anticancer effect of Silybum marianum on prostate cancer cells, skin, breast, colon, ovary and brain is reported. Recent studies have shown that Silybum marianum also has anti-inflammatory effects, immunity system regulatory, antioxidant properties and decreasing cholesterol levels and reduces risk of atherosclerosis (8-16). Flavonoids and polyphenolic compounds in Silybum Marianum have antioxidant activities which

neutralizes free radicals and reactive oxygen species in cells (17). According to disappointing results of current treatments for cognitive disorders, in Alzheimer's patients, more studies are needed. This study was conducted to determine the possible effect of Silybum marianum on Hypocampus GFPA and spatial memory in a mouse model of Alzheimer's disease.

Materials and methods

A total of 30 male Wistar rats weighing 200 - 250g in the range of 4 -5 months of age were selected. Two weeks before the experiment, the animals were housed in a room maintained at 22 ° C with a 12hr light, 12hr dark cycle and were allowed free access to water and food. The all procedures on animals have been approved by animal welfare committee of Yasuj University. The rats were randomly divided into sham, experimental and lesion groups. Lesion and experimental groups were anesthetized with a mixture of ketamine (100 mg/ kg) and xylazine (5 mg/ kg). After shaving and aseptic surgical preparation; the heads of animals were fixed on the stereotaxic instrument (Narishige Japanese Co). The Bregma and Lambda points based on the rat brain atlas stereotaxic surgery were placed on a surface. The Stereotaxic coordinates of Nucleus Basalis Magnocellularis (NBM) were AP=1.3, ML ± 2.2, and DV= -7 respectively. Then Ibotenic acid 8 µg/µl bilaterally were injected into the nucleus of Nucleus Basalis Magnocellularis (18). For injection we used Hamilton syringe 5 µl. Sham group rats were injected, but surgery was not performed. In order to correctly analyze the performance of Alzheimer animals, Y maze behavioral test was performed in all groups one week after surgery. The experimental rats were fed with200 mg per kg of silymarin extract diluted with1% gum Arabic as the solvent (prepared by the Institute of Medicinal Plants, Tehran Jahad e Daneshgahi) for four weeks. Lesion group wasn't fed any drug. The sham group was fed only1% gum Arabic. The Y maze behavioral test was performed in all groups one and four weeks after surgery. Y maze was made of Plexiglas and the dimension of each arm was40 × 30 × 15cm. During the test, each rat was placed at the end of one arm and allowed to move freely in the entire maze for eight-minutes and number of animals entering each arm was recorded. An arm entry was recorded when a rat moved all 4 feet into the arm. Alternation behavior was considered as successful and Consecutive entries into all three arms. The percent of alternation was calculated as the ratio of actual to possible alternations (defined as the total number of section entries – 2) × 100. The ability to alternate requires that the mice know which sections have been visited. Therefore, alternation behavior can be regarded as a measure involving spatial working memory (19). At the end of fourth week the animals of all groups were examined by immunohistochemistrical assessment and density of GFAP protein in brain astrocytes was determined. The different steps of this process are as follows:

Animals were anesthetized using intraperitoneal injection of ketamine (100 mg/kg) and xylazine(5 mg/ kg)
Perfusion in the heart ventricle
Removing the brain
The final fixation in 4% Paraformaldehyde
Processing - dehydration, clearing, and infiltration
Embedding
Trimming
Sectioning

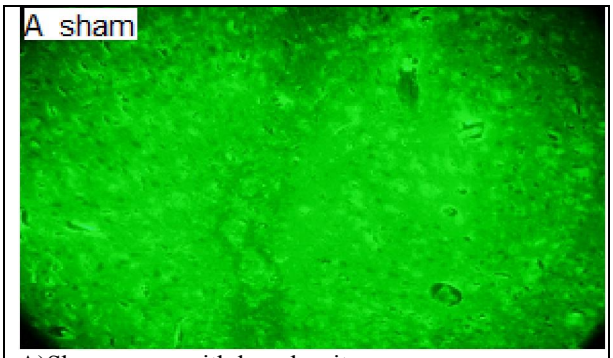
Study Of astrocytes with immunofluorescence techniques. Finally Hippocampal GFAP protein measured with ELISA method. (20). Collected data analyzed with SPSS. We used one way ANOVA and repeated Measure ANOVA for statistical data analysis.

Results

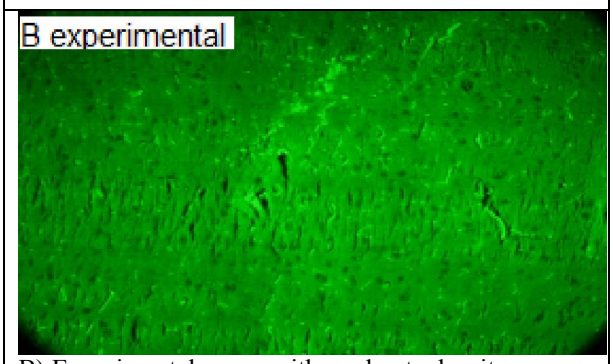
Immunohistochemistrical staining to assess the concentration levels of GFAP in brain astrocytes showed that this protein considerably has less density in sham and experimental groups compared with the lesion group (Figure 1).The results of Y maze test, in the first week showed that there are significant differences between the mean and standard deviation of the percent alternation in the sham, experimental and lesion groups ($p<0.01$). Also, differences between the mean and standard deviation of the percent of alternation in the fourth week in the sham, experimental and lesion groups were significantly meaningful ($p<0.001$) (table 1). The mean and standard deviation of the hippocampus GFAP were 3.28 ± 0.72, 4.60 ± 0.77 and 7.70 ± 0.60 ng/ml in the sham, experimental and lesion groups respectively. That shows significant difference ($p<0.001$) (Fig 2).

Conclusion

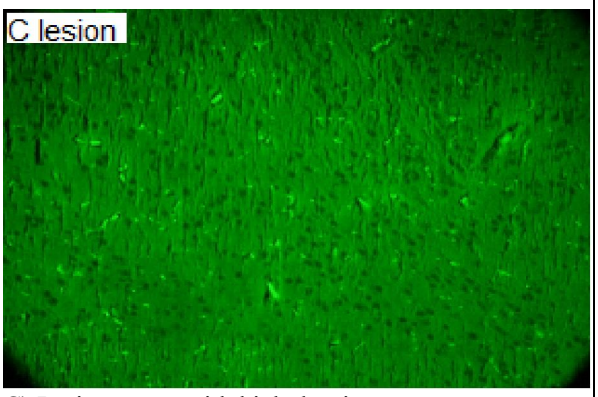
The brain requires a considerable amount of energy and oxygen, this tissue is susceptible to oxidative damage and increased oxidative stress in older people prepares them for Alzheimer's disease(1). Now a certain way to prevent Alzheimer's disease has not progressed, but epidemiological and experimental evidence shows that diets high in antioxidants may reduce the progression of Alzheimer's disease by reducing, neutralizing, and preventing the damage done to the body by free radicals (5). This study investigated the effects of Silybum marianum on the hippocampus GFAP and spatial memory in a mouse model of Alzheimer's disease.



A)Sham group with low density



B) Experimental group with moderate density



C) Lesion group with high density

Fig.1. Immunohistochemistrical staining to assess the concentration levels of GFAP in brain astrocytes

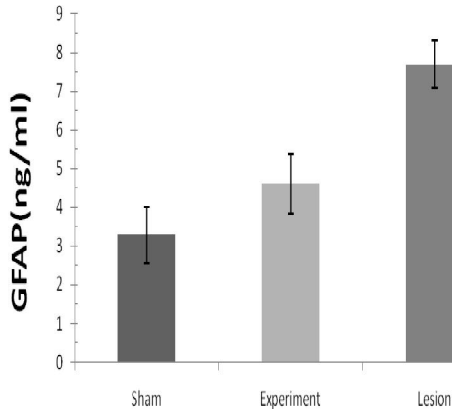


Fig. 2. the mean and standard deviation of hippocampus GFAP in study groups in fourth week.($p<0.001$)

This study showed that Silybum marianum extract significantly improved the percent alternation in Y maze test which is an indicator of spatial memory, in Alzheimer's mice groups than sham and lesions groups. Nencini et al (2007) in their study about the protective role of Silybum marianum on oxidative stress in the rats brain, showed that Silybum marianum protects the brain against oxidative damage and increasing the glutathione, ascorbic acid and superoxide dismutase activity in the brain have been noted as the mechanism of this action (21). Various studies have proved that Silybum marianum as an antioxidant with elimination of dangerous oxygen's free radicals, inhibited lipid peroxidation and stabilized the cell membranes (22, 23). Also, Silybum marianum with increasing in superoxide dismutase and glutathione oxidase activity and increasing the glutathione concentration shows its antioxidant properties (24). Although a certain way to prevent or reduce the progression of Alzheimer's disease has not been established, various studies have demonstrated the role of diet on the risk of getting.

Table1: the mean and standard deviation of percent alternation in study groups in the first and fourth weeks.

Group	percent alternation First week	percent alternation Fourth week
Sham	77.92 ± 5.22	78.5 ± 4.45
Experimental	60.34 ± 12.56	62.63 ± 13.68
Lesion	33.72 ± 5.12	44.14 ± 4.23
levels of significance	0.01	0.001

Alzheimer's. Foods with a high amount of antioxidants may reduce progression of Alzheimer's disease by preventing or neutralizing the destructive effect of free radicals. Long-term usage of vitamin E antioxidant diet, in transgenic mice that shows behavioral and neuropathological symptoms of Alzheimer's disease, reduces the level of beta amyloid. Epidemiological studies have shown that diets with vitamin E enrichment reduce the incidence of Alzheimer's disease in human (25). Other foods such as green tea, due to polyphenols, reduce beta-amyloid plaques in a mouse model of Alzheimer's disease (26, 27). In various studies the role of GFAP on the cognitive impairment has been proved. The level of this protein is increased in cerebrospinal fluid from Alzheimer's disease patients (28, 29). Also GFAP levels in astrocytes, under the influence of oxidizing agents such as nitric oxide increases (30). The results of this study showed that the extract of Silybum marianum significantly reduced the level of GFAP in rats of experimental group, which may be due to antioxidants property of Silybum marianumthat exerts its effects with elimination of oxygen free radicals and preventing the formation of nitric oxide. Recent results show that Silybum marianum having antioxidant properties may prevent the progression of Alzheimer's disease and improve cognitive and behavioral disorders, and also reduce the GFAP astrocytes in the brains of Mice Modeling Alzheimer's disease. In order to provide an appropriate treatment or prevention of Alzheimer's disease risk, it is suggested that more comprehensive studies should be done to identify effective compounds in the extract of Silybum marianum and its mechanism of action.

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