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*Research Article*

# Beneficial Effect of *Brassica nigra* Fixed Oil on the Changes in Memory Caused by B-Amyloid in an Animal Model

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**Abstract**

***Background:*** Dementia is a disease in which memory, thinking, and cognitive skills are impaired, with Alzheimer's being the most common type of dementia. *Brassica nigra* is useful for eliminating memory loss in traditional Persian medicine. This study aims to examine the effect of *B. nigra* fixed oil (BNO) on the changes in memory caused by β-amyloid.

***Methods:*** This research was conducted on 42 Wistar rats divided into 7 groups (n=6) including 1) control (received vehicle), 2) the group receiving BNO (925 and 462.5 mg/kg), 4) sham group 5) Alzheimer group (receiving 50 ng/μl/side β-amyloid in CA1 area of hippocampus) and 2 groups receiving β-amyloid along with two different doses of BNO. The daily gavage of BNO was done 2 to 21 days post amyloid injection. The spatial memory was evaluated in Morris water maze from day 21 to 26.

***Results:*** The results of this study revealed that the gavage of BNO (925 mg/kg) to rats receiving β-amyloid, as compared to those receiving β-amyloid alone, significantly decreased the traveled distance and the required time for finding hidden platform on the training days and increased the time of presence in the target quadrant on the test days. The analysis of BNO with GC-MS revealed that Erucic acid (24.79%) and 11-Eicosenoic acid (17.23%) had the highest content in the BNO.

***Conclusion:*** Regarding the presence of unsaturated fatty acids, it is likely that the consumption of BNO can play an important role in the prevention of memory degradation which warrants further clinical studies.

## Introduction

Dementia is a chronic or progressive brain disease which leads to loss of memory, thinking, and cognition skills whereby the ability to perform daily tasks and control sensory reactions is significantly diminished. Dementia can be caused by various factors, with Alzheimer's disease (AD) being known as the most common reason.[1] Although there are a wide range of reasons for the onset and progression of AD,[2] the β-amyloid (Aβ) 42 cascade hypothesis has remained the original theory. It suggests that changes in the production or accumulation of Aβ play a major role in the pathogenesis of AD.[3] Aβ is a type of peptide with 40 or 42 amino acids located out of neurons of some cerebral areas. It is formed by the cleavage of Amyloid Precursor Protein (APP) *via* three proteolytic enzymes. APP is expressed in the neural system cells and functions in the connection of cells, and connection to extracellular matrix and cell skeleton. Aβ 40 and 42 are made based on the effect of γ-secretase at C-terminal fragment of APP. The amount of Aβ40 and 42 in cells is generally low; however, the Aβ42/Aβ40 ratios seem to be crucial. Elevation of Aβ42/Aβ40 ratio causes AD and higher neuro-toxicity. Inversely, lowering Aβ42/Aβ40 ratios can reduce Aβ deposition.[2]

Nature and especially plants have always been one of the most available and richest sources of drug discovery and development tools leading to new medicinal compounds.[4,5] Black mustard is a one-year plant of Brassicacea family. Phytochemical analysis has shown that this plant contains alkaloids, flavonoids, glycosides, carbohydrates, sinapins, myrosins, sinigrin, inosite, albumins, and resins. Plant seeds include oil, protein, as well as phenylpropane derivatives such as sinapin and glucosinolate derivatives generally sinigrin. The fixed oil of seeds includes oleic,

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stearic, and brassica fatty acids.[6] Various pharmacologic effects have been reported for black mustard including antioxidant,[7,8] anti-inflammatory,[9,10] anti-convulsing,[11,12] and anti-diabetic[13] properties.

The benefits of black mustard have been frequently recommended in different Iranian traditional medicine (ITM) resources. Its oil has been particularly used for neurological disorders and dementia.[14] Different studies have also reported that the alcoholic extract of black mustard could offer moderate acetylcholinesterase inhibitory activity which may be important in memory enhancement.[15,16] Also, it has been proved that unsaturated fatty acids induce positive effects on the brain performance.[17] As black mustard contains huge amounts of fatty acids, in this study, the memory enhancement activity of *Brassica nigra* fixed oil (BNO) was evaluated in an animal model.

## Materials and Methods

### *Providing sample, plant, and oil*

*Brassica nigra* seeds were purchased from Tehran herbal Market which were confirmed at Herbarium of faculty of Pharmacy, Tehran University of Medical Sciences and was registered with PMP-788 code. Fixed oil was extracted by a cold press machine with the extraction yield of oil being 30%.

### *Analyzing BNO using GC-MS*

#### *Methylesterification of fatty acids*

Specifically, 1g of the obtained oil was reflexed in a balloon with 20 ml methanol potash (1% weight-mass) for 25 minutes and then, 12 ml Brome Fluorine was added to balloon and was boiled for 10 minutes. Once heating was stopped, sodium chloride solution was added to the aquoeus phase. The upper hexane phase which included methyl-esterized fatty acids was removed and 1µl was injected to gas chromatography -mass spectrophotometry immediately.[18]

#### *GC-MS analysis*

Gas chromatography apparatus used was Agilent 6890 which had a column with 30 m length, 0.25 mm internal diameter, and 0.25 µm layer thickness of BPX5 type. The mass spectrograph used was Agilent 5973 with 70 ev ionization voltages, EI ionization method, and ionization resource temperature 220 °C. The scan range for coppers was determined at 40-700. Further, an aliquot of n-alkane series (C10-C28) was injected for calculating the retention index and quality control. The spectra were detected using prevention indices and comparing them with indices in reference books and articles, using mass spectra of standard compounds, and based on information in computer libraries.

### *Animals*

Forty-two male Wistar rats weighing 180±20 g were purchased from the Central Animal House of the Pasteur Institute of Tehran. The animals were housed in a controlled environment at a constant temperature (24 ± 1 °C) under a 12-h light-dark cycle. The animals were fed standard food and water and were allowed to acclimatize to the conditions of the laboratory for one week before initiation of the experiments. All experimental protocols were approved by the Animal Research Ethics Committee of the Faculty of Science at Tehran University (IR.TUMS.PSRC.REC.1396.4357).

### *Grouping animals*

The current study is an experimental intervention study on male Wistar rats. Animals were divided into 7 groups (6 rats) through randomized sampling method. The studied groups were 1) control (healthy rats without surgery and or oil carrier administration), 2 and 3) receiving BNO in two different doses (462.5 and 925 mg/kg), 4) a sham on which surgery was done, 5) receiving β-amyloid (in form of infusion at CA1), and 6 and 7) receiving β-amyloid along with two different doses of BNO.

Amyloid (50 ng/µl/side) was injected bidirectionally in the hippocampus of rats on day 1. Daily gavage of BNO was done in two doses on days 2-21 after amyloid infusion. The spatial memory in Morris water maze (MWM) was evaluated from 21-26 days post β-amyloid infusion.

### *Preparing β-amyloid*

β-amyloid 1-42, rat (Gen Script, USA) was dissolved in phosphate-buffered saline (PBS) (pH 7.3) and incubated at 37 °C for 72 hours.

### *β-amyloid infusion in Hippocampus of a group of rats*

The animals were anesthetized through the intraperitoneal infusion of ketamine (90 mg/kg) and xylazine (5 mg/kg) and fixed in a stereotaxic frame. Once the head was fixed, skull skin was cut from middle of eyes to the end of occipital bone by a scalp, and bone was cleaned with cotton to detect bregma area. Then, coordinates of anteroposterior (AP), ML, and dorsoventral (DV) of bregma were recorded, and coordinates of the infusion area to bregma were determined. Since the infusion area in this study was CA1 of hippocampus, its coordinates were obtained according to Paxinus-Watson atlas.[19] Once made, the infusion area was drilled and then, the calculated dose of β-amyloid (50 ng/µl/side) was injected to CA1 by Hamilton syringe with the head stitched.[20]

### *Prescribing edible BNO*

In order to prepare appropriate doses of BNO (462.5 and 925 mg/kg body weight in 1 ml), the calculated amount of oil was dissolved in edible liquid paraffin and administered daily for the oil-receiving groups (2-3-6-7) through gavage. According to previous research,[20] administering oil for rats continued for 21 days. After this time, the MWM test was performed on groups to examine their memory.

### *Examining learning and memory in rats by Morris water maze method*

In order to assess learning and spatial memory, MWM

was used. MWM is used to examine learning, recall, and orientation in space whose location is determined only through its relation to spatial signs out of the maze.[21,22]

### *Stages of behavioral studies*

These stages include adaptation, training (4 days), and assessment (day 5) and are as follow:

#### *Visual test*

Before conducting the memory test (probe stage), eyesight test was done on all samples for recognizing rats with weakness in eyesight and excluding them. In this regard, MWM was transferred to the western south. Just as a training stage, 4 attempts (each 90 seconds) were done at different parts of the maze on one day. Finally, the results were analyzed by the software. Samples with eyesight problems were excluded.

#### *Adaptation to environment*

In order to become familiar with the environment, the rats were placed in the experiment environment before conducting the experiment. In order to familiarize the rats with MWM, the first rat was placed on the rescue area and then was allowed to swim for 2 minutes. Behavioral tests were conducted between 10 and 16 hours.

#### *Training*

As the learning was 4 days, rats were placed 4 days and 4 times a day in the maze. In every experiment, the rats were freed from one side of maze randomly (determined by computer), such that their face was on the wall of the pool. Each time rats should find the rescue area in 90 seconds. If the rat could not find the rescue area in 90 seconds, the researcher took him out of the water and after 30 seconds the rat was placed in water from another part. Once done, the rat was taken out of the pool, cleaned, placed in the cage, and transferred to the store. Movement speed and spent time were recorded by the computer. Note that spent time and distance traveled for finding the rescue area and swimming speed are used as spatial learning indices in Morris water maze model.

#### *Assessment*

After 4 days of learning, the spatial memory of rats was evaluated at day five. At this stage, a 90-second test was recorded for each rat such that the rat was placed in water just one time and from one direction where the time spent by the rat in the target quadrant (on which the rescue area was located throughout the training days) was recorded as memory index and analyzed further.

### *Biological examination*

#### *Assessing inhibition of acetyl and butyrylcholinesterase by Elman method*

Elman test was used to determine inhibition power of BNO against acetylcholinesterase.[23] Acetylcholinesterase, butyrylcholinesterase, acetylthiocholiniodis, and 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) were purchased from Sigma-Aldrich Co. and potassium dihydrogen phosphate, potassium diydrogen phosphate, potassium hydroxide, and sodium hydrogen carbonate were purchased from Fulica Co. Rivastigmine was used as a positive control drug in this examination. In order to prepare the DTNB solution, 7.8 mg of this material was dissolved in 13 ml buffer. Acetylthiocholine iodide was prepared by dissolving 13 mg of this material in 3 ml water. Note that acetylthiocholine iodide solution should be prepared fresh as it degrades at room temperature. A certain amount of BNO was dissolved and diluted in methanol and DMSO solvents at 1: 1 ratio. Then, 25 µl of the sample was poured to a 96-well plate. Although 50 µl buffer was poured in each cell, samples were assessed at four final concentrations (63.5, 125, 250, 500 µg/ml) with three repetitions. Also, 25 µl enzyme was added to certain cells in the plate. This system remained 15 minutes at ambient temperature. Initially, 125 µl of DTNB solution and then, 25 µl of acetylthiocholine iodide solution was added to all cells and after 15 minutes, the amount of UV absorption at 405 nm wavelength was read by ELISA plate reader with the percentage of acetylcholinesterase prevention determined. This method was also used for determining the inhibition of butyrylcholinesterase by BNO.

### *Statistical analysis*

GraphPad Prism V6.07 software was used for statistical calculations of behavioral data. the statistical test used was one-way variance (ANOVA). In cases where a significant difference was observed among the compared data, Newman Keules Multiple Comparison Test was used. Further, the two-way ANOVA test was applied to examine the interaction between the training day as well as material used independent variables and dependent variables.

## Results

### *Results of analyzing BNO by GC/Mass*

The results of analyzing BNO are shown in Table 1. Totally, 18 compounds were detected in *B. nigra* oil constituting 93.75% of total compounds of oil. Among these compounds, Erucic acid methyl ester with 24.79% had the highest amount followed by 11-Eicosenoic acid methyl ester (17.23%), 8,11-Octadecadienoic acid (14.92%), 15-Tetracosenoic acid methyl ester (7.28%), and oleic acid methyl ester (6.88%).

### *Results of behavioral tests*

#### *Examining the effect of β-amyloid on time, distance and speed in MWM*

Initially, memory degradation by β-amyloid (50 ng/µl/side) was assessed based on traveled distance and escape latency and probe test in comparison to the control (healthy rats without surgery) and sham groups. Figure 1 displays the effect of β-amyloid on escape latency, distance traveled for finding rescue area, swimming speed on four days of training, and time spent in the target quadrant in

**Table 1.** GC-Mass analysis of fixed oil of *Brassica nigra* seed.

| NO | RT[a] | %[b] | Components | KI[c] | Lipid name |
|---|---|---|---|---|---|
| 1 | 16.12 | 0.22 | Tetradecanoic acid methyl ester | 1731 | C14:0 |
| 2 | 17.20 | 0.05 | Pentadecanoic acid methyl ester | 1832 | C15:0 |
| 3 | 18.05 | 0.67 | (Z)-7-Hexadecenoic acid, methyl ester | 1914 | C16:1 (n-7) |
| 4 | 18.26 | 5.06 | Palmitic acid, methyl ester | 1935 | C16:0 |
| 5 | 18.89 | 0.02 | Palmitic acid, ethyl ester | 1997 | C16:0 |
| 6 | 19.22 | 0.10 | Margaric acid mcthyl cster | 2016 | C17:0 |
| 7 | 19.95 | 14.92 | 8,11-Octadecadicnoic acid, methyl ester | 2055 | C18:2 (n-7,9) |
| 8 | 20.05 | 6.88 | Oleic acid methyl ester | 2061 | C18:1 (n-9) |
| 9 | 20.10 | 4.56 | Linolenic acid, methyl ester | 2064 | C18:3 (n-9,11,13) |
| 10 | 20.21 | 3.22 | Stearic acid, methyl ester | 2070 | C18:0 |
| 11 | 21.77 | 17.23 | 11-Eicosenoic acid, methyl ester | 2258 | C20:1 (n-11) |
| 12 | 23.42 | 24.79 | Erucic acid methyl ester | 2458 | C22:1 (n-14) |
| 13 | 23.64 | 4.74 | Behenic acid, methyl ester | 2473 | C22:0 |
| 14 | 24.35 | 0.30 | Tricosanoic acid, methyl ester | 2616 | C23:0 |
| 15 | 25.05 | 7.28 | 15-Tetracoscnoic acid, methyl ester | 2655 | C24:1 (n-15) |
| 16 | 25.23 | 3.37 | Lignoceric acid niethyl ester | 2665 | C24:0 |
| 17 | 27.44 | 0.19 | Cerotic acid methyl ester | 2866 | C26:0 |
| 18 | 29.55 | 0.15 | y-Tocopherol | 3034 | |
| | | 76.33 | Unsaturated fatty acids | | |
| | | 17.27 | Saturated fatty acids | | |
| | | 93.75 | Total Identified | | |

[a]RT: Retention time; [b]%: percent of identified dcompound; [c]KI: Kovats indices

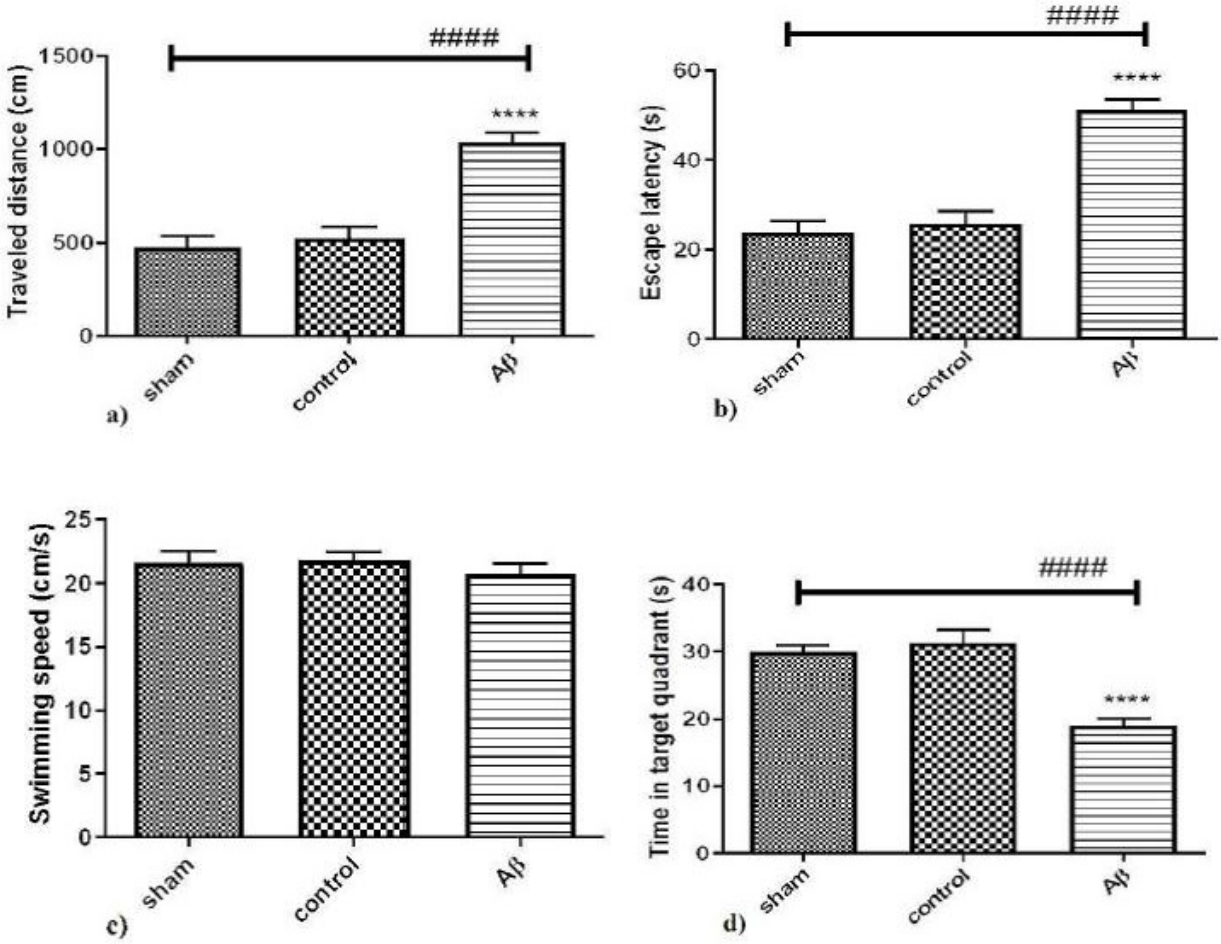


**Figure 1**. Effect of β-amyloid(Aβ) on a) traveled distance for finding rescue area; b)escape latency; c) swimming speed in four days of training and d) time spent at target quadrant at day five by rats in Morris water maze in comparison to the control group and sham group. Results are reported as Mean ± SEM (n=6). *: significant difference with control group (*p<0.05, **p<0.01, ***p<0.001), #: significant difference with Sham group (#p<0.05, ##p<0.01, ###p<0.001).

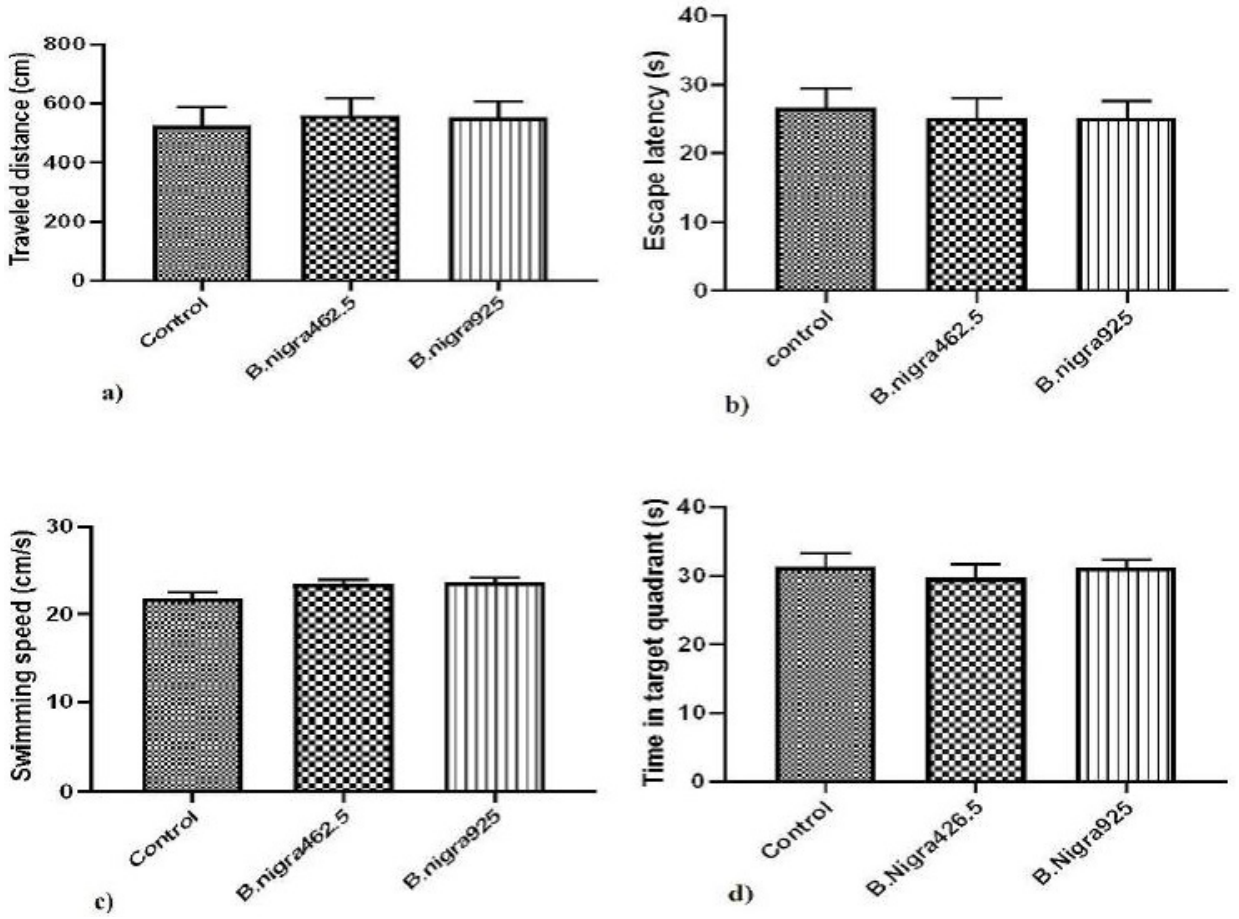


**Figure 2**. Effect of different concentrations of *B. nigra* fixed oil on a) traveled distance for finding rescue area; b) escape latency; c) swimming speed in four days of training and d) time spent at target quadrant at day five by a rat in Morris water maze in comparison to the control group. Results are reported as Mean ± SEM (n=6).

the assessment stage on day five.

According to the results, β-amyloid significantly increased the escape latency, distance traveled for finding rescue area ,and decreased spent time in the target quadrant time in comparison to the control group and sham group ($p<0.05$) suggesting the detrimental effects of this concentration of β-amyloid on the spatial memory. On the other hand, β-amyloid did not affect movement speed and the movement system of rats ($p>0.05$). Therefore, β-amyloid with 50 mg/µl/side concentration was used to impair the spatial memory and make the Alzheimer model.

### *Examining the effect of different doses of BNO on time, distance, and speed in MWM*

In order to examine BNO influence on improving memory, the effect of 462.5 and 925 mg/kg concentrations of the fixed oil was evaluated in healthy rats in comparison to the control group. Figure 2 reveals the effect of two concentrations of BNO on the escape latency, distance traveled for finding rescue area, swimming speed on four days of training, and time spent in the target quadrant in the assessment stage on day five. According to the results, two concentrations of BNO did not significantly affect the escape latency, traveled distance, spent time in the target quadrant, and swimming speed in comparison to control group ($p>0.05$) demonstrating that administering this oil has no effect on spatial memory promotion in comparison to the control group.

### *Examining the intervention effect of β-amyloid with different doses of BNO on time, distance and speed in MWM*

Figure 3 shows the intervention effect of β-amyloid with two doses of BNO on the escape latency, traveled distance, spent time in the target quadrant, and swimming speed in MWZ. The results showed that 925 mg/kg dose of BNO reduced the escape latency and traveled distance for finding the rescue area; increased the time in the target quadrant; and improved the detrimental effect of β-amyloid. According to the results, there was no significant difference between the above groups in the rat swimming speed. Thus, β-amyloid infusion and oil gavage did affect the speed and movement system of rats ($p>0.05$).

### *Examining the effect of four-day training on escape latency and traveled distance in MWM*

Examining the effect of four-day training on escape latency and traveled distance revealed a significant reduction between day 1 and day 4 in control and Aβ+ *B. nigra* 925 (mg/kg) groups (Figure 4).

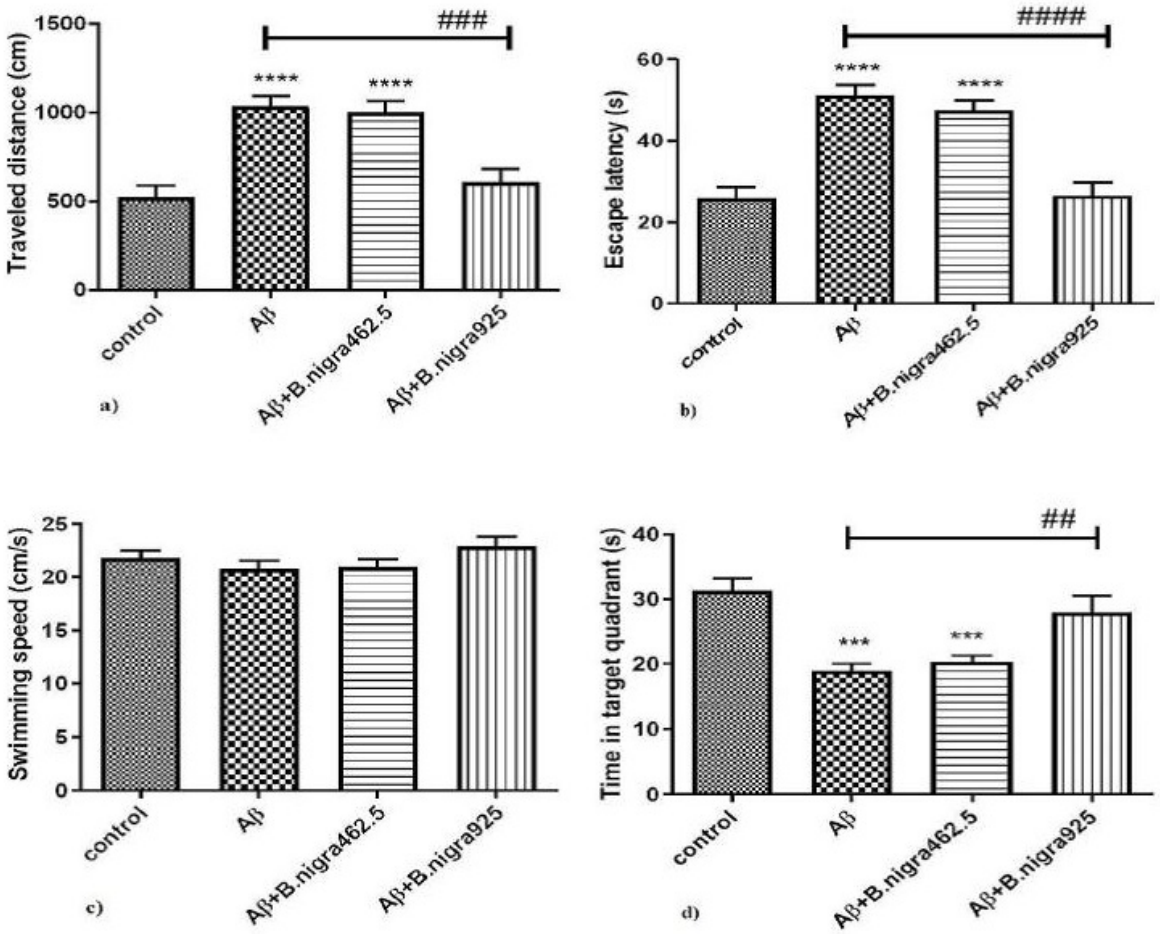


**Figure 3.** Comparison of rat a) traveled distance; b) escape latency; c) swimming speed and d) time spent at target quadrant in control, *B. nigra* fixed oil, and β-amyloid (Aβ) groups. Results are reported as Mean ± SEM (n=6). *: significant difference with control group (*p<0.05, **p<0.01, ***p<0.001); #: significant difference with Alzheimer (Aβ) group (#p<0.05, ##p<0.01, ###p<0.001).

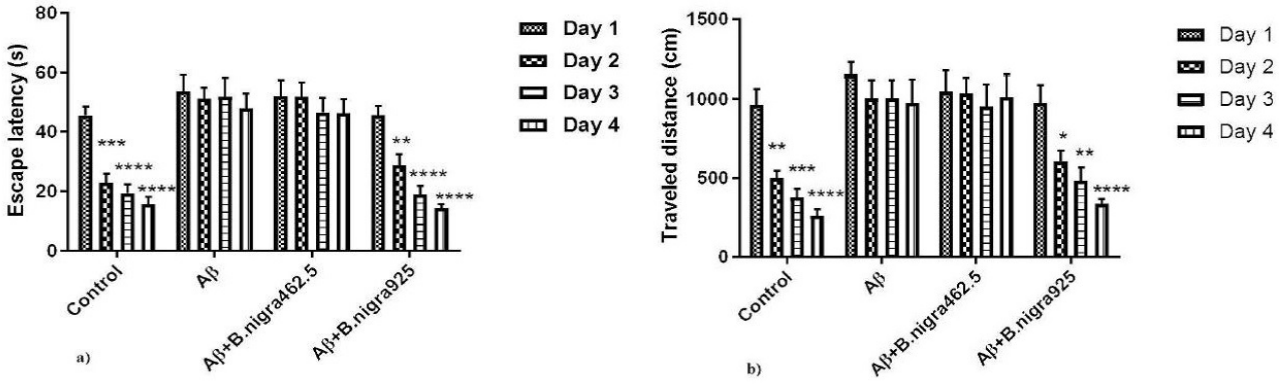


**Figure 4.** Comparison of the effect of four days training on a) escape latency and b) traveled distance. Results are reported as Mean ± SEM (n=6). * shows significant difference with day 1 (*p<0.05, **p<0.01, ***p<0.001).

### *Assessing the cholinesterase inhibitory effect of BNO*

The results of inhibition effect of cholinesterase of BNO showed that the inhibition percentage of AChE in 500 µg was 4.8% while that of BChE in 500 µg was 29.9%. Consequently no effect was seen at 500 µg of BNO, which was the highest dose examined for herbal samples.

## Discussion

β-amyloid accumulation in cerebral tissue, decreased acetylcholine in the brain, dysfunction of mitochondria, oxidative stress in neural cells and degradation of synapses, and neural cells apoptosis are among important factors contributing to memory disorders and Alzheimer's.[24] In recent years, various studies have been conducted on plants affecting the central nervous system.[6,25] It has been found that *B. nigra* has wide-range effects on the central nervous system. In this study, BNO was examined in order to prevent memory degradation caused by β-amyloid.

The animals' learning ability in finding a rescue area was expressed with escape latency, traveled distance, and spent time in the target quadrant. In order to examine dose-related effects, 462.5 and 925 mg/kg doses of *BNO* were used as daily gavage. The results of MWM behavioral test showed that 925 mg/kg dose prevents memory changes caused by β-amyloid, while 462.5 mg/kg dose does not have a significant protective effect.

Mediterranean diet which contains high Poly Unsaturated Fatty Acids (PUFAs) can have positive effects on brain functioning.[26] In a cross-sectional study on elderly people without signs of dementia, it was shown that receiving a diet with high ω-3 PUFA decreases Aβ-42 level in plasma. Further, it was determined that linoleic acid plays a role in the neural building of hippocampus and neural transfer.[27] In addition, oleic acid which is an 18-carbon fatty acid with an unsaturated bond (MUFA, ω-9) causes Long Term Potentiation (LTP) of healthy hippocampus through activating Protein Kinase C (PKC) and improves cognitive effects.[17] Since the chemical structure of Erucic acid is very similar to that of oleic acid, it may activate PKC. PKC has been recognized as an LTP mediator which continuously improves synapses and is considered as the learning and memory mechanism in the hippocampus. Protein Kinase C Zeta (PKCζ) is a sub-unit of PKC and has an important role in protecting LTP, and learning. PKCζ can be activated through phosphorylation by some kinases such as phosphoinositidecholine-3-kinase (PI3K). PI3K functions in synapse formation, cognitive memory stability, and long-term potentiation of the hippocampus.[28] Kim et al. studied memory promotion effects of Erucic acid (which existed in *Paphanus sativus* L.) on cognitive disorder caused by scopolamine through assessing behavioral tests (Morris water maze and Y-maze). Erucic acid enhanced the memory functioning in normal rats and improved memory disorders caused by scopolamine. Further, the results of this study showed that prescribing Erucic acid significantly increases PI3K phosphorylation level. Various studies reported that PKCζ takes part in Mitogen-Activated Protein Kinase (MAPK) flux which is related to transferring a signal to the memory. In particular, Extracellular Signal-Regulated Kinase (ERK) is a signaling molecule of PKCζ route where the signaling activation of PKCζ-ERK has an important role in learning and memory. Also, Erucic acid improved the phosphorylation level of ERK and CREB (cAMP response element-binding protein) in the hippocampus in comparison to the control group. Akt (additional protein kinase B) is another signaling molecule in PI3K route which can phosphorylate different substrata and as a result, can regulate various cellular processes in neural development such as cell infiltration and synapse formation. PI3K-Akt route has an important role in synaptic formation. According to Kim et al. study, Erucic acid enhanced the Akt phosphorylation in the hippocampus. Therefore, it is possible that Erucic acid activates PI3K-PKCζ-ERK-CREB signaling route or PI3K-Akt-CREB signaling route. Activation of such signaling fluxes may be accompanied by Erucic acid effects on cognitive functioning. It is not clear yet how these routes are affected by Erucic acid. However, Erucic acid can be a new remedial factor for diseases associated with memory disorder such as Alzheimer's.[29] With regard to analysis of fatty acids of *B. nigra* seed oil and presence of fatty acids such as oleic acid, linoleic acid and especially, Erucic acid which had the highest concentration in oil, it is likely that prescribing *B. nigra* seed oil activates PI3K-PKCζ-ERK-CREB signaling route or PI3K-Akt-CREB signaling route which requires further study.

With regard to the results of this study, *BNO* with dose up to 500 µg did not show significant inhibition effects on choline esterase enzymes. Previous studies examined the preventive effect of *B. nigra* methanol extract for choline esterase. Aparana et al. (2018) used *B. nigra* methanol extract at 1-3 ml doses. They examined the effect of this extract on choline esterase extracted zebrafish brain. They found that methanol extract at 1 ml dose had the greatest prevention (86.6%) for acetylcholine esterase activity. Further, they proved that *Brassica nigra* methanol extract at 1 ml dose amplifies α-amylase activity in the experiment environment. Alpha-amylase is a material that causes glucose production. Research on animal models shows that glucose has a positive effect on memory through facilitating acetylcholine production with its production diminishing in Alzheimer's.[30] Shereen et al. examined the effect of *B. nigra* methanol extract on Alzheimer's. They observed that the extract with 10 µl dose can prevent acetylcholine esterase. With regard to the results of these studies, preventing acetylcholine esterase by methanol extract can be attributed to other compounds in *B. nigra* seed.[25]

## Conclusion

Generally, this study revealed the effect of BNO on preventing memory degradation caused by β-amyloid. Considering the presence of unsaturated fatty acids such as oleic acid, linoleic acid, and especially erucic acid, the consumption of *B. nigra* fixed oil may play an important role in preventing memory loss caused by β-amyloid. Nevertheless, conducting further research seems necessary.

## Ethical Issues

All experimental protocols were approved by the Animal Research Ethics Committee of the Faculty of Science at Tehran University (IR.TUMS.PSRC.REC.1396.4357).

## Conflict of Interests

The authors claim that there is no conflict of interest.

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