

# Afghan Chehelghoza (*Pinus gerardiana* L.) Pine Nut Diet Enhances the Learning and Memory in Male Rats

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Kawsar Alami <sup>1</sup>  
Sayed Yousof Mousavi <sup>2</sup>

<sup>1</sup>Research and Technology Center, Khatam Al-Nabieen University, Kabul, Afghanistan; <sup>2</sup>Department of Physiology, Medical Faculty, Khatam Al-Nabieen University, Kabul, Afghanistan

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**Purpose:** The aim of this study is the evaluation of the effect of Afghan Chehelghoza as a constituent of diet on behavioral parameters, especially learning and memory of male rats.

**Methods:** Rats were divided into six groups: control group, diazepam group as the positive control, and 2, 6, 12 and 25% chehelghoza-treated groups, which received chehelghoza combined with their food for 28 days. After treatment, the rat's behavior was evaluated by Y-maze, elevated plus maze, Morris water maze and radial arm maze. In addition, their brain was extracted and weighed. The body weight of rats was also measured during the study.

**Results:** The results showed that the time spent in target quadrant and brain weight were increased in 12 and 25% chehelghoza-treated groups significantly ( $P < 0.05$ ). Also, the difference between the percentage of spontaneous alternation and the number of crossing, percentage of time spent in and the number of entries into open arms of only 25% chehelghoza-treated and the control groups was significant. Besides, on day 28, the body weight of all chehelghoza-treated groups was diminished as compared with the control group.

**Conclusion:** We can conclude that chehelghoza combination with food in high doses can improve the spatial working memory and reference memory, increase the brain weight, as well as reduction in anxiety behavior.

**Keywords:** anxiety, learning, memory, Chehelghoza, rat

## Introduction

Human and thus brain physiology like any other organs need nutrients. The brain uses these nutrients for development and maintenance of its structure, thereby functions in an appropriate and coordinate manner.<sup>1</sup> Research in last decades provides evidence of the effect of nutritional factors on specific molecular systems and mechanisms involved in mental function. Different constituents of diet and nutrients are effective on human cognition, so signals mediated by nutrients affect on the metabolism of energy, membrane fluidity, regulation of neurotransmitter pathways, signal transduction, synaptic transmission, synaptic plasticity and other brain processes.<sup>2</sup> Synaptic plasticity is considered as the cellular basis for learning and memory, which results from the interaction between organism and environment.<sup>3,4</sup> The learning and memory capability is also highly related to increase in brain weight or size, due to increase in cell size and the number of dendrites.<sup>5</sup> Search about factors that can increase the number of synapses and synaptic plasticity may develop new strategies for improvement of learning and memory functions.<sup>6</sup>

Correspondence: Sayed Yousof Mousavi  
Department of Physiology, Medical Faculty, Khatam Al-Nabieen University, 1th Street of Karte 4, Kabul, Afghanistan  
Tel +93 796850084  
Email Mousavi.knurtc@knu.edu.af

Nature gifts various plants, fruits and nuts to human.<sup>7</sup> Chehelghoza pine nut belongs to Pinaceae family. It is one of 25 pine nut types, which produces edible nuts and uses all over the world.<sup>8</sup> Chehelghoza was found in 2000–3350 meter heights,<sup>8–10</sup> in India, Pakistan, China and Afghanistan.<sup>8–12</sup> In Afghanistan, chehelghoza trees are found especially in Paktia, Paktika, Laghman and Khost Provinces.<sup>13</sup> People consume this kind of pine nut in both raw and roasted forms. It is used in combination with different loaves of bread, candies, desserts, cakes, and also in meat or vegetable foods.<sup>8,14</sup> Chehelghoza is known as an important and tasty dry fruit.<sup>9</sup>

Generally, pine nuts are considered as a reservoir of different nutrients including proteins, carbohydrates, fibers, minerals, and especially oil substances.<sup>8</sup> Chehelghoza pine nuts are a good reservoir of energy (628 kcal)<sup>12</sup> and contain unsaturated fatty acids, carbohydrates,<sup>8</sup> proteins,<sup>11</sup> minerals, vitamins,<sup>12</sup> antioxidants,<sup>15</sup> and phytosterols.<sup>14</sup> About 49.9% of chehelghoza is fat, mostly unsaturated fatty acids. Fatty acids that present in chehelghoza oil are stearic acid (0.3%), linoleic acid (51.3%), linolenic acid (1.5%), oleic acid (39.7), arachidic acid (2.1%) and palmitic acid (7.2%).<sup>8,9</sup> Chehelghoza also contains a large amount of plant protein. It is considered as a source of essential amino acids, especially arginine.<sup>11</sup>

Chehelghoza pine nuts have potent biological activity.<sup>8</sup> They have carminative, stimulant, expectorant,<sup>9,10</sup> lowering blood cholesterol,<sup>12</sup> and anti-diabetic properties.<sup>16</sup>

Despite these beneficial constituents and properties of chehelghoza and significant effect of diet on brain processes, there is not any evidence of study on the effect of dietary chehelghoza on learning and memory. Hence, this is the first study, which initiated to investigate the effect of chehelghoza as a constituent of diet on behavioral parameters, especially learning and memory of rats.

## Materials and Methods

### Materials

The main materials and equipment used in this study were chehelghoza (Afghanistan), diazepam (Caspian Tamin, Iran), Y-maze, elevated plus maze (EPM), Morris water maze (MWM), and radial arm maze (RAM), which are constructed in research and technology center of Khatam Al-Nabieen university (KNURTC).

### Chehelghoza and Diet

Raw seeds of chehelghoza pine nut from Alisheng, Laghman province of Afghanistan were purchased from the local market. The nuts were identified in Kabul university, Faculty of science, by Prof. Nasim Sediqi, Department of Pharmacognosy, Faculty of pharmacy, Kabul university, with herbarium code of KUFS017783. The nuts are pilled manually, ground and mixed with standard rodent food (Javaneh Khorasan, Iran).

### Animals

Thirty-six adult Sprague Dawley male rats, with body weight between 180 and 220 g, were randomly selected from KNURTC. Rats had at least 7 days' acclimatization period before starting of the actual experiments. Rats were housed in Plexy-glass cages, controlled-temperature (23±2°C) room and a 12-hour light/dark cycle (lights on at 7 a.m.). The ethic research board of Khatam Al-Nabieen University approved the experimental protocol (Approval No. AF.KNU.REB.1397.16) on 2018 and the study was conducted following the ethical guidelines set by the eighth edition of the National Institute of Health (NIH) guide for the care and use of laboratory animals. Rats were free in access to food and water, and were carefully handled to reduce the housing and experiment period unwanted stress to minimum. The body weight and food consumption of all rats were monitored throughout of study.

Rats divided into six groups (n=6) as following:

Group 1 (Control group): Rats of this group received standard rodent food without chehelghoza for 28 days.

Group 2 (2% group): Rats of this group received 2% chehelghoza combined with standard rodent food for 28 days.

Group 3 (6% group): Rats of this group received 6% chehelghoza combined with standard rodent food for 28 days.

Group 4 (12% group): Rats of this group received 12% chehelghoza combined with standard rodent food for 28 days.

Group 5 (25% group): Rats of this group received 25% chehelghoza combined with standard rodent food for 28 days.

Group 6 (Diazepam group): Rats of this group received standard rodent food without chehelghoza for 28 days. They considered as a positive control group for the EPM test and received 1.5 mg/kg diazepam as a standard anti-anxiety drug, 1 hour before the EPM test.<sup>17</sup>

The oral pretreatment design of the study was the same as described earlier.<sup>18</sup>

## Behavioral Tests

### Y-Maze

A black plexy-glass Y-maze (50×30×11) used in this study to evaluate the spatial working memory. Rats were placed in the Y-maze to explore for 8 min without any reward (eg food and water). The number of arm entries was counted and considered as an index of locomotor activity. Successive entries into three different arms are considered as a spontaneous alternation (%SA).<sup>17</sup> The maze was thoroughly cleaned with damp cotton after each rat's experiment to remove any smell cues.

### EPM

The behavior of rats was assessed in EPM for evaluation of exploring, anxiety and locomotor activity.<sup>17</sup> The basis of the EPM test is the principle that maze exposure causes an approaching conflict which is significantly greater than that caused by exposure to the closed arms.<sup>19,20</sup> A wooden EPM consists of a central area (10×10 cm wide), two closed arms (50×10×40 cm wide), and two open arms (50×10 cm wide) placed 50 cm above the ground. The rats were placed in the central area of EPM, facing one closed arm, to exploring the maze for 5 minutes. The number of entries into and time spent in open and closed arms was evaluated and the percentage of time spent in open arms was calculated. The number of entries into open and closed arms (number of the crossing) considered as a behavior of exploration.<sup>17,19,20</sup> The maze was cleaned with damp cotton after each rat's experiment to remove any smell cues.

### MWM

MWM was performed as a gold standard test for assessing spatial learning and memory.<sup>21</sup> A circular black stainless steel tank (diameter of 180 cm; height of 60 cm), filled with water (22±1 °C). The distance between circular platform and the surface of water was 1 cm. The colour of water became opaque by adding non-fat dry milk to make the platform invisible. Some spatial cues were placed outside of the tank in the same position throughout of experiment to facilitate spatial memory. The position of experimenter also remained consistent throughout of experiment. The tank was divided into four quadrants as northeast, northwest, southeast, southwest and platform placed in the northeast quadrant. Rats trained for 4 days with two blocks. Each block

consists of four trials with a rest of 5 minutes between two blocks. During training, each rat allowed swimming until 90 s to locating the platform and spent 30 s period between two trials. A video camera fixed above the tank and the time taking to locate the platform (escape latency) was recorded. On day 5, after removal of the platform, rats were allowed to swim for 60 s (probe trial), and the percentage of time spent in the target quadrant was recorded.<sup>22</sup> After each experiment, the rats were dried with a clean cloth and placed in a clean cage.

### RAM

RAM was used to evaluate the working and reference spatial memory. RAM is a reward-motivated model of spatial memory.<sup>23,24</sup> The maze consists of a central platform (32 cm wide), and eight-arm (58×12×38 cm), elevated 58 cm above the floor, with various visual cues in the surroundings. A circular food cup with 45 mg food pellet placed at the end of each arm as bait. The food of animals was restricted for 1 week before the RAM experiment to reach the body weight into 85% of their initial weight. Rats were trained for 10 minutes in 3 days, where all arms were opened and baited. On day 4, the performance tests, which consist of two phases (training and test) were conducted. In the training phase, the four arms were randomly blocked and the remaining arms were baited. The rats were allowed 5 min to retrieve the baits. After a 5-min delay, all arms were open, but the bait was only in the previously blocked arms. In this phase, rats were allowed to retrieve the baits for 5 min, the working memory errors (MWE, re-entries into baited and without bait arms) and reference memory errors (RME, entering without bait arms) were recorded.<sup>24,25</sup> The maze was thoroughly cleaned with damp cotton after each rat's experiment to remove any smell cues.

## Brain Extraction and Weighing

One day after the last behavioral test, rats were decapitated, brain extracted and placed in an ice-filled petri dish to clean. The cerebellum was rapidly removed and the remaining brain weighed (wet brain weight).<sup>26,27</sup>

## Statistical Analysis

Graph pad prism (6.07) software was used for statistical analysis. Parameters without considering the time were analyzed by One-way ANOVA test or Kruskal Wallis test and the parameters with consideration of time were analyzed by Two-way ANOVA (P<0.05).

## Results

### Effect of Chehelghoza on %SA and Number of Arm Entries

The %SA was considerably increased in 25% group (78.07 ±3.62), but not in 2% (76.20±3.99), 6% (71.12±4.13) and 12% (68.59±3.27) groups as compared with the control group (64.30±3.27) ( $P<0.05$ ) (Figure 1 and Table 1). Also, the difference in the number of arm entries between 2% (21.33±2.09), 6% (22.33±2.04), 12% (27.67±2.44) and 25% (25.50±2.14) groups and the control group (28.17 ±1.99) was not significant (Figure 2 and Table 1).

### Effect of Chehelghoza on the Percentage of Time Spent in and Number of Entries into Open Arms and Number of Crossing

The percentage of time spent in open arms was increased in diazepam (18.79±2.20) and 25% (15.22±2.91) groups significantly, in comparison with the control group (3.94 ±1.92). However, there was not a significant difference between 2% (4.41±1.90), 6% (4.96±2.06) and 12% (4.59 ±2.65) groups and the control group (3.94±1.92) (Figure 3 and Table 2). The number of entries into open arms in diazepam (4.00±0.52) and 25% (3.67±0.33) groups was significantly increased, as compared with the control group (0.67±0.33). However, there was not a significant difference between 2% (1.50±0.56), 6% (1.33±0.42) and 12% (1.17±0.65) groups and the control group (0.67±0.33) (Figure 4 and Table 2). The number of crossing was significantly increased in diazepam (13.67±2.43) and

**Table 1** Effect of Chehelghoza on %SA and the Number of Arm Entries

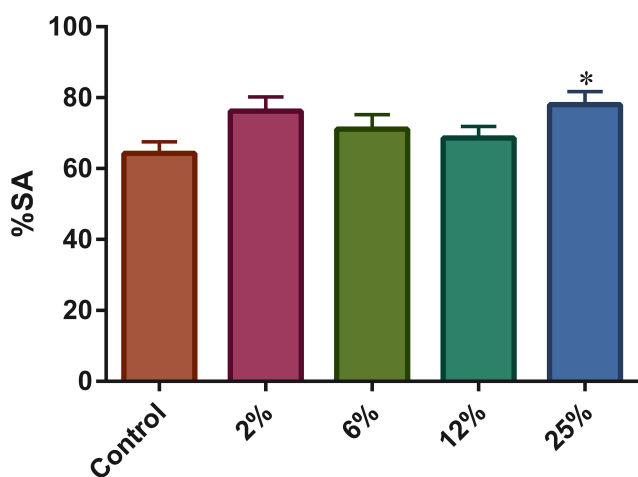
Groups	Parameters	
	%SA	Number of Arm Entries
Control	64.30±3.27	28.17±1.99
2%	76.20±3.99	21.33±2.09
6%	71.12±4.13	22.33±2.04
12%	68.59±3.27	27.67±2.44
25%	78.07±3.62*	25.50±2.14
<b>One-way ANOVA</b>	$F = 2.326$ $df = 4$ $P = 0.084$	$F = 2.055$ $df = 4$ $P = 0.117$

**Notes:** Data are shown as mean±SEM. \* $P<0.05$  as compared with the control group. **Abbreviations:** *df*, degrees of freedom; %SA, percentage of spontaneous alternation.

25% (11.50±1.02) groups, as compared with the control group (4.83±1.35). However, there was not a significant difference between 2% (7.33±1.73), 6% (8.50±1.93) and 12% (10.50±1.73) groups and the control group (4.83 ±1.35) (Figure 5 and Table 2).

### Effect of Chehelghoza on Escape Latency and Time Spent in the Target Quadrant

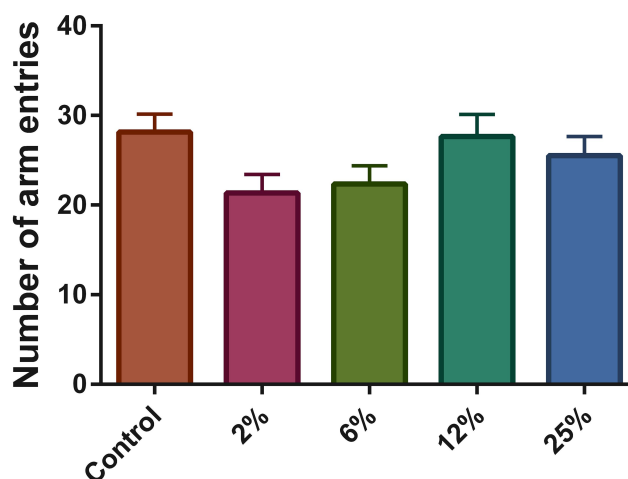
The difference between the escape latency of control and each chehelghoza-treated groups in either 1–4 days was not significant (Figure 6 and Table 3). Also, the time spent in the target quadrant was considerably increased by 12% (26.01±3.94) and 25% (26.85±4.55) groups, in comparison with the control group (11.21±2.47) ( $P<0.05$ ). However, the difference between the control (11.21±2.47) and each



**Figure 1** Effect of chehelghoza on %SA.

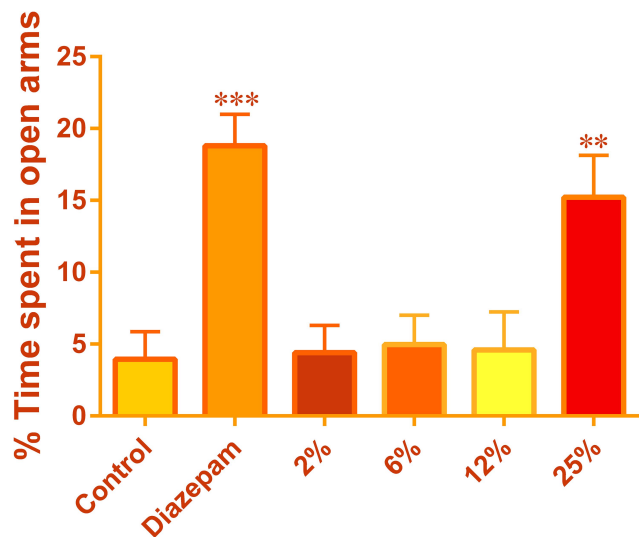
**Notes:** Data are shown as mean±SEM. \* $P<0.05$  as compared with the control group.

**Abbreviation:** %SA, percentage of spontaneous alternation.



**Figure 2** Effect of chehelghoza on the number of arm entries.

**Notes:** Data are shown as mean±SEM.



**Figure 3** Effect of chehelghoza on the percentage of time spent in open arms. **Notes:** Data are shown as mean±SEM. \*\*P<0.01, \*\*\*P<0.001 as compared with the control group.

2% (23.79±2.72) and 6% (20.70±4.46) groups was not significant (Figure 7 and Table 3).

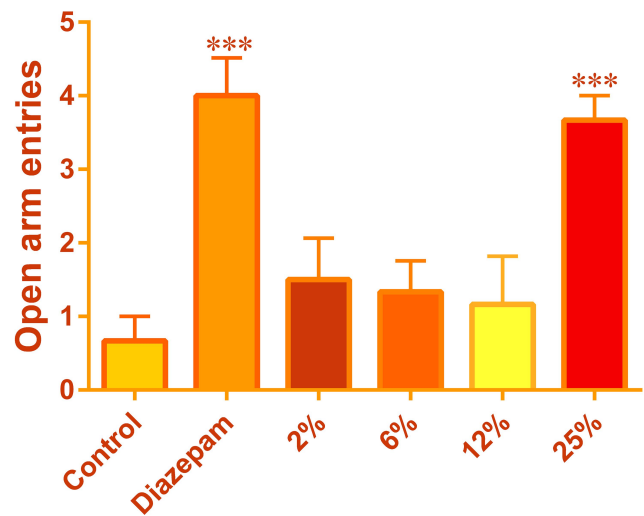
### Effect of Chehelghoza on WME and RME

There was not a significant difference between WME of control (6.83±0.87) and each 2% (3.83±0.48), 6% (8.67 ±1.89), 12% (5.17±0.91) and 25% (8.17±0.87) groups (Figure 8 and Table 4). There was also not a significant difference between RME of control (5.50±0.43) and each 2% (4.67±0.49), 6% (6.50±1.28), 12% (4.67±0.67) and 25% (6.17±0.83) groups (Figure 9 and Table 4).

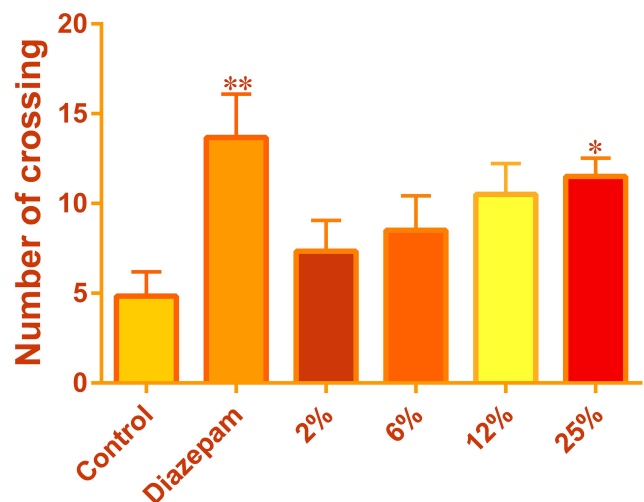
**Table 2** Effect of Chehelghoza on the Percentage of Time Spent in Open Arms, Open Arm Entries and a Number of Crossings

Groups	Parameters		
	% Time Spent in Open Arms	Number of Entries into Open Arms	Number of Crossing
Control	3.94±1.92	0.67±0.33	4.83±1.35
2%	4.41±1.90	1.50±0.56	7.33±1.73
6%	4.96±2.06	1.33±0.42	8.50±1.93
12%	4.59±2.65	1.17±0.65	10.50±1.73
25%	15.22±2.91**	3.67±0.33***	11.50±1.02*
Diazepam	18.79±2.20***	4.00±0.52***	13.67±2.43**
<b>One-way ANOVA</b>	F = 8.14 df = 5 P <0.0001	F = 8.44 df = 5 P <0.0001	F = 3.23 df = 5 P = 0.018

**Notes:** Data are shown as mean±SEM. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001 as compared with control group. **Abbreviation:** df, degrees of freedom.



**Figure 4** Effect of chehelghoza on the open arm entries. **Notes:** Data are shown as mean±SEM. \*\*\*P<0.001 as compared with the control group.



**Figure 5** Effect of chehelghoza on a number of crossings. **Notes:** Data are shown as mean±SEM. \*P<0.05, \*\*P<0.01 as compared with the control group.

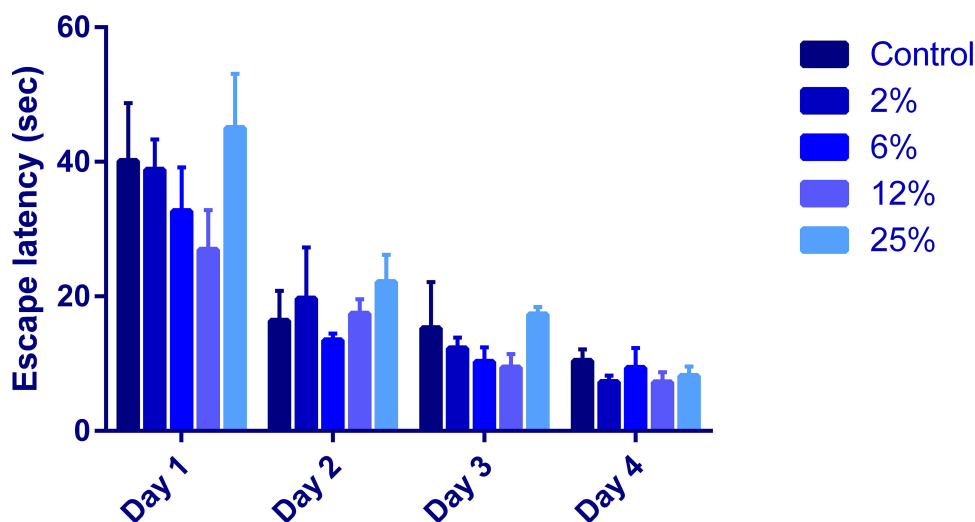
### Effect of Chehelghoza on Brain Weight

The wet brain weight was significantly increased in each 12% (1.39±0.05) and 25% (1.42±0.01) groups, as compared with the control group (1.29±0.01). However, there was not a significant difference between control (1.29 ±0.01) and each diazepam (1.33±0.02), 2% (1.36±0.01) and 6% (1.36±0.02) groups (Figure 10 and Table 5).

### Effect of Chehelghoza on Body Weight

The difference in the day 0 body weight between different groups was not significant. However, the day 28 body weight of each 2% (234.50±7.40), 6% (238.17±5.27),





**Figure 6** Effect of chehelghoza on escape latency.

**Notes:** Data are shown as mean±SEM.

12% (240.17±3.66) and 25% (242.17±5.19) groups was considerably decreased in comparison with the control group (269.17±4.50). There was not a significant difference between day 28 body weight of control (269.17±4.50) and diazepam (264.33±4.36) group (Figure 11 and Table 5).

## Effect of Chehelghoza on Brain:Body Weight Ratio

The difference in brain:body weight ratio between each 2% (0.0058±0.00014), 6% (0.0057±0.00007), 12% (0.0058±0.00014) and 25% (0.0059±0.00008) groups and the control group (0.0047±0.00005) was significant. However, the difference between brain:body weight ratio of the diazepam (0.0050±0.00002) and control (0.0047

±0.00005) groups was not significant (Figure 12 and Table 5).

## Discussion

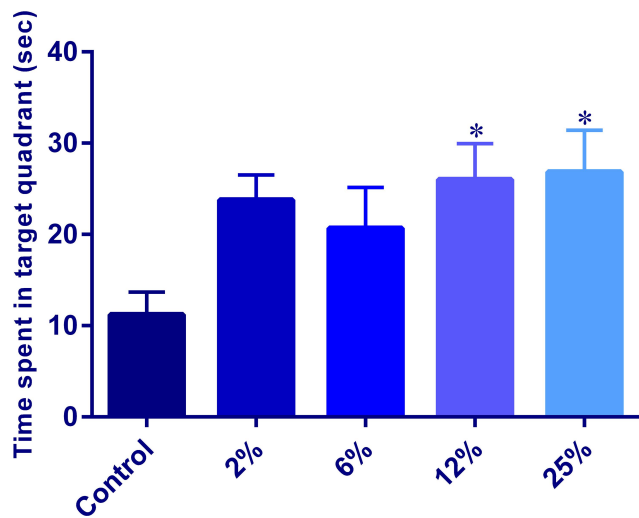
The nature gifts various plants, fruits and nuts to human.<sup>7</sup> Many researchers are focused on the use of natural compounds and medicinal plants.<sup>28</sup> There are many plants and nuts such as *Convulvulus pluricaulis*, *Evolvulus alsinoides*, *Canscora decussate*, *Juglans regia* which are used for enhancement of learning and memory.<sup>18,29–31</sup> In general, nuts have various beneficial constituents. Some of the nuts constituents may directly be related to brain function and physiology. For instance, walnut and pine nut have a large amount of unsaturated fatty acids, which excites the emotional experience-related brain areas.<sup>32</sup>

**Table 3** Effect of Chehelghoza on Escape Latency and Time Spent in the Target Quadrant

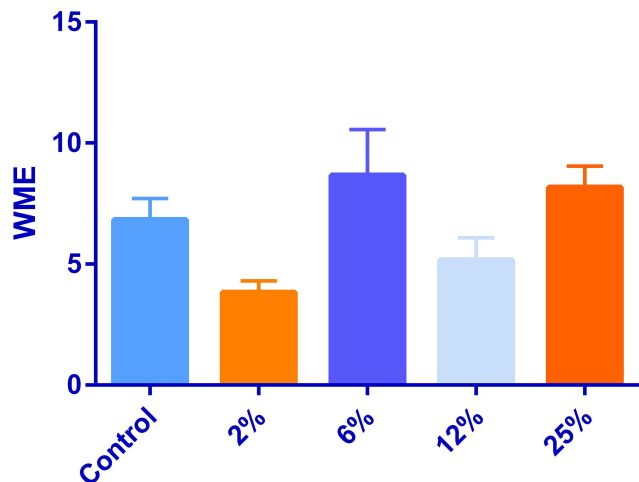
Groups	Escape Latency				Time Spent in Target Quadrant
	Day 1	Day 2	Day 3	Day 4	
Control	40.10±8.62	16.36±4.48	15.28±6.82	10.43±1.67	11.21±2.47
2%	38.85±4.48	19.68±7.58	12.28±1.58	7.26±0.98	23.79±2.72
6%	32.67±6.48	13.49±0.97	10.28±2.17	9.38±2.95	20.70±4.46
12%	26.94±5.88	17.40±2.16	9.42±1.99	7.22±1.50	26.01±3.94*
25%	45.04±8.06	22.13±4.02	17.31±1.09	8.19±1.37	26.85±4.55*
	<b>Two-Way ANOVA</b> F = 1.068 df = 4 P = 0.39				<b>One-Way ANOVA</b> F = 2.88 df = 4 P = 0.04

**Note:** Data are shown as mean±SEM. \*P<0.05 as compared with the control group.

**Abbreviation:** df, degrees of freedom.



**Figure 7** Effect of chehelghoza on time spent in the target quadrant.  
**Notes:** Data are shown as mean±SEM. \*P<0.05 as compared with the control group.



**Figure 8** Effect of chehelghoza on WME.  
**Notes:** Data are shown as mean±SEM.  
**Abbreviation:** WME, working memory errors.

In this study, the effect of Afghan chehelghoza combined with diet on learning, memory, anxiety, brain weight and body weight in rats was evaluated behaviorally. The results have shown that chehelghoza can have an effect on rat’s behavior as a dose-dependent manner. The administration of 25% chehelghoza combined with food could increase the %SA, an index of spatial working memory, whereas the 2%, 6% and 12% doses did not have a significant effect. Thus, chehelghoza can increase the spatial working memory only in high dose. The difference between the numbers of arm entries between different groups was not significant, so this excludes the false-positive results.

**Table 4** Effect of Chehelghoza on WME and RME

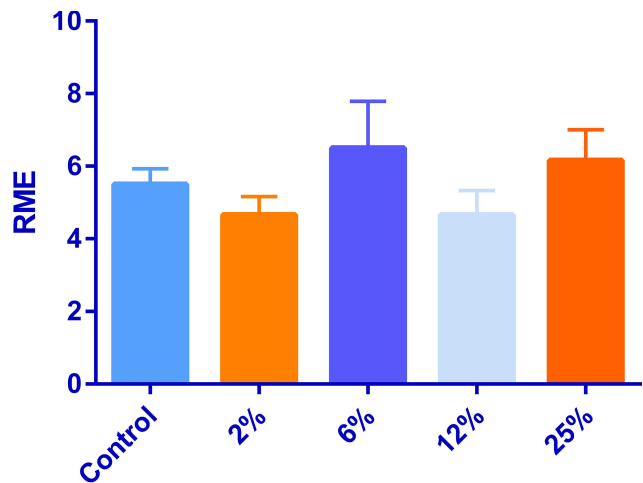
Groups	Parameters	
	WME	RME
Control	6.83±0.87	5.50±0.43
2%	3.83±0.48	4.67±0.49
6%	8.67±1.89	6.50±1.28
12%	5.17±0.91	4.67±0.67
25%	8.17±0.87	6.17±0.83
	<b>One-Way ANOVA</b> F = 3.344 df = 4 P = 0.025	<b>Kruskal–Wallis</b> P = 0.288

**Note:** Data are shown as Mean±SEM.  
**Abbreviations:** df, degrees of freedom; WME, working memory errors; RME, reference memory errors.

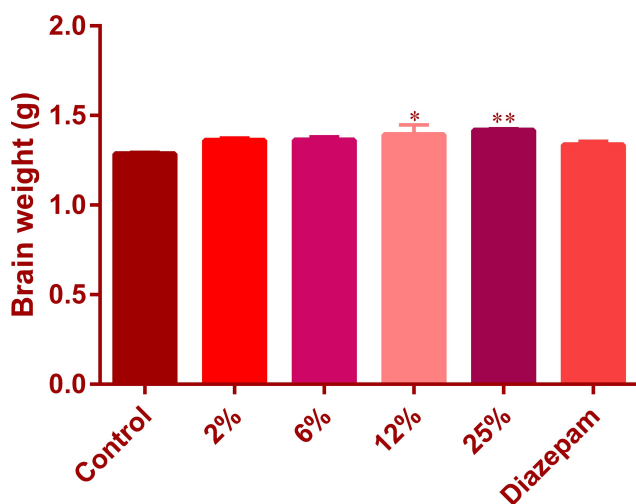
Also, the learning and reference memory was evaluated by MWM. Results have shown that neither doses of chehelghoza, which combined with food, could decrease the transfer latency as an index of learning. However, the 12% and 25% amounts of chehelghoza could significantly increase the time spent in target quadrant as an index of reference memory, whereas the 2% and 6% combinations did not have a significant effect.

Therefore, we can conclude that dietary chehelghoza can increase both short-term (working) memory and long-term (reference) memory in the rats.

As this is the first study conducted on the effect of chehelghoza on memory, we cannot compare the results of this study with any similar previous studies. However,



**Figure 9** Effect of chehelghoza on RME.  
**Notes:** Data are shown as mean±SEM.  
**Abbreviation:** RME, reference memory errors.



**Figure 10** Effect of chehelghoza on brain weight.

**Notes:** Data are shown as mean±SEM. \*P<0.05, \*\*P<0.01 as compared with the control group.

previous studies have been shown that different constituents of chehelghoza, such as polyunsaturated fatty acid (PUFA) and monounsaturated fatty acid (MUFA) have positive effects on memory-related brain areas and also on synaptic plasticity.<sup>33–36</sup>

Because the main constituents of chehelghoza pine nut are unsaturated fatty acids,<sup>8,9</sup> the positive effect of chehelghoza on memory in this study may result from the presence of unsaturated fatty acids. Previous behavioral studies have shown that there is a relation between polyunsaturated fatty acid (PUFA) and cognition in mammals.<sup>33</sup> Deficiency of PUFA in diet can affect ion channels activity, synaptic transmission and synaptic plasticity. In addition to multiple effects on inflammation, gene expression, apoptosis and

cell-protecting activity, PUFA metabolites can improve signal transduction related to memory process, induction and maintenance of long-term potentiation (LTP).<sup>33,34</sup>

Long-chain PUFAs, including eicosapentaenoic acid and also other types, can increase the intracellular calcium concentration.<sup>37</sup> Studies showed that an increase of calcium directly modulates the biochemical processes of synaptic vesicle exocytosis and therefore neurotransmitters release.<sup>38</sup> Levels of neurotransmitters is involved in building memory.<sup>29</sup> The increase in calcium also causes synaptic changes, which are important for short-term and also long-term memory.<sup>39</sup>

Besides, the presence of PUFA in the diet can improve membrane fluidity and modulate synaptic transmission. For instance, long-term consumption of arachidonic acid increases the fluidity of hippocampal neuronal membranes.<sup>33,40</sup> There is a feedback control between long-chain PUFAs and numbers of insulin receptors in neurons, so an increase in membrane fluidity by long-chain PUFAs increases the number of insulin receptors and affinity of the insulin to its receptors.<sup>37,40</sup> PUFAs either directly or through an increase in insulin receptors can accelerate the glucose uptake. It is known that arachidonic acid excites the glucose uptake by cerebral cortex astrocytes. Glucose, in turn, can increase the release of acetylcholine and therefore the memory.<sup>40</sup> Also, studies have shown that arachidonic and docosahexaenoic acid can increase the release of acetylcholine.<sup>37</sup> Acetylcholine in the hippocampus controls the balance of encoding and retrieval especially in pyramidal cells of the CA1 region, thereby modulates synaptic plasticity.<sup>41</sup>

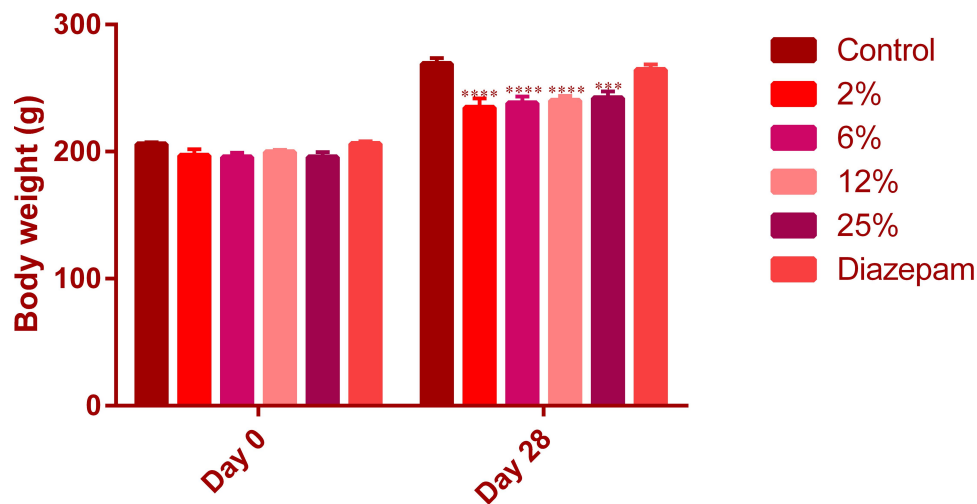
**Table 5** Effect of Chehelghoza on Brain Weight, Body Weight and Brain:Body Weight Ratio

Groups	Parameters			
	Day 0 Body Weight	Day 28 Body Weight	Brain Weight	Brain:Body Weight Ratio
Control	205.83±1.58	269.17±4.50	1.29±0.01	0.0047±0.00005
2%	196.83±5.00	234.50±7.40****	1.36±0.01	0.0058±0.00014**
6%	195.50±3.72	238.17±5.27****	1.36±0.02	0.0057±0.00007**
12%	199.50±1.86	240.17±3.66****	1.39±0.05*	0.0058±0.00014**
25%	195.67±3.94	242.17±5.19***	1.42±0.01**	0.0059±0.00008***
Diazepam	206.17±1.87	264.33±4.36	1.33±0.02	0.0050±0.00002
	<b>Two-way ANOVA</b> F = 7.08 df = 5 P = 0.0002		<b>One-Way ANOVA</b> F = 3.06 df = 5 P = 0.0239	<b>Kruskal-Wallis</b> F = 0.0001

**Notes:** Data are shown as mean±SEM. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001, \*\*\*\*P<0.0001 as compared with the control group.

**Abbreviation:** df, degrees of freedom.





**Figure 11** Effect of chehelghoza on body weight.

**Notes:** Data are shown as mean±SEM. \*\*\*P<0.001, \*\*\*\*P<0.0001 as compared with control group.

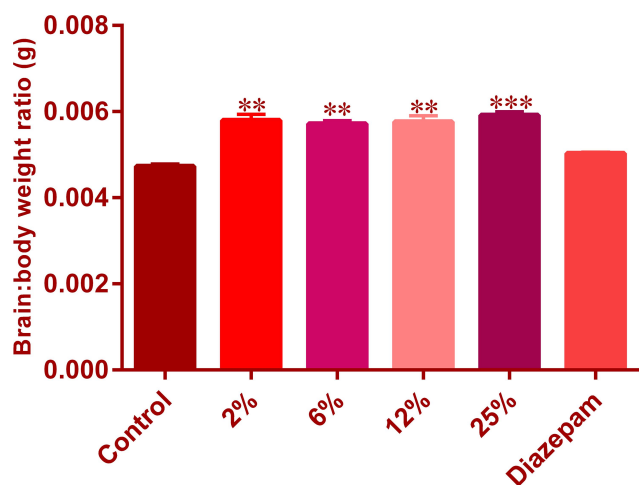
There is evidence that shows chehelghoza pine nut is rich in arginine.<sup>11</sup> Arginine is a semi-essential amino-acid, which acts as a precursor of nitric oxide.<sup>42</sup> Long-chain PUFAs also increase the synthesis of nitric oxide.<sup>37</sup> Nitric oxide-cGMP pathway has an important role in the induction of LTP and memory process.<sup>42,43</sup>

In addition, chehelghoza pine nut is also rich in oleic acid.<sup>8,9</sup> It is a monounsaturated fatty acid (MUFA), which is, considered as a neurotrophic factor in neurons.<sup>35,36</sup> Oleic acid causes neuronal migration and synapse formation by an increase in presynaptic and postsynaptic protein gene expression.<sup>36</sup> In general, it is believed that oleic acid can improve memory by an increase in LTP.<sup>44</sup>

By putting all the above matters together, one can conclude that chehelghoza pine nut because of containing most beneficial constituents such as PUFA, MUFA and arginine can improve memory through various mechanisms. Consumption of chehelghoza in the diet may cause an increase in calcium concentration, maintenance of membrane fluidity, increase in several insulin receptors and insulin affinity to its receptors, acceleration of glucose uptake by cell, increase in acetylcholine and nitric oxide neurotransmitters and therefore memory improvement.

Following other results, this study showed that high amounts of chehelghoza in the diet (12% and 25%) could increase the brain weight in rats, so the effect of 25% dietary chehelghoza was greater. Also, the brain:body weight ratio is increased in each 2, 6 and 12% groups, and especially in the 25% group. These effects of chehelghoza on brain weight and brain:body weight ratio is also explained by the presence of PUFAs and MUFAs in this type of pine nut. As PUFAs and also oleic acid as neurotrophic factors can increase the gene expression of pre-synaptic and post-synaptic proteins, they are effective in axonal and dendritic and thus brain growth.<sup>35,40</sup> The learning and memory capacity has been related to the absolute brain weight and its ratio with body.<sup>5,45</sup> Increase in brain weight due to increase in neuron size, dendrite numbers and neuronal interconnections results in the improvement of learning and memory.<sup>5</sup> So, the increase in brain weight resulting from chehelghoza consumption may be an indicator of brain growth and therefore memory enhancement.

In addition, results showed that neither amounts of dietary chehelghoza in this study could decrease the



**Figure 12** Effect of chehelghoza on brain:body weight ratio.

**Notes:** Data are shown as mean±SEM. \*\*P<0.01, \*\*\*P<0.001 as compared with the control group.

WME and RME. Because RAM is a reward-motivated model of spatial memory,<sup>23</sup> probably, chehelghoza pine nut does not have any effect on this type of memory by these doses, which used in this study. Higher doses may affect.

Besides, the current study also investigated the effect of chehelghoza on the behavior of anxiety and stress and in comparison with diazepam. Results showed that 25% chehelghoza combined with food could increase the percentage of time spent in open arms, some entries into open arms and number of crossing, so its effect was little less than the anti-anxiety effect of diazepam. However, a low amount of chehelghoza did not have a significant effect on the rat's anxiety.

Similarly, a previous study has shown that oleic acid can exert sedative effects in infant rats. It is suggested that oleic acid can bind to benzodiazepine sites in GABA-A receptors,<sup>46</sup> which modulates the opening of chloride channels. Most of the anti-anxiety compounds exert their anti-anxiety effect through acting on these receptors.<sup>46,47</sup> Thus, it is probable that chehelghoza pine nut, because of containing oleic acid, can reduce anxiety and stress behavior by acting on GABA-A receptors.

The body weight of different groups was also evaluated. Results indicate that consumption of chehelghoza with food has effects on body weight even by the lowest amounts; so all amounts of dietary chehelghoza could decrease the body weight of rats after 28 days' treatment period. Similarly, previous studies have been shown that nuts generally because of containing PUFAs, MUFAs, high protein and dense energy may cause early satiety and reduction in body weight.<sup>48–50</sup>

## Conclusion

One can conclude that high amounts of chehelghoza pine nut in combination with food can improve both short-term and long-term memory, reduce anxiety and stress behavior, and also increase in brain weight. The most effective dose percentage of chehelghoza pine nut combined with diet was 25%. Based on the present finding, we can suggest further evaluation of the effect of chehelghoza pine nut on memory impairment, especially Alzheimer disease and age-related cognitive decline by behavioral studies and electrophysiological analysis methods.

## Abbreviations

EPM, elevated plus maze; LTP, long-term potentiation; MWM, Morris water maze; MUFA, monounsaturated

fatty acid; NIH, National Institute of Health; PUFA, polyunsaturated fatty acid; RAM, radial arm maze; RME, reference memory errors; WME, working memory errors; %SA, the percentage of spontaneous alternation.

## Ethics Approval

This study was conducted on the ethical guidelines set by the eighth edition of the National Institute of Health (NIH) guide for the care and use of laboratory animals. The ethic research board of Khatam Al-Nabieen University approved the experimental protocol (Approval No. AF.KNU.REB.1397.16).

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## Author Contributions

All authors contributed to data analysis, drafting or revising the article, have agreed on the journal to which the article will be submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

## Disclosure

The authors report no conflicts of interest in this work.

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