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Original Article

Aegle marmelos Extract Can Enhance Memory in Rats

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Abstract

Diabetes mellitus is associated with disturbances of learning and memory and cognitive functioning. *Aegle marmelos* Corr. from Rutaceae family, is widely used in Iranian folk medicine for the treatment of diabetes mellitus. It decreases blood glucose level by improving glucose tolerance and also has lipid-lowering and antioxidant properties. Considering the beneficial antidiabetic potential of *A. marmelos*, this study was conducted to evaluate the effect of chronic oral administration of *A. marmelos* as cognitive enhancer, on learning and spatial memory in diabetic rats using Morris water maze test. Male Wistar rats were randomly divided into normal-control, diabetic-control, and *A. marmelos*-treated diabetic groups (100, 250 and 500 mg/kg, p.o., 4 weeks). Diabetes was induced by a single dose *i.p.* injection of streptozotocin (45 mg/kg). In each group of animals, spatial learning and memory parameters were analyzed. *A. marmelos* showed dose dependent improvement in spatial learning and memory parameters. Swimming time (Escape Latency) in normal-control and *A. marmelos*-treated diabetic animals rats was significantly lower than diabetic-control, while swimming speed was significantly higher. The study demonstrated that *A. marmelos* has a significant protective effect against diabetes-induced spatial learning and memory deficits. This effect can be attributed to hypoglycemic, hypolipidemic and antioxidant activity of *A. marmelos*.
Keywords: Aegle marmelos; Antihyperglycemic; Antioxidant; Diabetes; Spatial learning and memory.
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1. Intorduction

Diabetes mellitus is a common metabolic disorder, characterized by hyperglycemia due to an absolute or relative insulin deficiency. Diabetes is associated with functional and structural alterations in the peripheral, as well as the central nervous system [1, 2]. Moderate disturbances of learning and memory and complex information processing have been reported in both type 1 and 2 diabetic patients [3-5]. *Aegle marmelos* Corr. is commonly

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used as a folk medical plant in south of Iran, but it is indigenous to India. It is a medium sized, armed deciduous tree found wild, especially in dry forests and is also cultivated throughout Iranian subcontinent for its fruit. The fruit are globose with smooth, hard and aromatic rind. The ripe fruit is used for digestive and stomachic complications. Leaves, fruits, stem and roots of *A. marmelos* have been used in ethno-medicine for several medicinal properties such as astringent, antidiarrheal, antidysenteric, demulcent, antipyretic, antiscourbutic, haemostatic, aphrodisiac and as an antidote to snake venom [6-9]. *A. marmelos* is also known as herbal medicine for the treatment of diabetes mellitus [10, 11]. Preliminary reports indicated hypoglycemic effect of leaves, seeds and fruits of *A. marmelos* [12-15]. Ponnachan *et al.* [16, 17] have observed that the alkaloid extract prepared from leaves and crude aqueous leaf extract (1 g/kg for 30 days) exhibit hypoglycemic effect in alloxanized diabetic rats. Aqueous leaf extract reversed the increase in Km values of liver malate dehydrogenase enzyme [18] and improved histopathological alterations in the pancreatic and kidney tissues of streptozotocin (STZ) induced diabetic rats [19]. Moreover, change in glucose utilization, balance of cerebral lipid metabolism and oxidative stress that occur in diabetes are main reasons for cognitive dysfunction [20, 21]. Antioxidant and anti-dyslipidemic effects of *A. marmelos* also have been reported [22-25] and that is suggestive of its potential as cognitive enhancer.

Thus, treatment with herbal medicine that improve glucose utilization, decrease oxidative stress and modify lipid metabolism, may help to improve learning and memory impairment induced by diabetes. There are no available reports on the action of *A. marmelos* seeds on cognitive tests, therefore, the effect of aqueous extract of *A. marmelos* seeds on spatial learning and memory in streptozotocin induced diabetic rats has been investigated.

2. Materials and methods

2.1. Animals

Male Wistar rats, weighing 200-250 g, were provided by the Iranian Razi Institute and were housed in standard cages with free access to food (standard laboratory rodent's chow) and water. The animal house temperature was maintained at 23±3 °C with a 12-h light/dark cycle (light on from 06:00 to 18:00 h). The study was approved by animal research committee of Kermanshah University of Medical Science and the principles of laboratory animal care (National Institutes of Health publication No. 86-23, revised 1985) were followed in this study. The ethical guidelines for the investigation of experimental animals were followed in all tests. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Plant material and extraction

Seeds of *A. marmelos* from fresh fruits were collected in august 2007 from south of Iran (Bushehr) and authenticated by the School of Agricultural Sciences, Razi University, Kermanshah, Iran. The fruits were macerated in large amount of water and passed through large pore size sieve to separate seeds. The seeds were shade dried and rubbed vigorously to remove the last traces of fibre attached to it. Powdered seeds were extracted with boiling water for 10 h. The resulting extract was cooled and filtered using Whatman No. 1 filter paper. The filtrate was evaporated to dryness in an oven set at 40 °C. The dried extract was weighed and dissolved in normal saline to a concentration of 200 mg/ml. The extract was maintained at 4 °C throughout experiments.

2.3. Treatments

A freshly prepared solution of streptozotocin (Sigma Chemical Co.; St. Louis, USA; 45 mg/kg) in 0.1 M citrate buffer, pH 4.5 was injected intraperitoneally to

overnight fasted rats [26]. Fasting blood glucose level (FBG) was estimated at the time of induction of diabetes and FBG was checked regularly up to stable hyperglycemia, one week after streptozotocin injection. Depending on their FBG level the severe diabetic animals showing FBG above 250 mg/dl, were studied [27]. Diabetic animals were treated with *A. marmelos* extract (100, 250, 500 mg/kg, *p.o.*) or normal saline (diabetic-control), and normal-control group received the same volume of normal saline orally for 4 weeks. At the beginning and end of study, blood samples were collected and centrifuged. FBG level was measured by glucose oxidase method [28]. After 4 weeks, learning and spatial memory tests in normal and diabetic rats were carried out using Morris water maze (MWM) test. During the MWM test, animals fed as same as before. MWM was constructed from a circular black colored water tank, 140 cm in diameter and 80 cm in height that was located in the center of small room and was surrounded by numerous extramaze cues on the wall in the room. The tank was divided into four quadrants (N, E, W and S) was filled with water till it has reached 40 cm in depth. The experimenter stood in the southwest corner of the room. Invisible round disk platform (made of Plexiglas, 10 cm in diameter) were used and located 1cm beneath the surface of the water. In the first 4 days of experiment, location of platform was constant throughout the sessions (see below). An automated infrared tracking system (CCTV B/W camera, SBC-300 (P), Samsung Electronic Co., Ltd, Korea) recorded the position of the rat in the tank. The camera was mounted 2.5 m above the surface of the water [29].

2.3.1. Handling

Each rats received once daily, 10 min. handling period for three days, after which the animals were trained for two days to stand on the platform. On the first day, rats were placed on the platform which was at the center of the tank without water for 60 s, and on the second day, the rats were placed again on the platform under the same conditions but the tank was filled with normal saline, room temperature (20±2 ºC). When the rat climbed off the platform, the experimenter guided the rat to go back onto the platform [30].

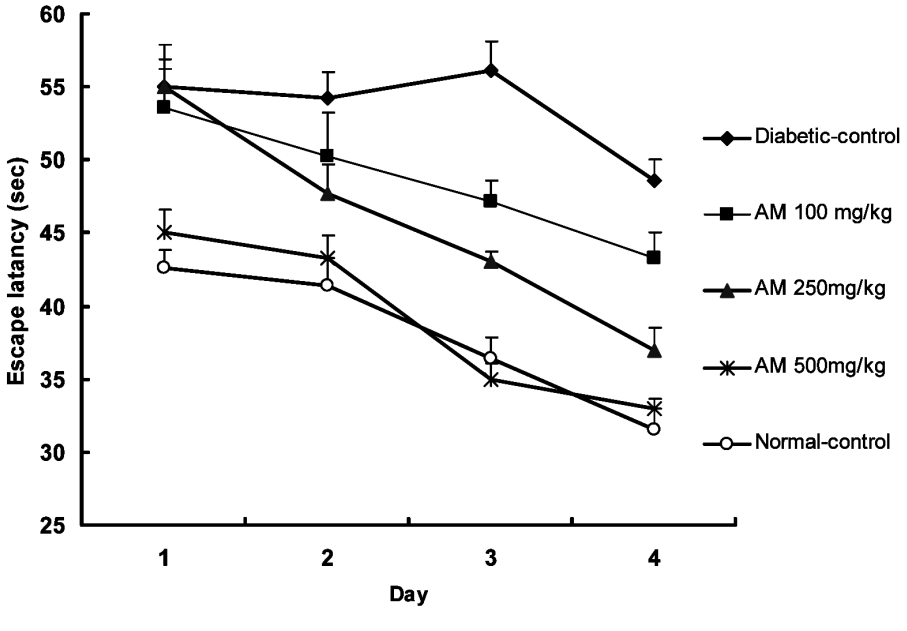

Figure 1. Escape latency in diabetic-control, *A. marmelos* and normal-control groups in the training days. Values are means±SEM.

Table 1. Blood glucose levels in diabetic-control, *A. marmelos* and normal-control groups at the beginning and end of experiment.

Group	Dose (mg/kg)	Blood glucose levels (mg/dl)	
		Pretreatment	Post treatment
Normal-control	-	92±7	88.4±2***
Diabetic-control	-	350±5	364±7
A. marmelos extract	100	339±8	287±10*
	250	344±4	192±6**
	500	341±5	126±6***

Values are means±SEM. *p<0.05, **p<0.01, *** p<0.001 vs. Diabetic-control group.

2.3.2. Training procedure

Extramaze landmarks (window, door, etc.) in the room were spatial cues for learning of platform's position for animals. The position of the platform was fixed throughout the experiments. The platform was located in the north-west quarter of MWM tank with 20 cm distance from the edge of the tank, and 1 cm beneath the surface of water. Each rat was tested for 5 sessions. Each session consisted of 4 trials in a day. In first sessions, a trial began by releasing the rat into the water facing the wall of the tank from one of the four quadrants (North, South, East or West). The sequence of starting location was chosen in a pseudorandom manner by computer in such a way that the starting location was different from the immediate preceding trial. The trial was concluded when the rat found the platform or at 60 s after start of the trial. If the rat could not reach the platform within 60 s, the experimenter led the rat to the platform and the rat remained on the platform for 30 s, then released into the water from the next starting location. After the last trial in each session, the rat was towel-wiped and placed in a drying chamber for 5 to 15 min. and then returned to the home cage. For evaluation of accuracy and validity of initial learning, probe trial was performed on the fifth day, in which, platform was expelled and animal during one session (consisting of 4 trials) was released into water exclusively from one of the above mentioned directions (East) that was determined by computer for all rats [29].

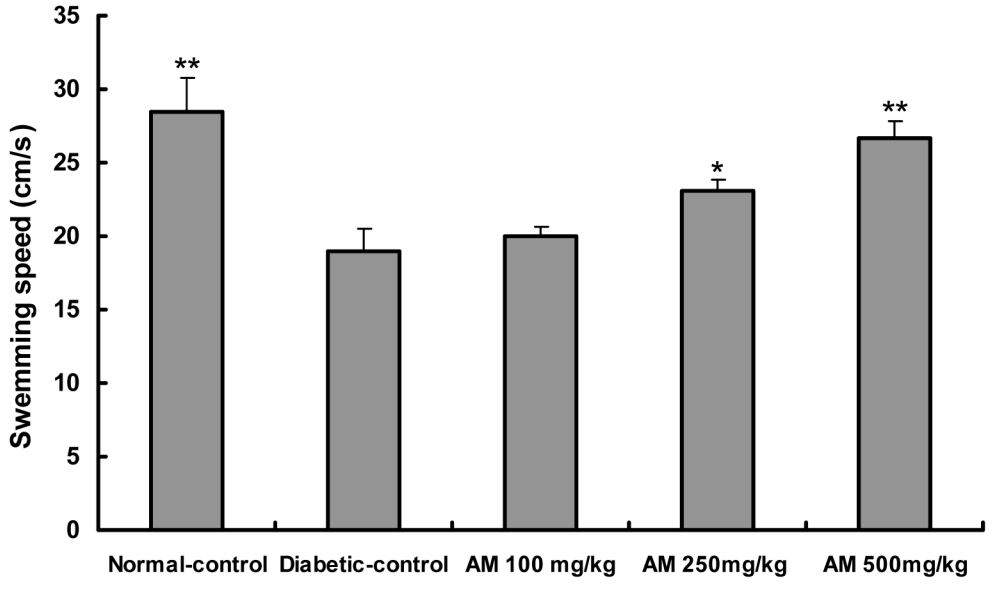

Figure 2. Swimming speed in diabetic-control, *A. marmelos* and normal-control groups in the training days. Values are means±SEM. *p<0.01, **p<0.001 *vs.* diabetic-control group.

2.4. LD_{50} experiments

For LD_{50} experiment, four groups of rats of both sex (six animals per group, three females and three males) and weighing about 200-250 g were administered orally a single dose of either 2.5, 5, 10 or 15 times of effective dose of aqueous extract of seeds of *A. marmelos*. Then rats were observed for gross behavioural, neurologic, autonomic and toxic effects at short intervals of time for 24 h. Food consumption, feces and urine were also examined at 2 h and then at 6 h intervals for 24 h [22].

2.5. Data analysis

The data were expressed as mean±SEM, subjected to analysis of variance (ANOVA) and followed by Tukey's test for multiple comparisons, and $p<0.05$ was the critical criterion for statistical significance.

3. Results

3.1. Evaluation of escape latency and swimming speed during training days

Results indicate that *A. marmelos* administration reduces escape latency during training days in a dose-dependent manner. Also, there were differences among experimental groups in third and fourth days of training. On these days, escape latencies in the normal-control and *A. marmelos*-treated groups were less than that of diabetic-control group. This difference was statistically significant in the third and fourth day of training ($p<0.001$), while there wasn't any statistically significant difference between *A. marmelos* 500 mg/kg and normal-control group in all four training days (Figure 1). Results also indicated that there was a difference in swimming speed among experimental groups. Post-hoc analysis showed that differences between normal-control ($p<0.001$), *A. marmelos* 250 mg/kg ($p<0.01$) and *A. marmelos* 500 mg/kg ($p<0.001$) in comparison with diabetic-control were significant. Differences in swimming speed between *A. marmelos* 500 mg/kg and normal-control group wasn't significant (Figure 2).

3.2. Evaluation of the percentage of presence in target quarter in probe trial

The percentage of the presence of animals in target quarter (quarter in which platform was located during training days) in probe trial

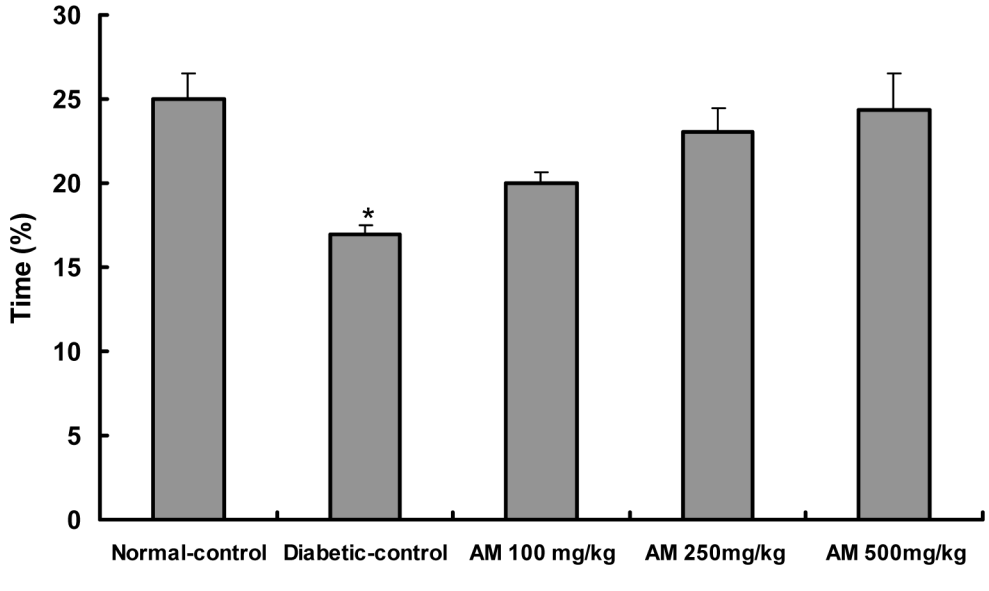

Figure 3. Percentages of time that animals spent in target quarter in probe trial in diabetic-control, *A. marmelos* and normal-control groups. Values are means±SEM. *$p<0.05$ *vs.* other groups.

session was investigated. This difference was significant (p<0.05) between diabetic-control group and other groups (Figure 3).

3.3. Effect of A. marmelos seed extracts on FBG

Effect of oral administration of the aqueous extract of *A. marmelos* seeds on FBG is shown in Table 1. *A. marmelos* administration, dose dependently, reduced FBG, and there were differences among experimental groups. Post-hoc analysis showed statistically significant differences between normal-control (p<0.001), *A. marmelos* 100 mg/kg (p<0.05), *A. marmelos* 250 mg/kg (p<0.01) and *A. marmelos* 500 mg/kg (p<0.001) in comparison with diabetic-control. Differences in FBG between *A. marmelos* 500 mg/kg and normal-control group wasn't significant.

3.4. LD_{50} studies

In LD_{50} experiment, the behaviour of the treated rats appeared normal. No toxic effect was reported up to 10 and 15 times of effective dose of the water extract and there were no death in any of these groups. Only the consumption of food was increased by 20% in 10 and 15 times doses during 4 h but remaining normal afterwards.

4. Discussion

Results of the present study suggest that chronic oral administration of *A. marmelos* has facilitating effects on spatial learning and memory in diabetic rats in Morris water maze. *A. marmelos* administration, during training days, leads to a decrease in escape latency and also an increase in the animals swimming speed as compared with the diabetic-control group. In this study, *A. marmelos* seed extract could reduce FBG that confirms hypoglycemic effect of this seed as reported earlier [22], but the most effective dose was 500 mg/kg.

In previous studies, moderate disturbances of learning and memory and complex information processing have been reported in both type 1 and 2 diabetic patients [3-5]. Previous experimental studies into cognitive functioning in animal models of diabetes mellitus, such as STZ-induced diabetic rodents, have used several learning tasks. In more complex learning tasks, such as an active avoidance T-maze, or a Morris water maze, diabetic rodents consistently displayed performance deficits [31-33]. According to the existing data, patients with Alzheimer's disease have a relatively high frequency of diabetes mellitus [34]. It seems that induced impairment of cognitive performance and the decrease of learning ability may not be restricted to Alzheimer's disease [35]. *A. marmelos* is known as a herbal medicine for the treatment of diabetes mellitus [10, 11]. Previous reports have indicated hypoglycemic effects of leaves, seeds and fruits of *A. marmelos* [12-15]. Pharmacological effects of *A. marmelos* particularly antidyslipidemic effect [1] is suggestive of its potential as a cognitive enhancer.

Patients receiving lipid-lowering drugs like statins have a reduced risk of dementia and cognitive dysfunction [36]. Lipids account for half of the dry matter of the brain and are integral to the myelin sheath and synapses. Anything that affects the balance of cerebral lipid metabolism could have profound effects on brain function. High cholesterol is also associated with elevated beta-amyloid, the hallmark of cognitive disorders [20]. Experimental studies have shown that cholesterol-fed wild-type rabbits develop memory dysfunction, which is supported by human studies, showing that statin therapy reduces the risk of memory impairment [37-39]. In addition, free radical generation and oxidative stress can affect all classes of macromolecules (sugar, lipids, proteins, and DNA), leading inevitably to neuronal dysfunction [21]. Moreover, in diabetic animals, impaired glucose utilization and insulin signaling has already been linked to

increased oxidative stress and mitochondrial dysfunction in neuronal cells [40, 41]. *A. marmelos* has antioxidant properties and effectively reduces oxidative stress. This was evident from a significant decrease in lipid peroxidation, conjugated diene and hydroperoxide levels in serum as well as in liver in diabetic rats after oral administration of *A. marmelos* [2-4].

The result of this study reveals that a regular administration of *A. marmelos* aqueous seed extract for 4 weeks improved learning and spatial memory in diabetic animals in Morris water maze test. The dose of 500 mg/kg was the most effective dose. The LD_{50} of the extract is high (no death even with 15 times of effective dose) indicating high margin of safety. The fall of FBG in diabetic groups treated with *A. marmelos*, after a period of study, further confirms our findings.

From this study, we can conclusively state that *A. marmelos* aqueous seed extract had beneficial effects on blood glucose levels as well as improving spatial memory impairment due to diabetes. This effect can be attributed to modification of lipid metabolism and blood glucose level and attenuation of the oxidative stress enhancement of diabetes mellitus induced by streptozotocin. Further pharmacological and biochemical investigations are underway to elucidate the mechanism of the learning and memory enhancement effect of *A. marmelos* seeds.

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