

The Effect of Chronic Administration of *Aegle Marmelos* Seed Extract on Learning and Memory in Diabetic Rats

*^{1,2,3}Amir Farshchi, ^{1,2,3}Golbarg Ghiasi, ⁴Samireh Farshchi, ⁵Amin Taleb Ghobadi

Abstract

Objective(s)

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. Considering the beneficial antidiabetic and antioxidant potential of *A. marmelos*, this study was conducted to evaluate the effect of oral administration of *A. marmelos* on learning and spatial memory in diabetic rats using Morris water maze test.

Materials and Methods

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.). Animals were treated for 4 weeks by *A. marmelos* or normal saline. 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.

Results

Clear impairment of spatial learning and memory was observed in diabetic group versus normal-control group. *A. marmelos* showed dose dependent improvement in spatial learning and memory parameters that swimming time (Escape Latency) in normal-control and *A. marmelos*-treated diabetic animals rats was significantly ($P < 0.01$) lower than diabetic-control, while swimming speed was significantly ($P < 0.05$) higher.

Conclusion

The study demonstrated that *A. marmelos* has significant protective affect against diabetes-induced spatial learning and memory deficits. This effect could be attributed to hypoglycemic, hypolipidemic and antioxidant activity of *A. marmelos*.

Keywords: *Aegle marmelos*, Diabetes, Morris water maze, Spatial learning and memory

1- School of Pharmacy, Kermanshah University of Medical Sciences, Kermanshah, Iran

2- Department of Pharmacoeconomics and Pharmaceutical administration, School of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran

3- Students Scientific Research Center, Tehran University of Medical Sciences, Tehran, Iran

*Corresponding author: Tel: +98-9188563290; Fax: +98-21-66566089; email: Farshchi_a@razi.tums.ac.ir

4-Department of otolaryngology, Amiralam Hospital, Tehran University of Medical Sciences, Tehran, Iran.

5-School of Medicine, Lorestan University of Medical Sciences, Khoramabad, Iran

Introduction

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. commonly used as folk medicinal 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 globose fruit has smooth, hard and aromatic rinds. The ripe fruit is used for indigestion and stomach pathologies. All parts of *A. marmelos* have been used in ethno-medicine for several medicinal properties: astringent, antidiarrheal, antidyenteric, demulcent, antipyretic, antiscorbutic, haemostatic, aphrodisiac and as an antidote to snake venom (6-9). Previous studies have discussed that *A. marmelos* is a herbal medicine for the treatment of diabetes mellitus (10, 11). Preliminary report indicates hypoglycemic effect in leaves, seeds and fruits of *A. marmelos* (12-15). Ponnachan et al. have reported that the alkaloid extract from *A. marmelos* leaves can exhibit hypoglycemic effect in diabetic rats induced by alloxan (16, 17). Extract of leaf can reverse the increase in K_m values of malate dehydrogenase enzyme in liver (18) and improved histopathological changes in the kidney and pancreatic tissues of diabetic rats induced by streptozotocin (19). Moreover, changing in glucose utilization, balance of cerebral lipid metabolism and oxidative stress that occurs in diabetes are main reasons for cognitive dysfunction (20, 21). Antioxidant and antidyslipidemic effects of *A. marmelos* also have been reported (22-25) and it suggests that *A. marmelos* could be cognitive modifier.

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 in diabetic rats, therefore, the effect of aqueous extract of *A. marmelos* seeds on spatial learning and memory in streptozotocin induced diabetic rats has been investigated.

Materials and Methods

The principles of laboratory animal care (National Institutes of Health publication No. 86-23, revised 1985) were followed in this study.

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 hr light/dark cycle (light on from 06:00 to 18:00 hr). 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 used animals.

Preparation of the plant extract

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 fiber attached to them. The seeds were ground mechanically to make powder. Powdered seeds were extracted with boiling water for 10 hr. 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. This extract solution was maintained at 4 °C throughout experiments.

Induction of diabetes in rats

Streptozotocin of Sigma Chemical Co. (St. Louis, USA) brands was purchased.

Streptozotocin solution (45 mg/kg) in 0.1 M citrate buffer, pH 4.6 was injected intraperitoneally to fasting rats (26). Fasting blood glucose level (FBG) was estimated at the time of induction of diabetes and checked regularly up to stable hyperglycemia, five days after streptozotocin injection. Depending on their FBG level the severe diabetic animals showing FBG above 250 mg/dl, were studied (27).

Treatment

Diabetic animals were treated with 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 levels were measured by glucose oxidase method (28). After 4 weeks, learning and spatial memory in normal and diabetic rats carried out using Morris water maze (MWM) test. During the MWM test, animals fed same as before but 30 min before the MWM test, animals were in fasting state.

Morris water maze task

MWM was constructed from a circular black colored water tank, 140 cm in diameter and 80 cm in height located in the center of small room and was surrounded by numerous extra-maze cues on the wall in the room. The tank was divided into four quadrants (N, E, W and S) and was filled with water till it has reached 40 cm in depth. The experimenter stood in the south-west corner of the room. Invisible round disk platform (made of plexiglas) 10 cm in diameter were used and was located 1 cm beneath the surface of the water. In the first 4 days of experiment, location of platform was constant throughout the sessions. 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).

A) *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 sec, and on

the second day, the rats were placed again on the platform under the same conditions but the tank was filled with water, at room temperature (25 ± 2 °C). When the rat climbed off the platform, the experimenter guided the rat to go back onto the platform (29).

B) *Training procedure*: Extra-maze 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 1cm 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 sec after start of the trial. If the rat could not reach the platform within 60 sec, the experimenter led the rat to the platform and the rat remained on the platform for 30 sec, 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).

LD₅₀ experiment

For LD₅₀ 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, 3 or 4 times of effective dose of aqueous extract of seeds of *A. marmelos*. Then rats were observed for gross behavioral, neurologic, autonomic and toxic effects at short intervals of time for 24 hr. Food

Effect of *Aegle Marmelos* on Memory

consumption, feces and urine were also examined at 2 hr and then at 6 hr intervals for 24 hr (22, 29).

Data analysis

Data was expressed as mean±SEM. Statistical analysis was performed using one way analysis of variance (ANOVA) and two-way repeated measures followed by Tukey's test for multiple comparisons. $P < 0.05$ was the critical criterion for statistical significance (29).

Results

Evaluation of escape latency and swimming speed during training days

Results indicate that diabetes induced deficiency in learning and spatial memory in animals during the experience. *A. marmelos* administration in a dose dependent fashion reduced escape latency during training days. Also there were differences among experimental groups in the third and fourth days of training. On these days, escape latencies in the normal-control and *A. marmelos* groups were less than diabetic-control group. This difference was statistically significant in the third and fourth day of training ($P < 0.001$), while there was no statistically significant difference ($P > 0.05$) between *A. marmelos* 500 mg/kg and normal-control group in either four training days (Figure 1). Results also indicated 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 ($P < 0.001$) versus diabetic-control were significant. Differences in swimming speed between *A. marmelos* 500 mg/kg and normal-control group was not significant ($P > 0.05$) (Figure 2).

Evaluation of percentage of presence in target quarter in probe trial

Presence percentage of animals in target quarter (quarter in which platform was located during training days) in probe trial session was investigated. Results showed that there was a significant difference among groups. This difference was significant ($P < 0.05$) between diabetic-control group and the other groups (Figure 3).

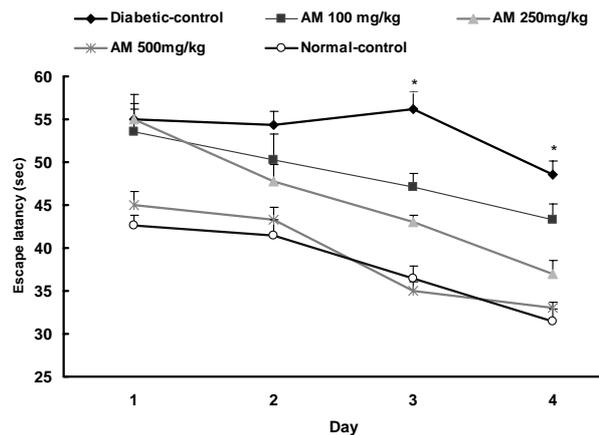


Figure 1. Escape latency in diabetic-control, *A. marmelos* and normal-control groups in the training days using Morris water maze in rats. Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons. Data are shown as means±SEM. * $P < 0.01$ vs. Diabetic-control group.

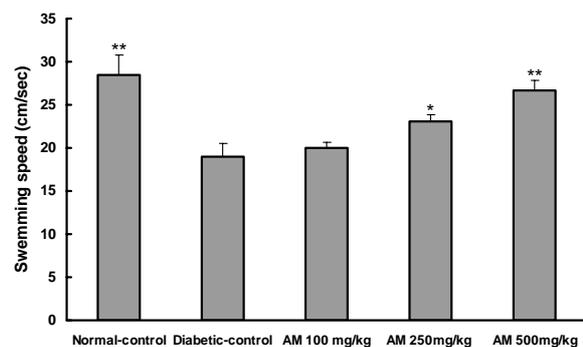


Figure 2. Swimming speed in diabetic-control, *A. marmelos* and normal-control groups in the training days using Morris water maze in rats. Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons. Data are shown as means±SEM. * $P < 0.01$, ** $P < 0.001$ vs. diabetic-control group.

Effect of *A. marmelos* on FBG of severely diabetic rats

Effect of *A. marmelos* oral administration on FBG has been 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 ($P < 0.001$) versus diabetic-control. Differences in FBG between *A. marmelos* 500 mg/kg and normal-control group was not statistically significant ($P > 0.05$).

Table 1. Blood glucose levels in study groups at the beginning and the end of experiment.

Group	Dose (mg/kg)	Blood glucose levels (mg/dl)	
		Pretreatment	Post treatment
Normal-control	-	92±7.1	88.4±1.9***
Diabetic-control	-	350±4.5	364±6.5
<i>A. marmelos</i> Extract	100	339±8.2	287±9.6*
<i>A. marmelos</i> Extract	250	344±3.6	192±5.9**
<i>A. marmelos</i> Extract	500	341±4.7	126±6.3***

Values are expressed as means±SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. diabetic-control group.

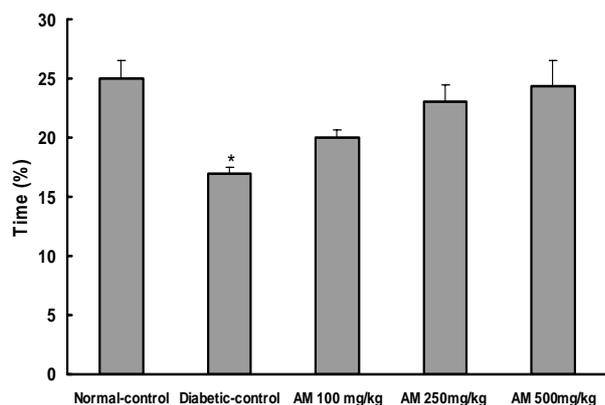


Figure 3. Percentages of time animals spent in target quarter in probe trial in diabetic-control, *A. marmelos* and normal-control groups using Morris water maze in rats. Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons. Data are shown as means±SEM. * $P < 0.05$ vs. other groups.

LD₅₀ experiment

In LD₅₀ experiment, the behaviour of the treated rats appeared normal. No toxic effect was reported up to 3 and 4 times of effective dose of the water extract and there were no death in either group.

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 test. *A. marmelos* administration, during training days, leads to 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* seeds extract could reduce FBG that confirm hypoglycemic effect of seeds as discussed 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 on cognitive functioning in animal models of diabetes mellitus, such as streptozotocin (STZ)-induced diabetic rodents, have used several learning tasks. In more complex learning tasks, such as active avoidance T-maze, or Morris water maze, diabetic rodents consistently displayed performance deficits (30-32). *A. marmelos* is known as herbal medicine for the treatment of diabetes mellitus (10, 11). Pharmacological effects of *A. marmelos* particularly antidiabetic and antihyperglycemic properties (1), suggest a cognitive enhancer trait. Patients receiving lipid-lowering drugs like statins have a reduced risk of dementia and cognitive dysfunction (33). Lipids account for half of the dry matter of the brain and are integral to the myelin sheath and synapses. Anything 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 and human studies showed that statin therapy reduces the risk of memory impairment (34-36). 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 (37, 38). *A. marmelos* has antioxidant properties and effectively reduces the oxidative stress (23, 24). This was evident from a significant decrease in lipid peroxidation, conjugated diene and hydroperoxide levels in serum as well as liver in diabetic rats after oral administration of *A. marmelos* (18, 25).

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 LD₅₀ of the extract is high (no death even with 4 times of effective dose) indicating high margin of safety. The fall of FBG in diabetic groups treated with *A. marmelos*, after period of study, further confirms our findings.

Conclusion

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 possibly 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.

Acknowledgment

This work was supported by department of physiology, School of Medicine, Kermanshah University of Medical Sciences.

References

1. Biessels GJ, Kappelle AC, Bravenboer B, Erkelens DW, Gispen WH. Cerebral function in diabetes mellitus. 1994; 37:643–650.
2. Vinik AI, Holland MT, Le Beau JM, Liuzzi FJ, Stansberry KB, Colen LB. Diabetic neuropathies. Diabetes Care 1992; 15:1926–1975.
3. Reaven GM, Thompson LW, Nahum D, Haskins E. Relationship between hyperglycemia and cognitive function in older NIDDM patients. Diabetes Care 1990; 13:16–21.
4. Ryan CM. Neurobehavioral complications of type I diabetes. Examination of possible risk factors. Diabetes Care 1988; 11:86–93.
5. Tun PA, Nathan DM, Perlmutter LC. Cognitive and affective disorders in elderly diabetics. Clin Geriatr Med 1990; 6:731–746.
6. Nandkarni AK. Indian Materia Medica. 3rd ed. Bombay: Popular Prakshan; 1976.
7. Kirtikar KR, Basu BD. Indian Medicinal Plant. Calcutta: Lalit Mohan Publication; 1935.
8. Zargari A. Medicinal Plants. Tehran: Tehran University Press; 1989. Vol. II.
9. Ave-Sina A. Law in Medicine. Tehran: Soroosh Press; 1988.
10. Alam MM, Siddiqui MB, Husain W. Treatment of diabetes through herbal drug in rural India. Fitoterapia 1990; 61:240–242.
11. Prakash V. Diabetes management-Some useful herbs. Shishir: Jeevaniya; 1992. Vol. 4.
12. Chakrabarti B, Mallick C, Battacharya S. Studies on the green leaves of *Aegle marmelos* and *Piper nigrum* on the glucose and cholesterol levels of blood in diabetes mellitus. Indian Med Forsch 1960; 9:285–286.
13. Karunanyake EH, Welhinda J, Sirimanne SR, Sinnadorai G. Oral hypoglycemic activity in some medicinal plants from Sri Lanka. J Ethnopharmacol 1984; 11:223–231.
14. Kamalakkannan N, Prince PSM. Hypoglycemic effect of water extract of *Aegle marmelos* fruits in streptozotocin diabetic rats. J Ethnopharmacol 2003; 87:207–210.
15. Kamalakkannan N, Prince PSM. Antidiabetic and antioxidant activity of *Aegle marmelos* extract in streptozotocin induced diabetic rats. Pharm Biol 2004; 42:125–130.
16. Ponnachan PTC, Paulose CS, Panikkar KR. Hypoglycemic effect of alkaloid preparation from leaves of *Aegle marmelos*. Amala Res Bull 1993; 13:37–41.
17. Ponnachan PTC, Paulose CS, Panikkar KR. Effect of leaf extract of *Aegle marmelos* in diabetic rats. Indian J Exp Biolo 1993; 31:345–347.
18. Seema PV, Sudha B, Padayatti PS, Abraham A, Raghu KG, Paulose CS. Kinetic studies of purified malate dehydrogenase in liver of streptozotocin-diabetic rats and the effect of leaf extract of *Aegle marmelose* (L) Correa ex Roxb. Indian J Exp Biolo 1996; 34:600–602.

19. Das AV, Padayatti PS, Paulose CS. Effect of leaf extract of *Aegle marmelose* (L) Correa ex Roxb. on histological and ultrastructural changes in tissues of streptozotocin induced diabetic rats. *Indian J Exp Biolo* 1996; 34:341–345.
20. Borek C. Garlic reduces dementia and heart disease risk. *J Nutr* 2006; 136:810–812.
21. Polidori MC, Mecocci P. Plasma susceptibility to free radical-induced antioxidant consumption and lipid peroxidation is increased in very old subjects with Alzheimer disease. *J Alzheimers Dis* 2002; 4:517–522.
22. Kesari AN, Gupta RK, Singh SK, Diwakar S, Watal G. Hypoglycemic and antihyperglycemic activity of *Aegle marmelos* seed extract in normal and diabetic rats. *J Ethnopharmacol* 2006; 107: 374–379.
23. Sabu MC, Kuttan R. Antidiabetic activity of *Aegle marmelos* and its relationship with its antioxidant properties. *Indian J Physiol Pharmacol* 2004; 48:81-88.
24. Upadhyaya S, Shanbhag KK, Suneetha G, Balachandra Naidu M, Upadhyaya S. A study of hypoglycemic and antioxidant activity of *Aegle marmelos* in alloxan induced diabetic rats. *Indian J Physiol Pharmacol* 2004; 48:476-480.
25. Kamalakkannan N, Stanely Mainzen Prince P. Effect of *Aegle marmelos* Correa. (Bael) fruit extract on tissue antioxidants in streptozotocin diabetic rats. *Indian J Exp Biol* 2003; 41:1285-1288.
26. Brosky G, Logothelopoulos J. Streptozotocin diabetes in the mouse and guinea pig. *Diabetes* 1969; 18:606–609.
27. Gupta RK, Kesari AN, Murthy PS, Chandra R, Tandon V, Watal G. Hypoglycemic and antidiabetic effect of ethanolic extract of leaves of *annona squamosa* L. in experimental animals. *J Ethnopharmacol* 2005; 99:75–81.
28. Triender P. Determination of glucose in blood using glucose oxidase with an alternative oxygen acceptor. *Ann Clin Biochem* 1969; 6:24.
29. Farshchi A, Ghiasi G, Farshchi S, Malek Khatabi P. Effects of *Boswellia papyrifera* gum extract on learning and memory in mice and rats. *Iran J Basic Med Sci* 2010; 13:9-15.
30. Biessels GJ, Kamal A, Ramakers GM, Urban IJ, Spruijt BM, Erkelens DW, *et al*. Place learning and hippocampal synaptic plasticity in streptozotocin-induced diabetic rats. *Diabetes* 1996; 45: 1259–1266.
31. Biessels GJ, Kamal A, Urban IJ, Spruijt BM, Erkelens DW, Gispen WH. Water maze learning and hippocampal synaptic plasticity in streptozotocin-diabetic rats: effects of insulin treatment. *Brain Res* 1998; 800:125–135.
32. Flood JF, Mooradian AD, Morley JE. Characteristics of learning and memory in streptozotocin-induced diabetic mice. *Diabetes* 1990; 39:1391–1398.
33. Jick H, Zornberg GL, Jick SS, Seshadri S, Drachman DA. Statins and the risk of dementia. *Lancet* 2000; 356:1627–1631.
34. Parale GP, Baheti NN, Kulkarni PM, Panchal NV. Effects of atorvastatin on higher functions. *Eur J Clin Pharmacol* 2006; 62:259–265.
35. Raja SG, Dreyfus GD. Statins: much more than just a lipid lowering therapy. *Indian Heart J* 2004; 56:204–209.
36. Masse I, Bordet R, Deplanque D, Al Khedr A, Richard F, Libersa C, *et al*. Lipid lowering agents are associated with a slower cognitive decline in Alzheimer's disease. *J Neurol Neurosurg Psychiatry* 2005; 76:1624–1629.
37. Lannert H, Hoyer S. Intracerebroventricular administration of streptozotocin causes long-term diminutions in learning and memory abilities and in cerebral energy metabolism in adult rats. *Behav Neurosci* 1998; 112:1199-1208.
38. Chander R, Khanna AK, Pratap R. Antioxidant activity of guggulsterone, the active principle of guggulipid from *Commiphora mukul*. *J Med Aromat Plant Sci* 2002; 24:370–374.