ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Neuroprotective Effect of *Moringa oleifera* in Scopolamine Induced Cognitive Impairment and Oxidative Stress in *Wistar Albino* Rats.

Rahmath A*, Nisha Rajan, Ahamed Shahal M, Seena TP and Sreekumaran E.

Department of Life Sciences, University of Calicut, Kerala, India.

ABSTRACT

The present study was to evaluate the anti - dementic effect of *Moringa oleifera* on scopolamine induced male *Wistar albino* rats. Animals were grouped into normal control, scopolamine control, moringa control, moringa + scopolamine, donepezil control, donepezil + scopolamine. Dementia was induced in rats by administration of scopolamine (1 mg/kg intraperitoneally). Aqueous extract of *Moringa oleifera* was fed to the rats at a dosage of 2500 mg per kilogram body weight of the animal for a period of 15 days. Donepezil was used as a standared nootropic agent. Learning and memory parameters were evaluated using the classical T-maze test. Biochemical estimations of acetycholinesterase, malondialdehyde, glutathione-S-transferase and superoxidedismutase were performed to estimate the level of cholinergic status, lipid peroxidation and degree of oxidative stress. It was observed that aqueous extract of *Moringa oleifera* had a significant role in the management of dementia. There was a significant difference between the control and moringa treated group in this study.

Keywords: *Moringa oleifera*, *Wistar albino*, Scopolamine, Donepezil and T-maze.

Corresponding author

ISSN: 0975-8585

INTRODUCTION

Learning is the process by which we acquire knowledge about the world and memory is the process by which that knowledge is encoded, stored and later retrieved [1]. Learning is classified into two - long term learning and short term learning. Accordingly, long term memories are those that we can recall days, months or years after they were originally stored. Short term memory- memories are those that last seconds to hours and are vulnerable to disruption [2].

Dementia refers to a syndrome that is characterized by progressive deterioration of cognitive functions. Neuropsychiatric symptoms such as apathy, agitation and depression are also common. Many diseases can cause dementia syndrome (hereafter called *dementia*). Alzheimer disease and cerebrovascular ischemia (vascular dementia) are the two most common causes and some cases of dementia involve both of these disorders. Although some potentially reversible conditions, such as hypothyroidism or vitamin B_{12} deficiency are often thought to cause dementia, no more than 1.5 % of cases of mild to moderate dementia are fully reversible [3].

In the last few decades there has been an exponential growth in the field of herbal medicine. It is getting popularized in developing and developed countries owing to its natural origin and lesser side effects. *Moringa oleifera*, an important medicinal plant is one of the most widely cultivated species of the family moringaceae. It is highly valued from time immemorial of its vast medicinal properties. *Moringa oleifera* (MO) is a small, fast-growing evergreen or deciduous tree that usually grows as high as 9 m, with a soft and white wood and corky and gummy bark. Roots have the taste of horse radish. Leaves are longitudinally cracked leaves, 30-75 cm long main axis and its branch jointed, glandular at joints, leaflets are glabrous and entire. The leaflets are finely hairy, green and almost hairless on the upper surface, paler and hairless beneath, with red tinged mid-veins, with entire (not toothed) margins and are rounded or blunt-pointed at the apex and short-pointed at the base. The twigs are finely hairy and green, flowers are white, scented in large axillary down panicles, pods are pendulous, ribbed and seeds are 3-angled [4, 5].

MO is a rich source of antioxidants [6]. It has been reported that aqueous extracts of leaf, fruit and seed of MO act as an antioxidant [7]. During a study reporting antioxidant property of freeze dried *Moringa* leaves from different extraction procedures, it was found that methanol and ethanol extracts of Indian origin MO have the highest antioxidant activity with 65.1 and 66.8 %, respectively [8, 9]. It was also reported that the major bioactive compounds of phenolics, such as quercetin and kaempferol are responsible for antioxidant activity [9, 10]. In another study, quercetin and kaempferol have shown good antioxidant activity on hepatocyte growth factor (HGF) induced Met phosphorylation with IC 50 value for 12 and ~6 µ M/L, respectively [11]. The oil from dried seed showed higher antioxidant activity than butylated hydroxyl toluene and alpha tocopherol [12].

Scopolamine is an anticholinergic drug, which produces dementia by decreasing the level of acetylcholine, which is considered to be an important neurotransmitter for learning and memory [13,14]. Therefore, the present study was aimed to investigate the anti-dementic effect of *Moringa oleifera* extract on scopolamine induced memory impairment and oxidative stress.

MATERIALS AND METHODS

Adult male *Wistar albino* rats weighing around 180 $\pm$ 20 grams were used for the study. The animals were housed in poly propylene cages (22.5 X 35.5 X 15 cm) and controlled temperature (25 $\pm$ 2^{o}C), humidity (50-55 %) and light (12 hr light and dark cycle) environment with food and water *ad libitum*. The experimental protocol was approved by the Institutional Animal Ethical Committee and experiments were conducted according to the CPCSEA guidelines on the use and care of experimental animals.

Moringa oleifera was collected from the surroundings of the Dept. of Life Sciences, University of Calicut, Kerala. The taxonomic identity of the plant was confirmed by comparing collected voucher specimen with those of known identity which are located in the herbarium (Acc. No. 88440) of the Dept. of Botany, University of Calicut, Kerala with the help of a pteridophyte taxonomist.

Fresh extract was made by grinding the leaves using a mortar and pestle, with a known amount of water. This was fed to the rats by orogastric feeding tube at a dosage of 2500 mg of *Moringa oleifera* per kilogram body weight of the animal for 15 days.

Scopolamine was procured from Kemwell Biopharma, Bengaluru, India. It was administered to the rats as intraperitoneal injection at a dose of 1 mg / kg body weight for 15 days, 30 minutes prior to behavioral experiments. Donepezil was obtained from Alkem laboratories, Mumbai, India and it was administered to the rat through orogastric feeding at a dose of 3 mg/kg body weight for 15 days and two hours prior to behavioral experiments. The other chemical reagents used in the study were obtained from HiMedia, Mumbai, India.

The experimental animals were divided in to six groups and each group consisted of seven animals. The different groups are

Group I	:	Normal control.
Group II	:	Scopolamine control.
Group III	:	Moringa control.
Group IV	:	Moringa + scopolamine.
Group V	:	Donepezil control.
Group VI	:	Donepezil + scopolamine.

Design of the behavioral experiments

T-MAZE: T-maze is used largely in preference and spatial learning tasks [15]. Animals learn to alternate between arms based on their memory of the previously visited arms or choose an arm based on the reward presented. The T-maze test was carried out on the 1^{st}, 8^{th} and 15^{th} day of treatment. The whole experiment was performed in a silent dark room under a dim red light and after 9.00 pm. Before performing this experiment, the animals were left without food for 24 hours with only water to drink. This makes the animal more active to find food during the experiment. The wooden T-maze apparatus consisted of a stem (35 X 12 cm), a choice area (15 X 12 cm), which is one of the two arms. The animal was left at the tail end. The presentation of the food in one of the arms is in such a way that it is concealed from the sight of the rat. The animal has to move forward and turn left or right of the T-maze. The move was considered to be a positive response if the rat reaches near the sealed food and if not, noted as a negative response. A total of 10 trials were given in each set with a gap of five minutes per set. The trials were continues till a set gets 90 % of positive response. The values obtained as percentage of positive response in different animal groups were statistically analyzed for significance using ANOVA followed by post-hoc test using SPSS version 16.

Biochemical Estimation

Preparation of brain homogenate

On day 15^{th} following the behavioral testing, animals were sacrificed and the brain tissues were quickly removed, cleaned in ice cold saline. After washing, the tissue samples were homogenized with 10 times (W/V) 0.1M phosphate buffer (pH 7.4). Aliquots of homogenates from the rat brains were separated and used to measure MDA. The remaining homogenates were centrifuged using Sigma Aldrich cooling centrifuge at 5400 rpm for 10 minutes at 4^{o}c twice to separate the supernatant. The supernatant was used for the quantification of acetyl choline, SOD and GST. A portion of the supernatant was mixed with 10 % TCA, shaken well and centrifuged at 4000 rpm for 10 minutes to estimate total protein.

Estimation of AChE

The cholinergic marker, acetylcholine esterase was estimated in the whole brain according to Ellman's method [16]. Briefly, 0.4 ml aliquot of the homogenate was added to a cuvette containing 2.6 ml phosphate buffer (0.1 ml pH 8) and 100 µl of DTNB. The contents of the cuvette were mixed thoroughly by bubbling air and the absorbance was measured at 412 nm in a spectrophotometer. When the absorbance reached a stable value, it was recorded as the basal reading. Following 20 µl of the substrate (acetylthiocholine iodide) was added and the change in absorbance was recorded for a period of 10 minutes at an interval of 2 minutes. Change in absorbance per minute was thus determined.

ISSN: 0975-8585

Estimation of MDA

About 1 ml of the homogenate was combined with 2 ml of the TCA-TBA-HCl reagent and mixed thoroughly. The solution was heated for 15 minutes in a boiling water bath. After cooling the flocculent precipitate was removed by centrifugation at 3000 rpm for 10 minutes. The absorbance of the sample was read at 535 nm against blank that contained no tissue homogenate [17].

Estimation of SOD

The reaction mixture contained 1.3 ml of 50 mM sodium carbonate solution with 0.1 mM EDTA (pH 10.0), 0.5 ml of 96 µM of NBT and 0.1 ml of 0.6 % triton-X-100. Reaction was initiated by the addition of 0.1 ml of 20 mM hydroxylamine hydrochloride (pH 6.0) to the reaction mixture and the rate of nitroblue tetrazolium reduction in the absence of the enzyme source was recorded for about 30 seconds. Following this, small aliquots of supernatant were added to the test cuvette as well as reference cuvette, which did not contain hydroxylamine hydrochloride. Finally, percentage inhibition in the rate of NBT reduction was noted and one unit of enzyme was expressed as inverse of the amount of protein (mg) required for inhibiting the reduction rate of NBT by 50 % [18].

Estimation of GST

To 2.75 ml 0.1 M phosphate buffer (pH 6.5) 0.1 ml of 1 mM 2,4-dinitrochloro benzene (CDNB freshly prepared) was added. After this, 0.1 ml of 1 mM glutathione (GSH-freshly prepared) and 50 µl samples were added to make up the volume to approximately 3 ml. Glutathione conjugate of CDNB absorbs maximum at 340 nm, using Jasco V double beam spectrophotometer and have the extinction coefficient of 9.6 Mm^{-1} cm^{-1}. Absorbance was noted after every one minute for a total duration of five minutes. The blanks in all the cases constituted non-enzymatic reaction of GSH with the respective substrate [19].

RESULTS AND DISCUSSION

Table 1: T-maze performance on dementia induced *Wistar albino* rats in different groups (n=7).

Sl.no	Groups	Mean ± SD		
		1st day	8th day	15th day
1.	Group I (Normal control)	73.14 ± 4.91	77.71 ± 2.92	77.42 ± 3.50
2.	Group II (Scopolamine)	37.43 ± 4.96	29.00 ± 4.12	21.71 ± 4.54
3.	Group III (Moringa control)	74.57 ± 3.36	78.85 ± 3.34	80.57 ± 2.88
4.	Group IV (Moringa + scopolamine)	71.43 ± 5.16	73.00 ± 3.11	75.57 ± 4.06
5.	Group V (Donepezil control)	77.28 ± 1.70	79.86 ± 3.76	85.57 ± 3.15
6.	Group VI (Donepezil + scopolamine)	71.71 ± 2.43	74.00 ± 3.41	77.71 ± 3,54

Table 1 shows the comparison of the performance in T- maze in percentage of different groups on 1st, 8th and 15th day. From the analysis the scopolamine treated group showed a decline in memory performance from 1st day to 15th day. The moringa treated and standard drug donepezil treated group showed significant increase in performance level, which is equal to the control group and it is better than scopolamine treated group.

ISSN: 0975-8585

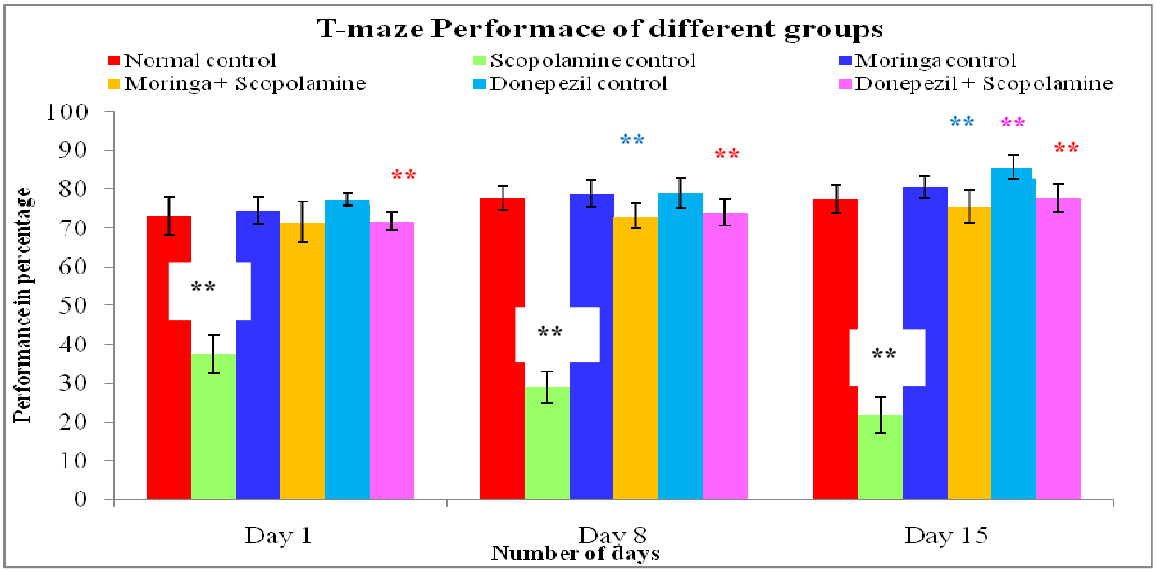

Figure 1: T-maze performance on dementia induced *Wistar albino* rats in different groups (mean ± SD, n=7).

* Comparison between Normal control & scopolamine
* Comparison between Moringa control and Moringa + scopolamine
***Comparison between normal control with donepezil control**
* Comparison between Donepezil control & Donepezil + scopolamine
** The difference is significant at 1 % level.

Table 2: Brain AChE, SOD, GST and MDA in dementia induced *Wistar albino* rats in different groups (n=6).

Sl.no	Groups	Mean ± SD			
		AChE	SOD	GST	MDA
1.	Group I (Normal control)	126 ± 41.59	103 ± 10.23	17.59 ± 4.24	0.3847 ± 0.061
2.	Group II (Scopolamine)	196 ± 44.12	88.9 ± 13.4	11.53 ± 3.94	0.04735 ± 0.068
3.	Group III (Moringa control)	114 ± 22.18	127 ± 25.96	19.74 ± 2.16	0.3526 ± 0.039
4.	Group IV (Moringa + Scopolamine)	139 ± 26.78	110 ± 24.83	18.93 ± 1.75	0.3708 ± 0.0522
5.	Group V (Donepezil control)	88.4 ± 18.24	161 ± 5.008	22.06 ± 1.32	0.2584 ± 0.026
6.	Group VI (Donepezil + Scopolamine)	125 + 40.97	122 ± 25.53	18.98 ± 3.78	0.3704 ± 0.0708

Table 2 shows the comparison of brain AChE, SOD, GST and MDA level. Scopolamine treated rats showed an increase in AChE and MDA levels and a decrease in SOD and GST levels. It indicates that *Moringa oleifera* treatment significantly decreased brain AChE activity and MDA level compared to their correrponding scopolamine treated groups. However, standard drug and *Moringa oleifera* extract treatment significantly increased SOD and GST level in brain compared to their corresponding scopolamine treated groups.

ISSN: 0975-8585

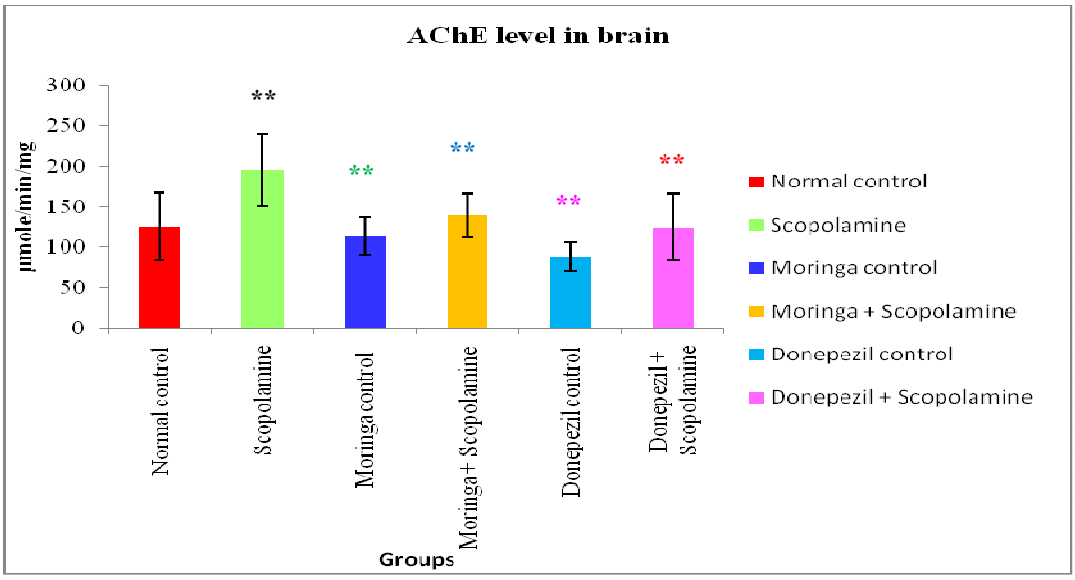

Figure 2: AChE level in brain in dementia induced *Wistar albino* rats in different groups (mean ± SD, n=6).

* Comparison between Normal control & scopolamine
* **Comparison between normal control and Moringa control**
***Comparison between normal control with donepezil control**
* Comparison between Moringa control and Moringa + scopolamine
* Comparison between Donepezil control & Donepezil + scopolamine

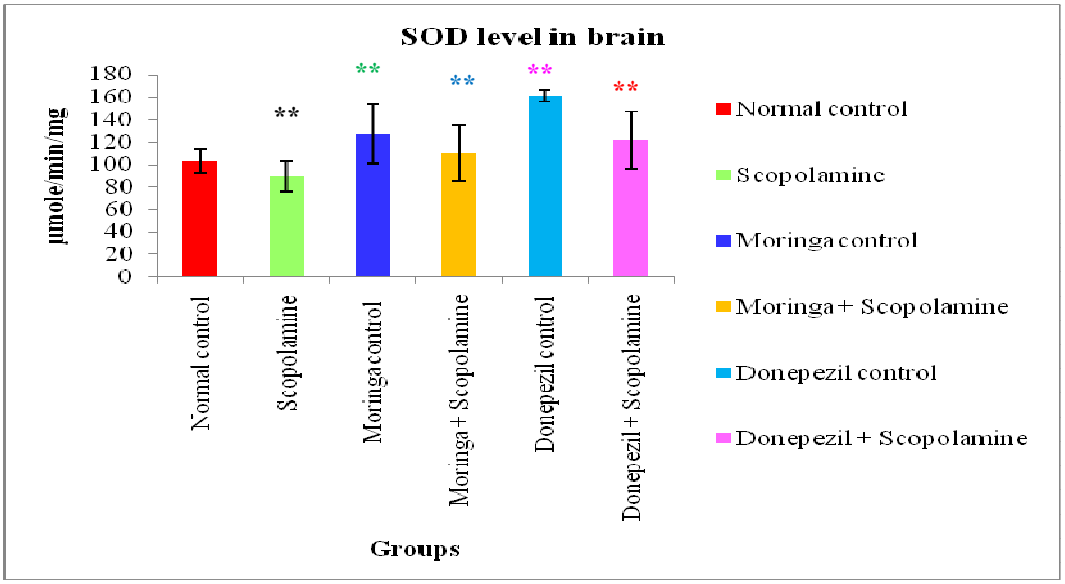

Figure 3: SOD level in brain in dementia induced *Wistar albino* rats in different groups (mean ± SD, n=6).

* Comparison between normal control & scopolamine
* **Comparison between normal control and Moringa control**
***Comparison between normal control with donepezil control**
* Comparison between Moringa control and Moringa + scopolamine
* Comparison between Donepezil control & Donepezil + scopolamine
** The difference is significant at 1 % level.

ISSN: 0975-8585

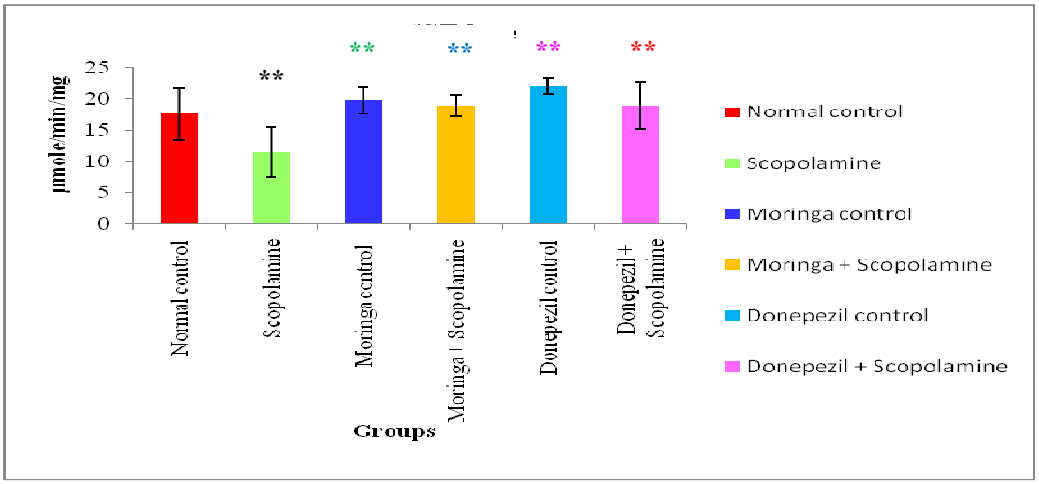

Figure 4: GST level in brain in dementia induced *Wistar albino* rats (mean ± SD, n=6).

* Comparison between Normal control & scopolamine
*** Comparison between normal control and Moringa control**
***Comparison between normal control with donepezil control**
* Comparison between Moringa control and Moringa + scopolamine
* Comparison between Donepezil control & Donepezil + scopolamine
** The difference is significant at 1 % level.

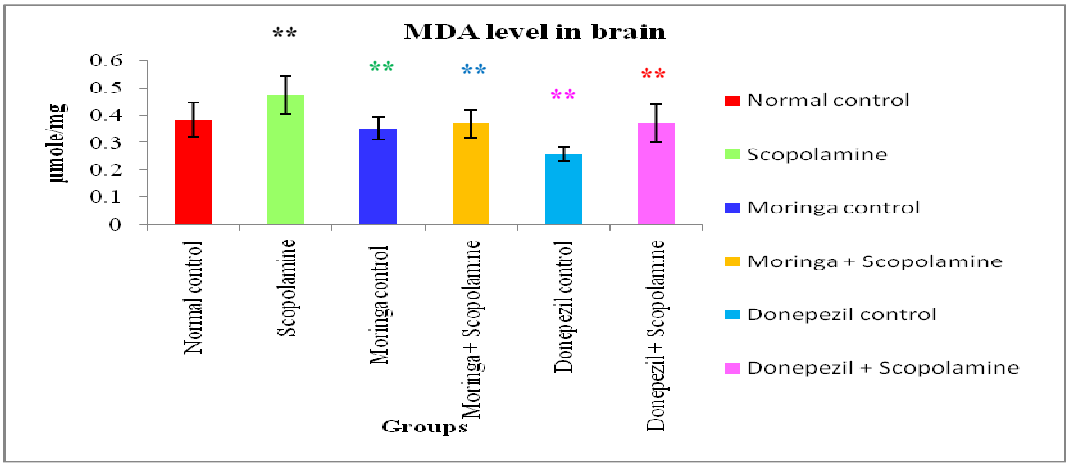

Figure 5: MDA level in brain in dementia induced *Wistar albino* rats in different groups (mean ± SD, n=6).

* Comparison between normal control & scopolamine
*** Comparison between normal control and Moringa control**
***Comparison between normal control with donepezil control**
* Comparison between Moringa control and Moringa + scopolamine
* Comparison between Donepezil control & Donepezil + scopolamine
** The difference is significant at 1 % level.

In this study, the effect of *Moringa oleifera* and the modern medicine donepezil were compared in scopolamine induced dementia in rats. This study also focused on different parameters like memory test and enzyme activity related with oxidative stress including estimation of SOD, GST and lipid peroxidation and acetylcholinesterase.

Moringa oleifera has an impressive range of medicinal uses with high nutritional value and medicinal benefits. Different parts of Moringa contain a profile of important minerals and are a good source of protein, vitamins, beta-carotene, amino acids and various phenolics. Moringa provides a rich and rare combination of zeatin, quercetin, beta-sitosterol, caffeoylquinic acid and kaempferol [20]. Nowadays donepezil is widely

accepted as a modern drug for dementia. This study compares the effect of *Moringa oleifera* and modern medicine donepezil in scopolamine induced dementia in rats.

Table 1 shows the comparison of the performance in percentage in T maze in percentage of different group of animals. In case of control rat fed with *Moringa oleifera* there was an increase in the performance from 1st to 15th day (Figure 1). Scopolamine induced group showed a decline in memory performance. When the scopolamine induced group treated with moringa was studied, there was a drastic increase in memory. It indicates that the leaves of *Moringa oleifera* possess nootropic effect in view of its facilitatory effect on retention and acquired learning. In case of normal rats fed with donepezil, it showed an increase in memory performance than moringa fed group. Donepezil improved learning and memory in multiple sclerosis patients with initial cognitive difficulties in a single-center clinical trial [21, 22].

This study also focused on different parameters like enzyme activity related with oxidative stress including estimation of SOD, GST and lipid peroxidation and AChE. The results mentioned in table 2 were compared in each of the groups. Figure 2 represents the level of AChE in different groups of animals. In scopolamine induced group, a drastic elevation in AChE level was found. Scopolamine is a muscarinic receptor antagonist that blocks cholinergic neurotransmission, leading to memory impairment in rats. Recent studies have reported that scopolamine is a muscarinic cholinergic receptor antagonist which causes cholinergic dysfunction, impaired cognition and oxidative stress in rats [23, 24]. When the scopolamine induced group was treated with moringa, it decreased the AChE level significantly. MO may have a protective action on neurons in the cortical and hippocampal regions and thus may support memory process the possible mechanism might occur partly *via* the decreased oxidative stress and the enhanced cholinergic function [25, 26]. In case of moringa treated group, there was a decline in AChE level than the control group which suggests that the aqueous extract of *Moringa oleifera* produces an acetylcholinesterase inhibiting activity. Retention of acetylcholine in the brain, the essential part for cognitive functions, learning and memory is greatly increased by this activity [27].

Release of oxygen radicals in brain as a result of oxidative stress, greatly enhances lipid peroxidation as these free radicals act on polyunsaturated fatty acids in brain, thereby proliferate the process of oxidative degradation of lipids [28]. The major oxidative free radical scavenging enzymes like SOD and GST plays an important role to reduce oxidative stress in brain. In the present study, rats after moringa treatment showed a significant increase in the SOD and GST level in the brain (Figure 3 & 4). In case of scopolamine induced group, the SOD level was highly reduced because of oxidative stress. It has been reported that aqueous extracts of leaf, fruit and seed of MO act as an antioxidant [29, 30, 31]. *Moringa oleifera* significantly prevented the reduction of SOD and GST activity in brain during scopolamine treatment.

Malondialdehyde is one of the final products of lipid peroxidation in the cells. An increase in free radicals causes over production of MDA. High MDA level is a marker of lipid peroxidation and oxidative stress [32]. Rats after scopolamine treatment significantly increased in the brain MDA level (Figure 5), which is the measure for lipid peroxidation. The brain is more vulnerable to oxidative stress due to its high oxygen expenditure and high levels of polyunsaturated fatty acids [33, 34]. The level of MDA is increased in scopolamine induced group but the rise in the MDA level was controlled by *Moringa oleifera* treatment. The results suggest that *Moringa oleifera* reduces oxidative stress by inhibiting free radical generation, proliferating endogenous antioxidant enzymes and decreasing acetylcholine esterase activity in the brain.

CONCLUSION

In the present study, it was observed that the *Moringa oleifera* improved memory of young *Wistar albino* rats probably by lowering MDA, increased anti oxidant enzymes and inhibiting AChE enzyme. We recommend further detailed study to explore therapeutic potential of *Moringa oleifera* in the management of dementia.

ACKNOWLEDGEMENT

The authors gratefully acknowledge the financial and other support provided by the University Grants Commission, New Delhi. We extend our gratitude to the teaching and non teaching staff of the Department of Life Sciences, University of Calicut, Thenhippalam, Malappuram.

REFERENCES

[1] Kandel ER, Schwartz JH, Jessell TM, Siegelbaum S, Hudspeth AJ. Principles of Neural Science. Elsevier, 2012; pp. 433-443.

[2] Bear MF, Connors BW, Paradiso MA. Neuroscience : exploring the brain. Lippincott Williams & Wilkins, Baltimore, MD, 2011; pp.15-22.

[3] Boustani M, Peterson BH, Harris R, Krasnov C. Screening for Dementia. Rockville , MD, 2002; pp. 1-164.

[4] Roloff A, Weisgerber H, Lang UB. Enzyklopädie der Holzgewächse- Handbuch und Atlas der Dendrologie. Wiley, 2009; pp. 1-8.

[5] Gupta RK. Medicinal & Aromatic Plants. CBS publishers & distributors, 2010; pp. 151-152.

[6] Chumark P, Khunawat P, Sanvarinda Y, Phornchirasilp S, Morales NP, Phivthong-ngam L, Ratanachamnong P, Srisawat S, Pongrapeeporn KS. Journal of Ethnopharmacology 2008; 116: 439-446.

[7] Singh BN, Singh BR, Singh RL, Prakash D, Dhakarey R, Upadhyay G, Singh HB. Food and Chemical Toxicology 2009; 47 (6): 1109-1116.

[8] Lalas S, Tsaknis J. Journal of Food Composition and Analysis 2002; 15 (1): 65-77.

[9] Siddhuraju P, Becker K. Journal of Agricultural and Food Chemistry 2003; 51 (8): 2144-2155.

[10] Bajpai M, Mishra A, Prakash D. International Journal of Food Sciences and Nutrition 2005; 56 (7): 473-481.

[11] Labbe D, Provencal M, Lamy S, Boivin D, Gingras DRB. Journal of Nutrition 2009; 139 (4): 646-652.

[12] Mohammed AH, Naglaa AG, Mahitab HEB. International Journal of Pharma Sciences 2014; 4 (3): 532-430.

[13] Abhinav K, Jogender M, Madhusudana K, Vegi GMN, Yogendra KG, Sistla R. Pharmacology & Pharmacy 2010; 1: 1-8.

[14] Kumar SS , Jissa G, Setty PV, Mukkadan JK. Journal of Universal College of Medical Sciences 2013; 1(3): 46-52.

[15] Aswathy Gopinath, Archana R, Kumar Sai sailesh, Mukkadan J K. Effect of caloric vestibular stimulation on memory. Int J Pharm Bio Sci 2015 July; 6(3): (B) 453 – 459.

[16] Ellman GL, Courtney KD, Andres V, Feather-Stone RM. Biochemical pharmacology 1961; 7 (2): 88-95.

[17] Niehaus WG, Samuelsson B. European Journal of Biochemistry 1968; 6 (1) ; 126-130.

[18] Kono Y. Archives of Biochemistry and Biophysics 1978; 186 (1): 189-195.

[19] William HH, Michael JP, William BJ. Journal of Biological Chemistry 1974; 249 (22): 7130-7139.

[20] Fozia F, Meenu R, Avinash T, Abdul AK, Farooq S. Journal of Medicinal Plants Research 2012; 6 (27): 4368-4374.

[21] Christodoulou C, Melville P, Scherl WF, Macallister WS, Elkins LE, Krupp LB. Journal of the Neurological Sciences 2006; 245 (1): 127-136.

[22] Puchchakayala G, Akina S, Thati M. International Journal of Alzhiemr's Disease 2012; 2012: 1-8.

[23] Oh JH, Choi BJ, Chang MS, Park SK. Neuroscience Letters 2009; 461 (1): 41-44.

[24] Zaki HF, Abdel FMA, Attia AS. Bulletin of Faculty of Pharmacy 2014; 52 (1): 15-25.

[25] Ganguly R, Guha D. Indian Journal of Medical Research 2008; 128: 744-751.

[26] Chatchada S, Jintanaporn Wm Supaporn M and Wipawee T. Oxidative Medicine and Cellular Longetivity 2013; 2013: 1-9.

[27] Edward DL and Barbara BS. Psychopharmacology 1998; 138: 217-230.

[28] Coyle T and Puttfarcken P. Science 1993; 262 (5134): 689-695.

[29] Abdul RAF, Ibrahim MD and Kntayya SB. Asian Pacific Journal of Cancer Prevention 2014; 15 (20): 8571-8576.

[30] Kumbhare MR, Guleha V and Sivakumar T. Asian Pacific Journal of Tropical Disease 2012; 144-150.

[31] Shahid I and Bhanger MI. Journal opf Food Composition and Analysis 2006; 544-551.

[32] Debasree D, Veena N, Bairy KL, Mohandas RKG, Jeevan S, Mangala VH, Salini SK, Research Journal of Pharmaceutical, Biological and Chemical Sciences 2013; 4 (1): 1174-1182.

[33] Floyd RA and Carney JM. Annals of Neurology 1992; 32: S22-27.

[34] Aparecida VA, Monique M, Florentino D, Zapelini ND, Tezza RG, Dimer LD, Jeremias JF et al. Current neurovascular research 2015; 12 (2): 147-154.