

NEUROPROTECTIVE EFFECT OF EPALRESTAT ON MEMORY IMPAIRMENT IN STREPTOZOTOCIN-INDUCED TYPE-2 DIABETIC RATS USING DIFFERENT BEHAVIORAL MODELS

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Received: 25 October 2017, Revised and Accepted: 23 November 2017

ABSTRACT

Objective: The present study was designed to evaluate the protective effects of epalrestat (EPS) on memory and learning in type-2 diabetes.

Methods: Sixty percent high-fat diet for 2 weeks and a single dose of streptozotocin (35 mg/kg, ip) was used to induce memory impairment in rats. Once the diabetes is confirmed, test drug (EPS - 13.5, 27, and 54 mg/kg, oral) and donepezil (1 mg/kg, oral) were administered to different groups of rats for 4 weeks followed by an assessment of memory and learning deficit using behavioral paradigms: Elevated plus maze (EPM), Morris water maze (MWM), and passive avoidance test.

Results: EPS and donepezil showed significant improvement in learning and memory of rats, as indicated by markedly decreased escape latency to reach a hidden platform and increased time spent in target quadrant using MWM task, reduced transfer latency in EPM, and also there is a significant increase in the transfer latencies using passive avoidance test were noted. Memory-enhancing activity of EPS (13.5, 27, and 54 mg/kg) was comparable with the diabetic control group.

Conclusion: The study findings suggest that memory-enhancing effect of EPS may be mediated by its antioxidant and anti-inflammatory activities. This recommends the potential effect of EPS therapy as a useful memory restorative agent in the treatment of neurodegenerative disease seen in type-2 diabetes rat.

Keywords: Type-2 diabetes, Epalrestat, Donepezil, Learning and memory, Morris water maze, Passive avoidance, Elevated plus maze, High-fat diet.

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INTRODUCTION

Diabetes mellitus (DM) is the most common endocrine disorder characterized by increased blood glucose levels, resulting from defective insulin secretion, resistance to insulin action, or both. DM is often associated with severe complications, and there is an increasing appreciation that cognitive function declines in DM [1-3]. Chronic hyperglycemia is associated with disturbance of the blood sugar level which leads to nerve cells damage in the brain causing cognitive impairment [4].

Animal studies have shown that high-calorie diets impair the structure and function of the hippocampus, a brain region critical for learning and memory [5,6]. In animal models, both type-1 and type-2 diabetic animals are reported to induce severe memory deficits [7,8]. The increased oxidative stress in diabetes produces oxidative damage in many regions of rat brain including hippocampus.

Oxidative stress and harmful free radicals play an important role in the development of memory impairment. Free radicals and reactive oxygen substances generated by living cells as a result of physiological and biochemical processes and accumulation of these free radicals in the body can cause oxidative damage to lipids, proteins, and DNA, which leads to diabetes and neurodegenerative diseases [9,10].

Epalrestat (EPS) (5-[(1Z,2E)-2-methyl-3-phenyl propenylidene]-4-oxo-2-thioxo-3-thiazolidine acetic acid; EPS) is an inhibitor of aldose reductase, well proven to have beneficial effects for the treatment of diabetic neuropathy and is easily absorbed by neural tissue and inhibits

aldose reductase with minimum adverse effects [11]. It is reported for anti-inflammatory and antioxidant effects using rat Schwann cell and human neuroblastoma cell line [12-14]. Therefore, the anti-inflammatory and anti-oxidant properties of EPS can be beneficial in the management of neurodegenerative disease by improving the memory and learning in an animal model. However, there are no study reports regarding the effect of EPS such as compound on memory and learning in diabetes. In the present study, the effect of EPS was evaluated experimentally with regard to learning and memory in rats.

METHODS

Animals

Male Wistar rats (150–200 g) were procured from the central animal house facility of Jawaharlal Nehru Medical College, Belagavi. The rats were acclimatized to 12:12 h light-dark cycle for 7 days, before an animal study. They were maintained at constant room temperature (22°C–25°C) and on standard chow pellet (Amrut Brand) with water *ad libitum*. The animals were housed in polypropylene cages with 3 animals per cage. The study was approved by the Institutional Animal Ethics Committee constituted as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (Resolution No.: 7/D; dated 18.05.2016).

Drugs and solutions

EPS (Micro Labs Ltd., India), streptozotocin (STZ) (Enzo Life Sciences, UK), donepezil hydrochloride (Alkem Laboratories Ltd., India), and Glucometer/strips (Accu Chek: Roche Diagnostics India Pvt., Ltd., Mumbai) were used. EPS was suspended in 1% gum acacia, whereas,

STZ and donepezil hydrochloride were dissolved in citrate buffer (pH 4.4) and distilled water, respectively. Drug solutions/suspensions were prepared fresh before administration. EPS at doses of 57, 27, and 13.5 mg/kg, p.o. and donepezil hydrochloride 1 mg/kg, p.o. were selected on the basis of previous literature and as per human dose [11,15,16-18]. The clinically equivalent human doses of drugs were converted into rat equivalent doses by the conversion table devised by Paget and Barnes [19].

Experimental induction of diabetes

Type-2 diabetes was induced in rats by previously described methods [20,21]. The rats were fed with a high-fat diet (HFD) (VRK Nutritional Solutions, Pune, India) for the initial period of 2 weeks followed by low dose of STZ (35 mg/kg; i.p) was administered. All diabetic rats were given 5% glucose solution to prevent from hypoglycemic shock, whereas, normal control (NC) rats were administered with vehicle (1 ml/kg; p.o.). The fasting blood glucose was measured 48 h after STZ injection. The rats injected with STZ showing fasting blood glucose > 200 mg/kg noted as diabetic and used for further study. The HFD composition is given in Table 1.

Treatment schedule

The confirmation day of diabetes was taken as day 1 of diabetic condition. A total of 60 rats (10 normal; 50 HFD-STZ induced diabetic rats) were used and divided into six groups (n=10) and received the following treatment:

- Group I: NC received known volume of vehicle (1 ml/kg, p.o)
- Group II: Diabetic control (DC) - vehicle only (1 ml/kg, p.o)
- Group III: Diabetic rats + Donepezil hydrochloride (1 mg/kg, p.o)
- Group IV: Diabetic rats + EPS (57 mg/kg, p.o)
- Group V: Diabetic rats + EPS (27 mg/kg, p.o)
- Group VI: Diabetic rats + EPS (13.5 mg/kg, p.o).

All the drugs were administered orally for 4 weeks. Behavioral tests were carried out at the end of the treatment.

Assessment of cognitive function [7,22,23]

Elevated plus maze (EPM)

The test was used to evaluate spatial long-term memory. The maze has two open and two enclosed arm (OA and EA), and it was elevated 25 cm above the ground. The behavioral tests were conducted in a quiet room illuminated by a dim light.

Learning protocol

EA divided into two equal parts. Each individual rat was kept at the end of the OA on day 1 and initial transfer latency (TL) was recorded. To become habituated with the maze, the animals were allowed to explore the plus maze for 90 s after reaching the EA. On the 2nd day, 24 h after the first exposure, TL was again noted. A long latency period to reach EA indicates poor retention compared with significantly shorter latencies

Passive avoidance test (step through test)

The passive avoidance apparatus have two chambers, light and dark compartment with grid floor 50 × 50 cm and 35 cm high walls, separated

by a wall with a guillotine door 6 × 6 cm. One of the two chambers was illuminated with 100 V bulb placed at 150 cm height, and the other was dark. The test was conducted on 3 consecutive days at the same time of the day. On day 1 (trial 1) and day 2 (trial 2, 3, and 4), acquisition trial was conducted, and individual rat was kept in illuminated chamber of the apparatus. At the end of the 3rd trial as soon as rat entered the dark compartment, it received an electric shock on the feet (50 V, 50 Hz, 1 s) through the stainless steel grid floor. The time when rat entered in the dark chamber was noted as step-through latency (STL). Retention was tested after 24 h, and STL was recorded. Cutoff time allotted was 300 s. Increase in the STL was considered as an index of improvement of memory.

Morris water maze test (MWM)/spatial discrimination

During spatial discrimination, the hidden platform was kept at 1.5 cm below the water level changing the area of the pool from that used during cue discrimination training. We added milk to make the pool water opaque, in which platform shown invisible. The platform has fixed in one place. Rats had trained to four consecutive trials each day for 4 consecutive days. Each trial was given 120 s. Rats were allowed to start swimming in each trial from one of the four locations (north, south, east, and west); the choice of the location was random for each rat and each trial. The rat should escape to the platform within 120 s, and if that did not occur, we guided them gently toward the hidden platform where they remained for 10 s. Probe trial conducted on the 5th day in which platform was removed from the swimming pool and allowed the rat to swim freely for 120 s, and time spent in target location was noted as a function of memory.

Statistical analysis

The study results were expressed as mean ± standard error of mean. Data were analyzed using one-way ANOVA followed by Dunnett's multiple comparison test. p < 0.05 was considered as statistically significant.

RESULTS

Blood glucose levels

At the onset of the study, all experimental animals had equivalent blood glucose levels (Table 2). At the conclusion of the experiment, glucose concentrations were highly significantly elevated in donepezil 1mg/kg and EPS-treated animals relative to those in the NC.

Effect of EPS on performance of EPM

The effect of donepezil and EPS treatments in diabetic rats on mean transfer latencies in the EPM test is shown in Fig. 1. Statistical analysis revealed that chronic treatment with donepezil 1 mg/kg and EPS (57, 27 mg/kg) had a significant effect on the transfer latencies on day 1 (day 29) as compared with DC (p < 0.001). On the day 2 (day 30), HFD-STZ induced TL was drastically decreased when compared to the day 1. This clearly indicates the learning behavior of animals on the day 2, whereas there was no difference in TL tested on day 1 and 2 in DC animals. On the other hand, cholinesterase inhibitor, donepezil showed a significant reversal of HFD-STZ induced deficits. However,

Table 2: Blood glucose levels (means ± SEM) in the six groups of rats at the onset and at the end of the experiment

Treatment	Glucose mg/dl	
	Onset of the study	End of the study
NC	96.50 ± 1.48	100.2 ± 3.16
DC	98.11 ± 4.69	456.4 ± 11.44***
DC+Donepezil 1	100.8 ± 4.78	411.6 ± 14.72***
DC+EPS 54	97.20 ± 3.83	396.1 ± 20.85***
DC+EPS 27	100.2 ± 3.99	404.4 ± 17.43***
DC+EPS 13.5	99.89 ± 4.67	413.7 ± 10.94***

DC: Diabetic control, NC: Normal control, EPS: Epalrestat, SEM: Standard error of mean, n=10, Data expressed in mean ± SEM, statistical analysis by one-way ANOVA followed by Dunnett's test. Significance at ***p < 0.001 versus NC group

Table 1: Composition of HFD

Ingredients	Diet (g/kg)
Powdered NPD	365
Lard	310
Casein	250
Cholesterol	10
Vitamin and minerals mix	60
DL-methionine	03
Yeast powder	01
Sodium chloride	01

HFD: High-fat diet, NPD: Normal pellet diet

donepezil and EPS-treated animals (57, 27, and 13.5 mg/Kg) showed significant decreased TL when compared to DC on day 2.

Effect of EPS treatment on TL (passive avoidance test)

In the present study, we used passive avoidance task to assess short-term/long-term memory for evaluating the effect of EPS. Memory performance was correlated with the latency to escape from the light compartment, the better the recollection, and the greater the latency.

Acquisition

There was no significant difference in the STLs among the treatments and DC group of rats in the first acquisition trial (before receiving the electrical shock) (Table 3). This observation designates that the exploratory behavior of the different groups of rats in the dark did not differ. However, significant differences were observed among the different experimental groups with respect to the number of trials to acquisition criterion ($p < 0.001$, $p < 0.01$) (Fig. 2 and Table 3). Specifically, the number of trials to acquisition in donepezil-treated group ($p < 0.001$, $p < 0.01$) and EPS-treated group ($p < 0.001$, $p < 0.01$) were significantly less than DC group. Consistent with the presence of a cognitive deficit, the number of trials to acquisition in DC group was significantly greater than NC group ($p < 0.001$).

Retention

In the retention test which was conducted 24 h after the aversive stimuli (foot shock), retention of memory was significantly decreased in DC group ($p < 0.001$) as compared to NC group, whereas significantly enhanced TLs were observed in the animals treated with donepezil and EPS (54, 27 mg/kg; $p < 0.001$, $p < 0.01$) in comparison to DC group.

Effects of EPS treatment on spatial memory deficits in the MWM tasks

The memory impairment was assessed using MWM task. The mean escape latency time was measured to assess spatial memory in the experimental rats. The treatment with donepezil and EPS during training session significantly influenced the escape latency (Table 4 and Fig. 3). Further, statistical analysis revealed that donepezil and higher dose of EPS (54 mg/Kg) significantly reduced ($P < 0.05$) escape latency over the course of the training sessions when compared to the DC group. In the spatial probe test, performed on day 5 (day 38), the time spent within the target quadrant by the DC group was decreased as compared to those of the NC group ($p < 0.001$) (Fig. 3). Moreover, animals treated with EPS (27 and 54 mg/kg) resulted in enhanced time spent within the target quadrant as compared to DC groups ($p < 0.001$). These effects of EPS were similar to that shown by donepezil treatment ($p < 0.001$).

DISCUSSION

The study was designed and evaluated for the effect of EPS in diabetes-induced memory impairment: Memory and learning in rats. Type-2 diabetic rats exhibited marked impairment in memory that was revealed with behavioral parameters: EPM, passive avoidance test, and MWM task. Concomitant treatment with EPS, an aldose reductase inhibitor, responds the behavioral changes induced by diabetes.

Earlier studies directed that HFD in combination with a low dose of STZ induces type-2 diabetes [21]. Results of the present study showed the similar effect as well as diabetic rat indicates spatial memory and learning deficits in MWM task.

This was substantiated by the study results in the EPM and passive avoidance task, which was associated with avoided and reversed impairment by EPS treatment in rats. In the present study, we used MWM task to test spatial memory by observing the escape latency to reach a hidden platform. The diabetic rats were severely impaired as compared with NC rats, confirming earlier findings [24]. Furthermore, rats treated with donepezil and higher dose of EPS learned the platform location faster than diabetes control rats, and these findings were persistent throughout the trials. In addition, enhanced time

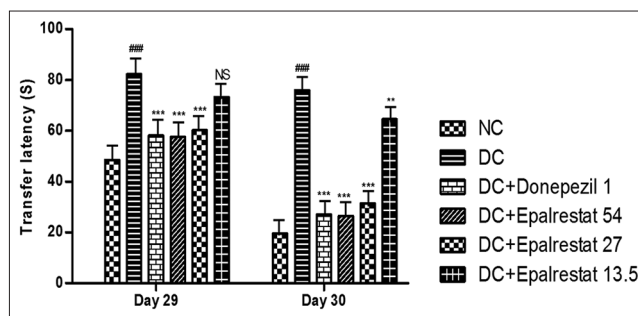


Fig. 1: Effect of EPS on transfer latency in high-fat diet streptozotocin-induced memory impairment on elevated plus maze: DC: Diabetic control, NC: Normal control, EPS: Epalrestat, S: Seconds SEM: Standard error of mean, n=10, Data expressed in mean±SEM, statistical analysis by one-way ANOVA followed by Dunnett's test. Significance at ** $p < 0.01$, * $p < 0.001$, ns: Not significant versus DC group and ### $p < 0.001$ versus NC group**

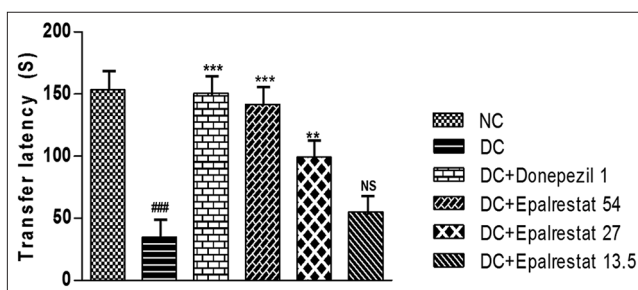


Fig. 2: Effect of EPS on transfer latency in high-fat diet streptozotocin-induced memory impairment on passive avoidance test: DC: Diabetic control, NC: Normal control, EPS: Epalrestat, S: Seconds, SEM: Standard error of mean, n=10, Data expressed in mean±SEM, statistical analysis by one-way ANOVA followed by Dunnett's test. Significance at ** $p < 0.01$, * $p < 0.001$, ns: Not significant versus DC group and ### $p < 0.001$ versus NC group**

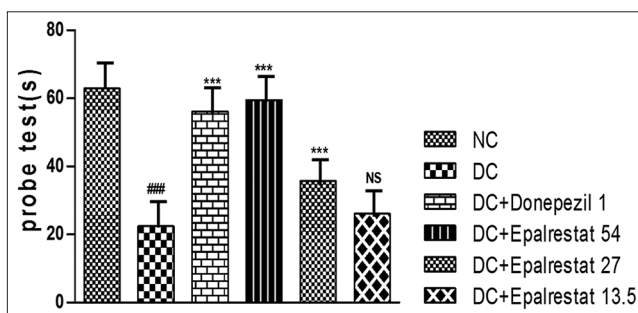


Fig. 3: Effect of EPS on probe test in Morris water maze task in high-fat diet streptozotocin-induced memory impairment rats: DC: Diabetic control, NC: Normal control, EPS: Epalrestat, S: Seconds, SEM: Standard error of mean, n=10, Data expressed in mean±SEM, statistical analysis by one-way ANOVA followed by Dunnett's test. Significance at * $p < 0.001$, ns: Not significant versus DC group and ### $p < 0.001$ versus NC group**

spent in target quadrant in experimental rats treated with donepezil and higher dose of EPS was also revealed in the similar fashion suggesting their motor performance (ability to swim) was unaffected by diabetic condition. In a passive avoidance test, a significant increase in TL time (TLT) as compared with DC (STZ treated) directs successful learning and memory function in EPS-treated rats, whereas STZ-treated rats failed to demonstrate an increase in TLT in retention

Table 3: Effect of EPS on HFD-STZ-induced memory impairment in the passive avoidance test

Treatment	Time taken to enter small compartment (s)				Day 33
	Day 31		Day 32		
	Trial 1	Trial 2	Trial 3	Trial 4	
NC	55.50±6.77	36.10±5.42	27.90±5.31	18.40±3.18	153.5±15.93
DC	114.4±5.86	99.89±6.85 ^{###}	73.33±6.27 ^{###}	66.89±6.48 ^{###}	34.78±3.97 ^{###}
DC+Donepezil 1	99.56±3.31	55.56±5.40 ^{***}	35.22±4.58 ^{**}	23.33±2.90 ^{***}	150.6±17.86 ^{***}
DC+EPS 54	101.5±2.63	52.70±8.40 ^{***}	33.50±7.89 ^{***}	19.10±3.31 ^{***}	141.6±13.49 ^{***}
DC+EPS 27	107.7±17.62	66.10±8.47 ^{**}	56.20±9.13 ^{ns}	39.70±5.86 ^{***}	99.20±12.56 ^{**}
DC+EPS 13.5	124.9±3.52	100.2±4.98 ^{ns}	68.33±4.12 ^{ns}	57.00±4.87 ^{ns}	54.89±6.52 ^{ns}

DC: Diabetic control, NC: Normal control, EPS: Epalrestat, S: Seconds, SEM: Standard error of mean, n=10, Data expressed in mean±SEM, statistical analysis by oneway ANOVA followed by Dunnett's test. Significance at ^{**}p<0.01, ^{***}p<0.001, ns: Not significant versus DC group and ^{###}p<0.001 versus NC group, HFD: High-fat diet, STZ: Streptozotocin, TL: Transfer latency

Table 4: Effect of EPS on spatial memory in MWM task in HFD-STZ induced memory impairment rats

Treatment	Time taken to reach target platform (s)				Probe test: Time spent in target quadrant (s)
	Day 34	Day 35	Day 36	Day 37	
	Day 34	Day 35	Day 36	Day 37	
NC	45.80±7.79	36.80±5.68	30.23±5.25	17.63±2.78	62.90±2.90
DC	85.00±2.79 [#]	82.22±6.05 [#]	72.81±12.49 [#]	63.25±10.58 [#]	22.44±1.28 ^{###}
DC+Donepezil 1	50.53±10.08 [*]	43.28±9.74 [*]	34.86±12.07 [*]	27.50±7.18 [*]	56.11±1.91 ^{***}
DC+EPS 54	48.58±9.48 [*]	40.45±10.31 [*]	31.33±7.17 [*]	29.30±5.89 [*]	59.50±2.80 ^{***}
DC+EPS 27	57.40±9.85 ^{ns}	52.75±10.88 ^{ns}	38.35±9.18 ^{ns}	35.73±8.40 ^{ns}	35.70±1.46 ^{***}
DC+EPS 13.5	82.69±3.39 ^{ns}	79.56±7.64 ^{ns}	70.44±9.38 ^{ns}	53.44±7.61 ^{ns}	26.11±1.70 ^{ns}

DC: Diabetic control, NC: Normal control, EPS: Epalrestat, S: Seconds, SEM: Standard error of mean, n=10, Data expressed in mean±SEM, statistical analysis by one-way ANOVA followed by Dunnett's test. Significance at ^{*}p<0.05, ^{**}p<0.01, ^{***}p<0.001, ns: Not significant versus DC. [#]p<0.05, [#]p<0.01, ^{###}p<0.001 versus NC. HFD: High-fat diet, STZ: Streptozotocin, MWM: Morris water maze

trial. As per earlier findings, donepezil administrations before trials in STZ-induced rats attenuated the memory impairment from 2nd trial onward [25]. In similar manner, treatment with EPS also attenuated the memory impairment from 2nd trial onward. These results confirm the previous study findings that have shown cognitive impairment in STZ-induced diabetic rats, which is associated with hippocampal dysfunction [26-28].

In our study, the EPM, passive avoidance, and MWM were used for the assessment of learning and memory. We evaluated the EPS treatment orally for 4 weeks improved learning and memory. All the doses of EPS improved the memory, as reflected by the diminished TL compared with diabetic rat, and decreased latency time in all repeated trials in MWM indicates learning and memory function, whereas decreased TL in EPM and increased TL during retention trial in PAT test showed improvement of memory.

Previous studies reported that decreased TLT in the 2nd day compared to 1st day in EPM indicated retention of memory [29,30]. The treatment with donepezil and EPS in experimental rats revealed that significant decreased TL on 2nd day trial (30th day) as compared to 1st day trial (29th day). This finding indicated memory retention in treated rats. In several studies, these cognitive deficits in diabetes were correlated for probable mechanisms such as hyperglycemia-induced end-organ neuronal damage, dyslipidemia, amyloidopathy, and tauopathy [31,32].

In the present study, EPS treatment significantly improved learning and memory impairment in HFD-STZ-induced diabetic rats using behavioral parameters. On the other hand, EPS treatment had no effect on blood glucose levels during the study, suggesting that there is no antidiabetic effect of EPS in experimental rats. This is also supported by earlier reports for blood glucose levels on EPS treatment [18]. Oxidative stress is considered to play a fundamental role in the development of memory impairment in diabetes [33,34]. EPS increases the intracellular levels of glutathione (GSH) which plays a crucial role in protecting endothelial cells from oxidative stress, thereby preventing several

vascular diseases caused by oxidative stress [13]. These observations suggest that EPS have significant antioxidant activity against STZ-induced oxidative stress [14].

STZ-induced diabetes was used in the present study because it is a well-known model of experimental diabetes and provides a good and relevant example of chronic oxidative stress due to hyperglycemia [26,35]. In addition, there are several potential explanations for polyol pathway-induced increase in oxidative stress. Hyperglycemia activates the polyol pathway, and reduction of glucose to sorbitol through aldose reductase may lead to NADPH consumption [36]. As NADPH is used in several critical reductive metabolic steps, a large drain on the NADPH pool could compromise the ability of the cell to protect itself from oxidative stress. NADPH is also required for GSH reductase to regenerate GSH [37-40]. Considering the importance of oxidative stress in the pathophysiology of diabetic state and development of cognitive impairment, reduction of oxidative stress by EPS may produce a beneficial effect on diabetic-induced cognitive impairment.

Neuroinflammatory mediators and oxidative stress markers are capable in causing cognitive alterations through several mechanisms that could possibly affect the neuronal properties and cell survival. Several studies were conducted earlier to conceptualize the possible cause and link between neuroinflammation, oxidative stress, behavior, and cognitive impairments [34,41-43].

According to the previous findings, heme oxygenase (HO)-1 has potent antioxidant and anti-inflammatory functions; however, EPS upregulates HO-1, dismutase, and catalase by activating Nrf2 and suggests that EPS has the beneficial effect on improvement of several neurological disorders [12].

Interestingly, a recent *in vitro* study reported that EPS treatment on rat Schwann cells and human neuroblastoma cell line upregulates HO-1 suggesting the potential of EPS to prevent neurological diseases. Therefore, a pilot study was conducted by us to analyze behavioral effects in EPS-treated rats using STZ-HFD induced model.

CONCLUSION

Based on our results obtained in the present study, the EPS has shown enhanced learning and memory activity. In particular, it was more significant at the dose of 27 and 57 mg/kg. However, further extensive studies are needed to establish its exact mechanism of action for potent and efficacious agent in the treatment of memory deficit.

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