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Original article

Aqueous Ajwa dates seeds extract improves memory impairment in type-2 diabetes mellitus rats by reducing blood glucose levels and enhancing brain cholinergic transmission

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ABSTRACT

Diabetes is a metabolic disorder prevalent across the globe and is known to cause brain dysfunction, especially memory and cognitive decline. The current study investigates the effect of aqueous Ajwa seeds extract (AASE) on type-2 diabetes mellitus (T2DM)-induced memory deficits using a rat model. T2DM was induced by an administration of nicotinamide (120 mg/kg, i.p.) and streptozotocin (STZ) (60 mg/kg, i.p.). AASE (200 and 400 mg/kg, p.o.) were treated to T2DM rats for 30 days and the results were compared with the metformin (200 mg/kg). Elevated plus maze (EPM), Y-maze, and novel object recognition (NOR) tests were performed to assess the memory functions. The blood glucose and plasma insulin levels were estimated to assess the anti-diabetic effects of AASE. Acetylcholine (ACh) and acetylcholinesterase (AChE) levels were estimated from brain homogenates to assess cholinergic transmission. Treatment with AASE resulted in the reversal of behavioral deficits. EPM showed, a significant reduction in transfer latency (TL) among T2DM rats. High exploration time with a novel object and improvement in discrimination index were observed among treated groups during the NOR test. The Y-Maze test improved the entries and also time spent in the novel arm. Moreover, treatment of AASE reversed hyperglycemic and enhanced plasma insulin levels (200 mg/kg: 3.81 ± 0.08 ng/ml and 400 mg/kg: 4.09 ± 0.10 ng/ml) among T2DM rats (2.81 ± 0.15 ng/ml). Improved ACh levels (200 mg/kg: 186.6 ± 9.51 pg/mg protein and 400 mg/kg: 165.5 ± 9.25 pg/mg protein) and reduced AChE levels (200 mg/kg: 0.29 ± 0.02 ng/mg protein and 400 mg/kg: 0.32 ± 0.03 ng/mg protein) were also noted in the brain of AASE treated groups as referred to diabetic group (ACh: 107.1 ± 7.16 pg/mg protein and AChE: 0.51 ± 0.03 ng/mg protein). The above results were found to be comparable with the metformin-treated groups. From the results, it can be concluded that AASE has the potential to improve T2DM associated cognitive deficits.

1. Introduction

T2DM is a combination of metabolic disorders and a chronic disease characterized by hyperglycemia and has affected the population globally. Either impaired insulin secretion or defective insulin secretion or both are responsible for the above-mentioned metabolic disorder. Particularly, T2DM is distinguished by a progressive reduction in insulin activity because of insulin resistance and which is further results in the incapable of pancreatic β-cells to regulate insulin resistance (Tahara et al., 2011). Across the world, over 176 million people were reported to be affected by diabetes in the year 2000 and by the year 2011, this number increased to>366 million. The estimated number to reach by the year 2030 is expected to be 366 million (Akushevich et al., 2018). Saudi Arabia is listed as sixth among the top ten countries of the world with the highest prevalence of diabetes and around 23.9% of the total population are affected with diabetes. Because of the high prevalence rate of diabetes, the financial burden on national healthcare is likely to exceed $0.87 billion in the Kingdom

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and is projected to increase in the next 10 years (Marwa, 2015; Robert and Al Dawish, 2021). Furthermore, diabetes is a chronic disorder and it causes many typical complications that make it more hazardous to the patient's health, and difficult to treat. For example, cardiovascular disease, damage of the retina, nephropathy, neurological defects, cognitive disorders, and Alzheimer's disease (AD) are linked with DM (Forbes and Cooper, 2013). Consider the types of diabetes, type 2 is a major type and has a rapidly growing etiology among the population. AD is a foremost cause of dementia leading to aberrations at the molecular, histopathological, and biochemical levels. The evidence suggests that the development of AD increases by 50 to 60 percent among patients suffering from T2DM (Talbot et al., 2012).

There's significant number of evidence, indicating that T2DM raises the risk of cognitive and memory decline. Diabetes, as well as memory and cognitive impairment, are very common among people of the older age group (Srikanth et al., 2020). T2DM is a considerable risk factor associated with dementia because of its harmful effects on the brain and microvascular system (Velayudhan et al., 2010). In addition, decreasing insulin production in the pancreas has an impact on human memory and learning development. Insulin serves unique functions in CNS and it crosses the blood–brain barrier (BBB) utilizing a specific transporter, altering the cognitive functions through CNS mechanisms that are essentially unrelated to glucose utilization. Also, insulin dysregulation may contribute to neurodegeneration via modulating vascular functions through regulating vasoreactivity, inflammation, and lipid metabolism (Kellar and Craft, 2020). Furthermore, studies have shown that higher acetylcholinesterase (AChE) or decreased acetylcholine (ACh) levels or activities are linked to cognitive impairment in diabetics due to impairment of cholinergic transmission (Kim, 2019). It is also reported that central cholinergic dysfunctions are highly linked to the cognitive deficit and memory impairment through decrement of ACh in the synaptic cleft (Mohd Azahan et al., 2020). STZ-induced hyperglycemia is known in animal experiments to cause severe alterations and damage to the brain, including the loss of nerves in the cortex and, as a result, lower ACh levels. It also causes reduction in transportation of glucose across blood–brain barrier and reduction of glucose availability subsequently leading to neuroglycopenia and decrease in the ACh generation respectively (Welsh and Wecker, 1991; Kumari et al., 2000).

Date palm (*Phoenix dactylifera* L. Arecaceae) is a common fruit crop in Saudi Arabia. The dates production from Kingdom accounts for 12 to 13 % of world production. It is widely spread around the Arab World and is well-known for its nutritional and medicinal properties. From the literature, date fruits and seeds are used as powder, pulp, and infusion for the treatment of atherosclerosis, asthenia, throat disease, pulmonary disease, expectorant, and mouth hygiene (Chao and Krueger, 2007). Ajwa dates from the district of Al Madinah, Saudi Arabian is considered unique due to its abundance in vitamin contents, dietary fiber, and proteins (Sheikh et al., 2016). Regarding minerals, phosphorus, iron, potassium, and a large amount of calcium are all found in dates. According to phytochemical research, Ajwa dates contain high levels of flavonoid and phenolic chemicals, which are the major constituents for its free radical scavenging and antioxidant abilities (Hamad et al., 2015). Ajwa dates have several pharmacological qualities, including antioxidant, antimicrobial, anti-inflammatory, anti-mutagenic, and anti-tumor capabilities. Its extracts were also found to have hepatoprotective, nephroprotective, hypolipidemic, and gastroprotective properties (Khalid et al., 2017). The aqueous extracts of Ajwa date seeds were reported to control the plasma glucose levels and glycosylated haemoglobin (HbA1c). It was also reported to elevate serum insulin levels in normal as well as in diabetes-induced models. Reference to a recent report, administration of an aqueous extract from Ajwa dates seeds saved the liver tissues from carbon tetrachloride (CCl_4) by inhibiting hepatic lipogenesis and oxidative stress (Mesalam et al., 2021). The other report also supported the cholesterol-lowering and hepatoprotective potential effects of Ajwa date seed extract on the high-fat diet-induced hyperlipidemic rat model (Khan et al., 2018). Additionally, the extract restored the kidney as well as liver functions and balanced the oxidative stress in diabetes-induced rats (Hasan and Mohieldein, 2016). Similarly, in alloxan-induced diabetic rats, Ajwa seed extract was able to regulate glucose and liver enzyme levels to some extent. (Sarfraz et al., 2017). In male rats, the treatment of the date seed extract was reported to protect cortical neurons against cerebral-induced injuries, and that mechanism was supported by its antioxidant properties (Kalantaripour et al., 2012). Recently, the dietary supplementation of date fruits reported a significant reversal of memory deficit, anxiety-related behavior, and motor coordination in a transgenic mouse model for AD. Besides, similar treatment significantly lowered Aβ proteins ($A\beta_{40}$ and $A\beta_{42}$) in plasma (Subash et al., 2015). Because there is limited evidence about the effects of Ajwa date seeds on the CNS, especially against neuroprotection, the current aims to assess the effect of AASE on the improvement of memory deficit in T2DM rat models. The study also intends to explore the effect of AASE on plasma glucose and insulin levels. Besides levels of acetylcholine and acetylcholinesterase have also been estimated to explore the effect of AASE on brain cholinergic transmission of T2DM rat models.

2. Material and method

2.1. Drugs and chemicals

Fine chemicals such as streptozotocin, metformin hydrochloride, and nicotinamide were procured from Cayman Chemical, USA. ELISA kits for estimation of insulin, acetylcholine, acetylcholinesterase were procured from Cloud-Clone Corp., USA. Other chemicals and solvents were purchased locally with analytical grades.

2.2. Plant material and extraction

Ajwa dates were directly acquired from the date farm of Al-Madinah. The collected dates were identified with PCR analysis by Prof. Mohamed Motawei, Professor in Genetic Molecular, Department of Plant Production and Protection in College of Agriculture and Veterinary, Qassim University from College of Agriculture and Veterinary, Qassim University, Saudi Arabia. The seeds were separated washed with an adequate amount of water and were kept for drying at room temperature for 2–3 days. After drying the seeds were prepared as coarse powder by using a coffee grinder. Each 100 g of the collected seed powder was soaked in one liter of distilled water for three days at normal temperature. Then, the extract was collected and concentrated in a rotary evaporator and used for further studies (Hasan and Mohieldein, 2016).

2.3. Experimental animals

In this experiment, a total number of thirty male adult Sprague Dawley (SD) rats with the age of and body weight of three months and 200–250 g respectively were used. Rats were obtained from the Animal Facility, College of Pharmacy, Qassim University, Saudi Arabia. For acute toxicity study, SD rats of female gender with similar age-matched were used. Animals were subjected to standard laboratory conditions with a 12-hour light–dark cycle for one week. The animals were assigned randomly into five groups and each group consist of six animals. Three rats were housed in one

polypropylene cage, and maintained free of viruses and pathogens. The animals were provided standard rodent pellet food (First Milling Company, Jeddah, Saudi Arabia) and allowed water *ad libitum*. The experimental protocols were approved by the Institutional Animal Ethical Committee of College of Pharmacy (approval ID 2020- CP –6) and Deanship of Scientific Research of Qassim University under the grant number Pharmacy-2019–2-2-I-5643.

2.4. Vehicle

Aqueous Ajwa seeds extract (AASE) was solubilized in distilled water and was administered orally to the animals. Standard drug metformin hydrochloride was dissolved in 0.9 % (w/v) normal saline and was used for the treatment through oral route The STZ and nicotinamide were solubilized in 0.1 M cold citrate buffer (pH 4.5) and 0.9 % (w/v) normal saline respectively. Both of the solutions were injected intraperitoneal (i.p) for inducting T2DM.

2.5. Acute toxicity study

Acute toxicity experiments were executed in compliance with the guidelines laid by Organization for Economic Co-operation and Development 423. In this experiment, for each step, three female rats were allocated using a random sampling technique. Animals were kept on overnight fasting with access to water. Initially, AASE was administered orally at 5 mg/kg and was closely observed for toxic symptoms and mortality for the first four hours and another three days respectively. If two of three animals were found dead, the considered dose is toxic. However, if one of the three animals was found dead, the considered dose is repeated to confirm toxicity. And If none of the animals is dead is observed at the above dose, higher doses can be tried, and thus AASE at the dose level of 50, 300, and 2000 mg/kg were employed to evaluate the toxic effects (Mani et al., 2021).

2.6. Experimental design and drug treatments

The total number of five groups were employed with each consisting of six animals were employed. The control group and only diabetic-induced groups were administered 0.5 % w/v CMC, p.o. i.e. vehicle only. The remaining three groups were subjected to the treatment of standard drug metformin (200 mg/kg, p.o.) and AASE at the dose of 200 and 400 mg/kg for 30 days after the T2DM induction, respectively. During treatment, the spatial memory assessments of animals were performed using various behavioral tests which include elevated plus-maze on 26th and 27th days. NOR was performed on 28th and 29th days of treatment while Y-maze test on 30th day of treatment) (Fig. 1). End of the 30th-day animals were sacrificed and fresh brains were collected for further biochemical and ELISA analysis. During treatment, the body weight of each rat was recorded every 15th day. The plasma glucose levels were also measured using a glucometer during treatment at the gap of fifteen days.

2.7. Induction of diabetes

Rats were kept of overnight fasting before the injection of nicotinamide (120 mg/kg) followed by STZ (60 mg/kg) within fifteen minutes to induce T2DM (Aboonabi et al., 2014). The hyperglycemic induction was confirmed by quantifying blood glucose levels after 72 h, and on the 7th day using glucometer Accu-Chek glucometer (Roche, Germany). The blood glucose levels developed>126 mg/dl in rats under fasting conditions were considered to be diabetic and were used for further studies (Aboonabi et al., 2014). Furthermore, the blood glucose levels were measured on the 15th and 30th days of treatment.

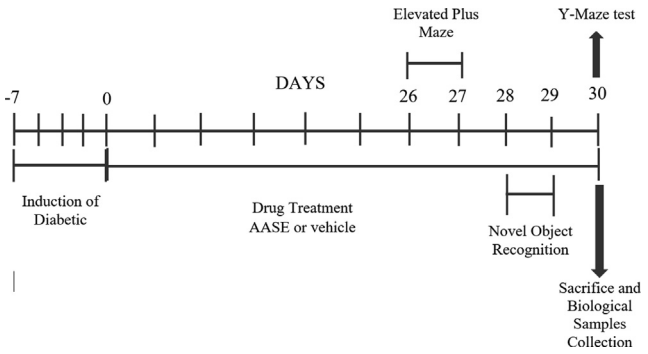

Fig. 1. Timeline administration of drug, behavioral assessments, and isolation of brain samples.

2.8. Behavioral assessments

2.8.1. Elevated plus maze (EPM) test

EPM made of wood contains two open and two enclosed arms on the opposite side. TL was recorded from the EPM test which is defined as "time taken (in seconds) by the rat to move from the open arm to either one of the closed arms" (Ahmad et al., 2014; Mani, 2021). On the 26th day of treatment, each of the rats was trained by placing them at one end of the open arm, away from the central area. If the animal fails to move into a closed arm within 90 s, the animal was guided towards a closed arm and was allowed to stay in the maze for two minutes. After 24 h (on the 27th day of treatment), the TL was recorded as retention of learned–task memory.

2.8.2. Novel object recognition (NOR) test

NOR was performed using an open wooden box of dimensions 80 X 60 X 40 cm apparatus using the procedure designated by Malik et al with minor modifications (Malik et al., 2013; Mani, 2021). Discriminating objects were selected with two dissimilar shapes (rectangle box as a familiar object (FO) and cylindrical box as a novel object (NO). The objects were tall and firm, so that they cannot be moved by the animals during the test). The experiments were performed in three phases such as habituation, training, and test phases (the 28th and 29th days of drug treatment). In the habituation process, each animal was permitted freely to explore the empty box i.e. without FO and NO for 5 min. The training session (T1) was performed after 24 h (the 28th day of drug treatment). In this session, each rat was allowed to explore with two similar rectangle shape samples [Familiar Object 1 (FO1) and Familiar Object 2 (FO2)] for five minutes during the familiarization process, and the exploration times of FO1 and FO2 were recorded. The exploration time is defined as the time spent by an animal pointing its nose to an object placed at a distance of $\leq$ 2 cm. To avoid being coerced into exploring the items, rodents are released against the opposite wall's center, with their backs to the objects. Following the T1 session, the test session (T2) was scheduled after a gap of 4 h. During the familiarization and test phases, the experimental context was not significantly different. During T2, the rat was again allowed to explore with two objects. The first object was the same as used in training session T1 i.e rectangular box (FO1) and the second was a novel cylindrical box (NO). The test phase was conducted for five minutes. The time for exploring the FO as well as the NO was recorded. The discrimination index (DI) was calculated by: D = N-F/N + E; N – Exploration time of the novel object and F- Exploration time of the familiar object (Mani, 2021).

2.8.3. Y-Maze test

Y-maze made of wood with three arms at a 120° angle (50 × 10 × 18 cm) was used on the 30th day. Each arm end was pasted with a picture containing a different pattern. The apparatus was set on the ground. To ensure the even distribution of light, the lighting objects were placed right above the maze. The procedure of the test was followed by Tripathi et al. with minor modification (Tripathi et al., 2017; Mani, 2021). In the training session, one of the arms (novel arm) was closed and the animals were allowed to explore the remaining two arms of the maze freely for 5 min. After 4 h the test session was scheduled. During the test session, rats were allowed to explore all three arms of Y-maze for 5 min. The number of entries in two arms during the training session and in three arms during the test was recorded. During the test session, the time spent in the novel arm was also recorded. An animal is considered to have entered an arm if it enters 85% of its body. The proportion of time spent in the novel arm was calculated as the total time spent in the novel arm/time spent in all the arms during the test session (Mani, 2021).

2.9. Blood and brain sample collection

After the end of the 30th-day brain and samples of blood were collected. The blood was collected in the heparin-coated tube and the plasma was collected by running the centrifuge at 4000 rpm for 10 min, then aliquot into a 4-ml vial and stored at − 80 °C. The collected plasma samples were used to measure insulin levels. The entire fresh brain was homogenized using a homogenizer in ice-cold phosphate buffer (4 °C, pH 7.6) followed by centrifugation for 10 min at 4000 rpm. The cloudy supernatant aliquot was transferred into 4-ml vials and stored at − 80 °C. The total protein content of the samples was quantified using the biuret colorimetric method (Crescent Diagnostics, Saudi Arabia). The ACh and AChE levels were quantified using brain homogenate.

2.10. Estimation of plasma insulin level

Insulin levels of plasma were estimated by using an ELISA kit procured from Cloud-Clone Corp., USA. The protocol of this experiment was followed according to the manual from the manufacturer. The assay was performed in duplicate and measurements of absorbance were performed at 450 nm by using a Microplate Reader (BioTek Instruments, Inc.).

2.11. Determination of brain acetylcholine (ACh) and acetylcholinesterase (AChE) levels

ACh and AChE levels were estimated in brain homogenate using ELISA kits. These assays were based on the sandwich enzyme immunoassay method. The procedure of this assay was followed as per the manufacturer's instructions. Finally, the absorbance was recorded at 450 nm using a Microplate Reader (BioTek Instruments, Inc.) after 20 min of incubation.

2.12. Statistical analysis

Results were indicated as mean ± SEM. Results were evaluated using a one-way ANOVA analysis and Tukey–Kramer post hoc test to calculate the significance level. In the NOR test, the student's unpaired 't'-test was utilized to compare corresponding each group of exploration times. Graph Pad version 9.0 (GraphPad Software Inc., United States) was used for analyzing variations between two groups. The level of p-value < 0.05 was accepted as statistically significant.

3. Results

3.1. Acute toxicity study

As per the guidelines of OECD 423, no evidence of toxicity or animal death was noted at the doses of extract given orally maximum at 2000 mg/kg. For subsequent memory testing and biochemical analysis, two lower dosages of AASE (200 and 400 mg/kg, p.o) were chosen.

3.2. Administration of AASE did not alter the body weight of diabetic-induced rats

Fig. 2 shows the effect of AASE on the body weight of animals under study. The body weight was measured every 5th day starting from day 0. No variations were found among the groups regarding body weight until the end of the experiment.

3.3. Administration of AASE reduced blood glucose levels in diabetic-induced rats

Fig. 3 refers to the effects of two doses of AASE on glucose levels in rat's blood. The results showed significant differences among the groups on day 1st [F (4,25) = 34.88, p < 0.001)], day 15th [F (4,25) = 35.38, p < 0.001], and day 30th [F (4,25) = 34.52, p < 0.001] using One-way ANOVA. The diabetic group exhibited extensively higher (p < 0.001) glucose levels in blood on 1st day (7th day after the STZ-nicotinamide injection), 15th and 30th days of drug treatment confirming hyperglycemic condition in experimental animals. However, treatment with doses of AASE considerably (p < 0.001) declined the glucose levels on days 15 and 30, when matched to the diabetic-induced group. Also, the blood glucose levels of AASE and metformin-treated animals were found significant (p < 0.001) high on 15th and 30th days, as a comparison with control.

3.4. Administration of AASE shortens the transfer latency of diabetic-induced rats in the EPM test

Fig. 4 shows the effects of AASE on TL using the EPM test. Statistical analysis among the groups revealed a significant difference [F (4,25) = 42.66, p < 0.001] in TL values. Referred to control, the diabetic group showed significantly (p < 0.001) longer TL in the EPM test thus confirming the memory impairment in the diabetic group. Thirty days of treatment of AASE considerably (p < 0.001) restored the memory deficit by declining the TL time as compared to the diabetic group. The AASE effects on TL were comparable with the effect of metformin using the EPM test.

3.5. Administration of AASE improved cognitive functions of diabetic-induced rats in NOR test

The effect of AASE on targeted behavioral parameters of diabetes-associated cognitive deficits in rats was examined with the NOR test is revealed in Fig. 5. A considerable difference among the groups was recorded in the mean exploration time for familiar objects FO1 [F (4,25) = 9.100, p < 0.001] and FO2 [F (4,25) = 9.658, p < 0.001] during the training session (T1). Further, analysis between the selected two groups explained a significant decrease (p < 0.001) in exploration time of objects FO1 and FO2 in the diabetic-induced group as parallel to the control group (Fig. 5a). The exploration times during the training session for the two doses of AASE treatment groups showed a considerable difference (p < 0.01) as associated with the control group. In addition, com-

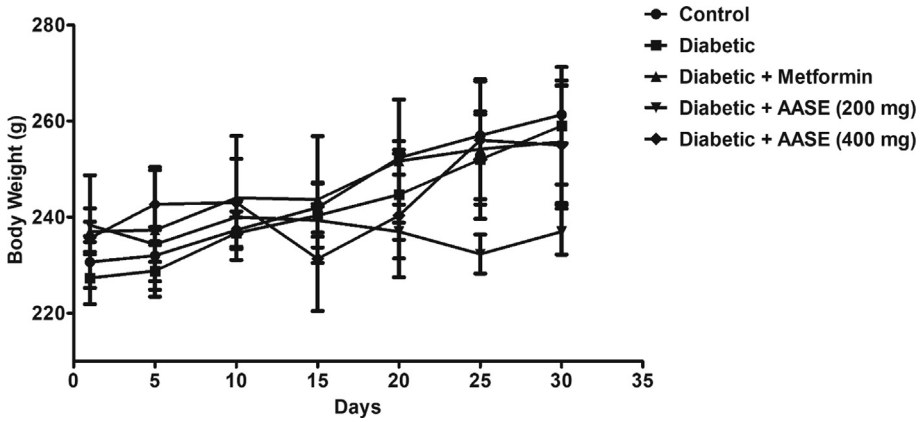

Fig. 2. Effect of AASE and metformin on body weight. The values are mean ± SEM (n = 6). One-way ANOVA [$F(4,25) = 0.4876$, p > 0.05 for day 1; $F(4,25) = 0.4887$, $p > 0.05$ for day 5; $F(4,25) = 0.1865$, $p > 0.05$ for day 10; $F(4,25) = 0.3028$, $p > 0.05$ for day 15; $F(4,25) = 0.499$, $p > 0.05$ for day 20; $F(4,25) = 1.065$, $p > 0.05$ for day 25; $F(4,25) = 0.8811$, $p > 0.05$ for day 30] followed by Tukey-Kramer multiple comparisons test. There were no statistically significant differences found between the groups in body weight.

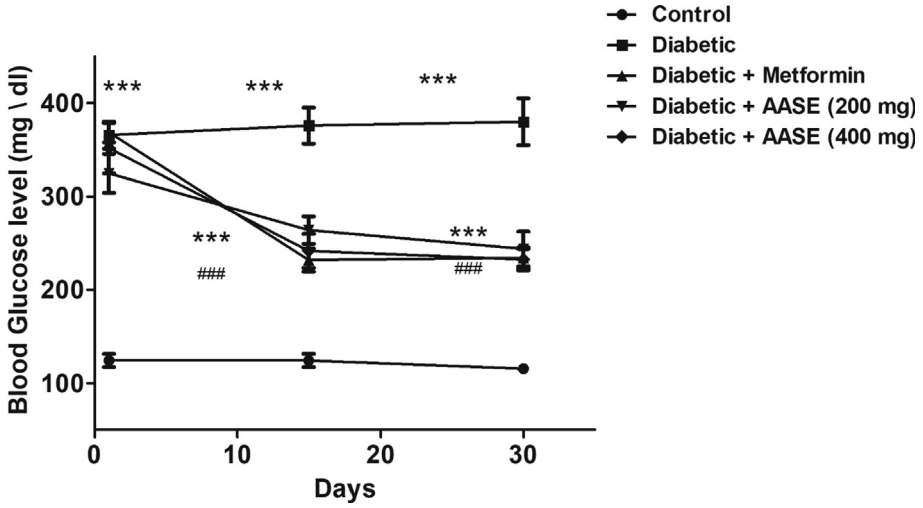

Fig. 3. Effect of AASE and metformin on blood glucose levels of streptozotocin-nicotinamide-induced diabetic rats. The values are mean ± SEM (n = 6). One-way ANOVA [$F(4,25) = 34.88$, $p < 0.001$for day 1; $F(4,25) = 35.38$, $p < 0.001$ for day 15; $F(4,25) = 34.52$, $p < 0.001$ for day 30] followed by Tukey-Kramer multiple comparisons test. $^{***}p < 0.001$ as compared to the control group; $^{###}p < 0.001$ as compared to diabetic -induced group.

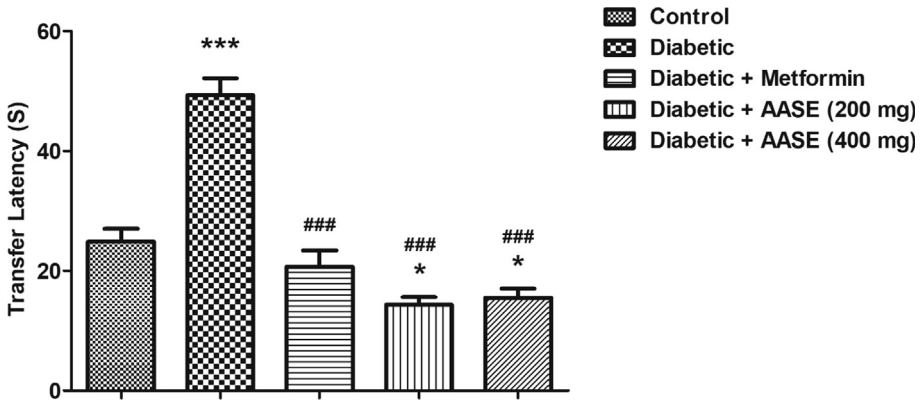

Fig. 4. Effect of AASE and metformin on transfer latency (s) of streptozotocin-nicotinamide-induced diabetic rats using elevated plus-maze. The results are expressed by mean ± SEM (n = 6). One-way ANOVA [$F(4,25) = 42.66$, $p < 0.001$] followed by Tukey-Kramer multiple comparisons test. $^{*}p < 0.05$ and $^{***}p < 0.001$ as compared to the control group; $^{###}p < 0.001$ as compared to the diabetic-induced group.

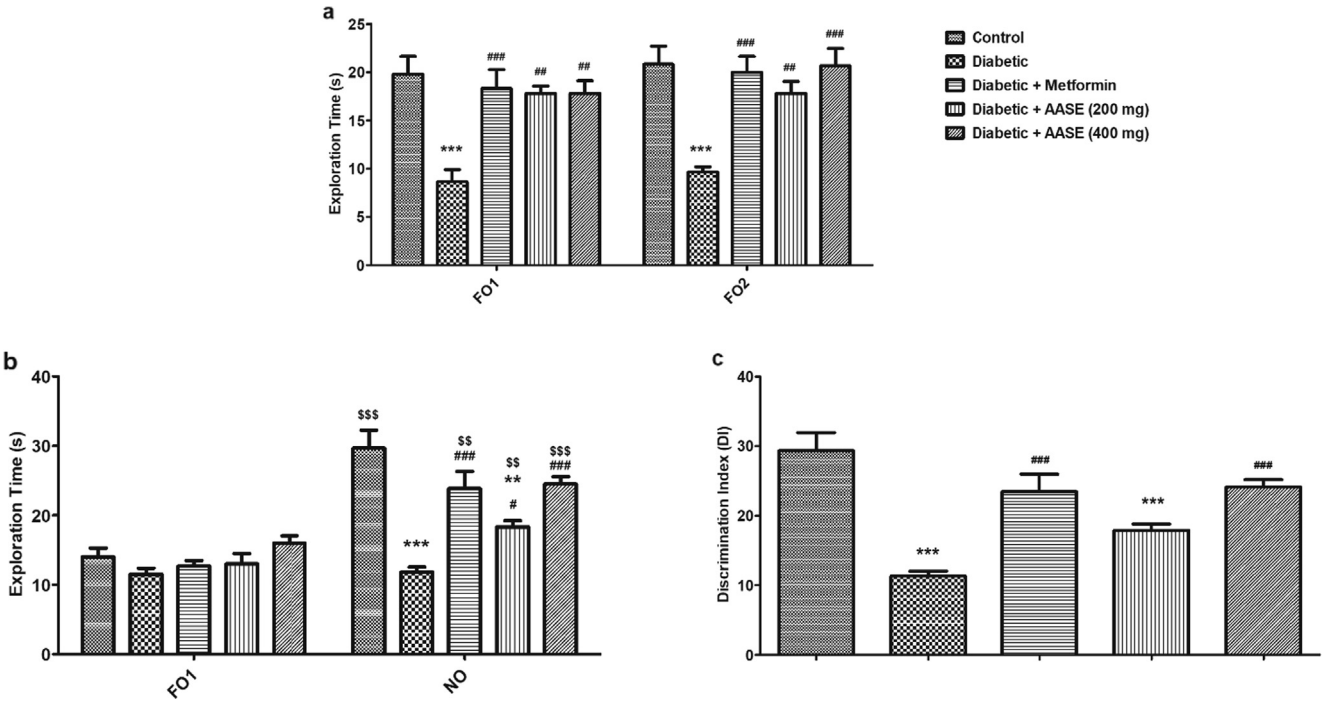

Fig. 5. Effect of AASE and metformin on (5a) exploration time of two familiar objects (FO1 and FO2) during the training session (T1), (5b) exploration time of familiar (FO1) and novel (NO) objects during the test session (T2), (5c) discrimination index of streptozotocin-nicotinamide-induced diabetic rats model using novel object recognition test. The results are expressed by mean ± SEM (n = 6). One-way ANOVA [$F(4,25) = 9.100$, $p < 0.001$ for FO1 and $F(4,25) = 9.658$, $p < 0.001$ for FO2 during T1; $F(4,25) = 2.268$, $p > 0.05$ for FO1 and $F(4,25) = 13.95$, $p < 0.001$ for NO during T2; $F(4,25) = 15.13$, $p < 0.001$ for discrimination index] followed by Tukey-Kramer multiple comparisons test for comparisons of within the groups. Student's unpaired 't' test was used to comparisons of corresponding each group of exploration time. $^{\$\$}p < 0.01$ and $^{\$\$\$}p < 0.001$ as compared to the corresponding group; $^{**}p < 0.01$, and $^{***}p < 0.001$ as compared to the control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ as compared to diabetic -induced group.

parisons between objects FO1 and FO2 groups revealed similarities in exploration duration.

During the test session (T2), the objects were FO1 and NO, the group of rats was spent substantially more exploration time ($p < 0.01$) with NO than the FO1 (Fig. 5b). The analysis between all groups presented a variation in the exploration time for NO [$F(4,25) = 13.95$, $p < 0.001$] and but not with FO1 [$F(4,25) = 2.268$, $p > 0.05$]. The comparison of groups for exploration time during a test session for NO revealed an excellent reduction ($p < 0.001$) in the diabetic group as matched to the control group. For AASE treated groups displayed an increase in the exploration time ($p < 0.05$ and $p < 0.01$, respectively for 200 and 400 mg/kg) for NO were observed concerning the diabetic-induced group.

The effect of AASE on the discrimination index (DI) is depicted in Fig. 5c. One-way ANOVA showed a difference [$F(4,25) = 15.13$, $p < 0.001$] in DI among the groups. The diabetic group exhibited a considerable decrease ($p < 0.001$) in DI value as related to the control group. Treatment of animals with two doses of AASE ameliorated the effect of hyperglycemia on cognitive deficits. 400 mg/kg of AASE showed significantly improved ($p < 0.001$) DI values as compared to the diabetic group. Unfortunately, the reversal of the DI values of AASE at 200 mg/kg was not comparable with control animals.

3.6. Administration of AASE improved cognitive functions of diabetic-induce rats in Y-maze test

Fig. 6 illustrates the results of behavioral parameters studied using Y-maize. Fig. 6a and b display the number of entries for known and novel arms respectively During the test session, significant variations in the number of entries for the known [$F(4,25) = 5.138$, $p < 0.01$] as well as a novel [$F(4,25) = 13.760$, $p < 0.001$] arms were observed. The diabetic group established a notable reduction in the number of know arm entries ($p < 0.05$) and novel arm entries ($p < 0.01$) as matched to respective control animals. Oral administration of AASE at the two dose levels exhibited a significant increase ($p < 0.01$) in the number of entries for the know arm as compared to the diabetic group. An improvement in the number of entries for novel arms ($p < 0.01$ and $p < 0.001$, respectively) was also noted for the two doses of AASE treated groups.

The percentage of time spent by rats in a novel arm of the Y-maze during the test session is shown in Fig. 6c. The time stays in the novel arm was exposed to a statistical variation [$F(4,25) = 6.270$, $p < 0.01$] among all the groups. The diabetic group of rats showed a significant reduction ($p < 0.01$) in the percentage of time spent in the novel arm when compared to the control group. However, the AASE treated group showed a higher percentage ($p < 0.05$ and $p < 0.01$, respectively) of time spent in the novel arm as paralleled to the diabetic group.

The total number of entries to the arms in a trial as well as in test sessions using the Y-maze are shown in Fig. 6d and 6e respectively. The analysis between all the groups established notable differences in the total number of entries during trial [$F(4,25) = 21.80$, $p < 0.001$] and test [$F(4,25) = 7.820$, $p < 0.001$] sessions, when compared among the groups. The comparison between control and diabetic selective showed a significant difference ($p < 0.001$) in the total number of entries during the trial session (Fig. 6d). However, the two doses of AASE showed improvement ($p < 0.001$) in entries number during the trial session as paralleled to diabetic group. In the test session, the total number of entries in arms considerably declined ($p < 0.05$) for the diabetic group of rats as associated to control (Fig. 6e). Remarkably, there were good improvements in the number of arm entries ($p < 0.001$) during the test session for the group of animals treated with the oral 200 as well as 400 mg/kg of AASE as considering to the diabetic-induced rats.

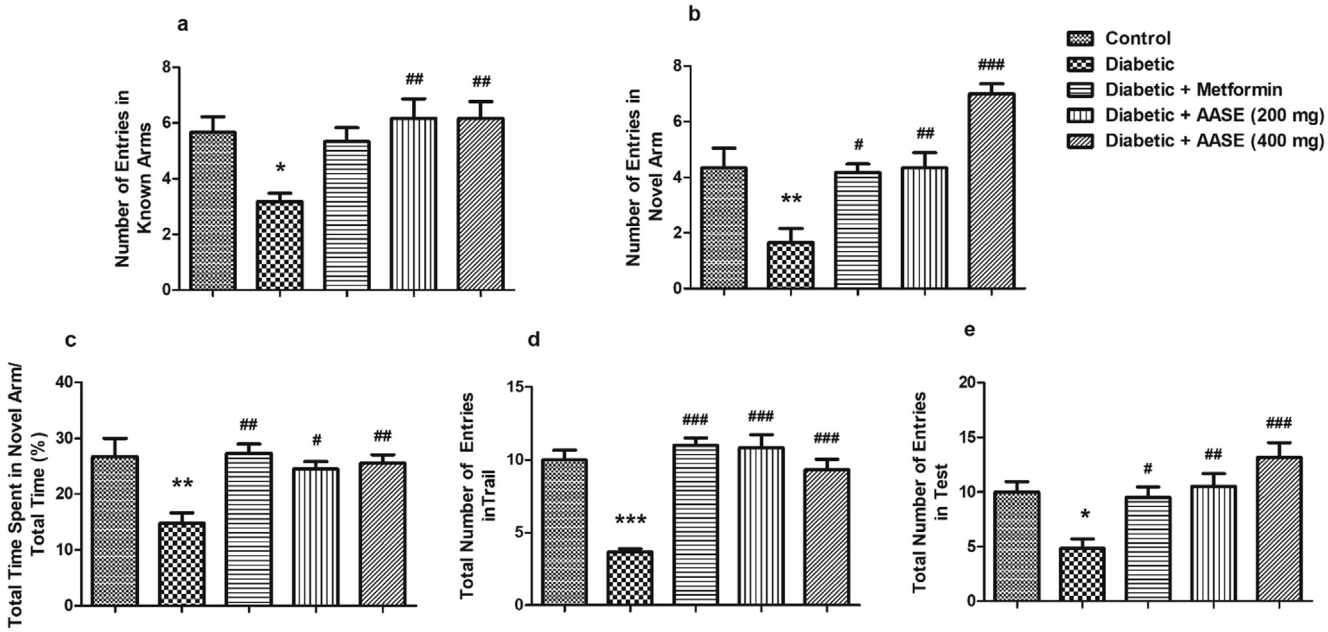

Fig. 6. Effect of AASE and metformin on (6a) number of entries in known arms in test, (6b) number of entries in novel arm in test, (6c) percentage of time spent in the novel arm in test, (6d) the total number of entries in the trail, and (6e) total number of entries in streptozotocin-nicotinamide-induced diabetic rats model using Y-maze test. The results are expressed by mean ± SEM (n = 6). One-way ANOVA [$F(4,25) = 5.138$, $p < 0.01$ for the number of entries in known arm; $F(4,25) = 13.760$, $p < 0.001$ for the number of entries in novel arms; $F(4,25) = 6.270$, $p < 0.01$ for the percentage of time spend in novel arm; $F(4,25) = 21.800$, $p < 0.001$ for the total number of entries in the trail; $F(4,25) = 7.820$, $p < 0.001$ for the total number of entries in test] followed by Tukey-Kramer multiple comparisons test. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ as compared to the control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ as compared to the doxorubicin-induced group.

3.7. Administration of AASE increased plasma insulin levels in diabetic-induced rats

Fig. 7 highlights the results of AASE and metformin on plasma insulin levels. A variation in plasma insulin levels [$F(4,25) = 16.09$, $p < 0.001$] was recorded among all the groups. As related to the control group a significant drop ($p < 0.001$) in the plasma insulin level was recorded in the diabetic group. Thirty days of treatment of animals with administration with both doses of AASE considerably ($p < 0.01$ and $p < 0.001$; respectively) elevated the insulin levels plasma as compared to the diabetic group. The effects were comparable with the group of animals treated with the standard drug metformin.

3.8. Administration of AASE elevated acetylcholine (ACh) and reduced acetylcholinesterase (AChE) activities in the brain of diabetic-induced rats

Fig. 8 refers to the effect of AASE on ACh and AChE levels in brain homogenate for the group of animals under study. A significant variation in ACh levels [$F(4,25) = 10.77$, $p < 0.001$] as well as AChE levels [$F(4,25) = 15.86$, $p < 0.001$] in between all the groups. The diabetic group of rats revealed a decline ($p < 0.001$) in ACh levels as related to the control group. Treatment with metformin and AASE, however, considerably ($p < 0.01$, $p < 0.001$, and $p < 0.01$, respectively) elevated the ACh levels in the diabetic group of rats (Fig. 8a). On the other hand, diabetic-induced rats exhibited significantly ($p < 0.001$) higher AChE levels in rat's brain. Nevertheless, administration of AASE considerably ($p < 0.001$) restored the AChE levels to that of the baseline control level (Fig. 8b).

4. Discussion

T2DM is considered the world fastest growing disorder and is reported with a high number of morbidity and mortality. Furthermore, cognitive functional defects, including challenges in memory functioning are commonly associated with diabetic patients. Until now, the underlying mechanisms of diabetes-associated impairment in cognitive functions have remained unknown (Kodl and Seaquist, 2008). Scientific shreds of evidence are also highlighting

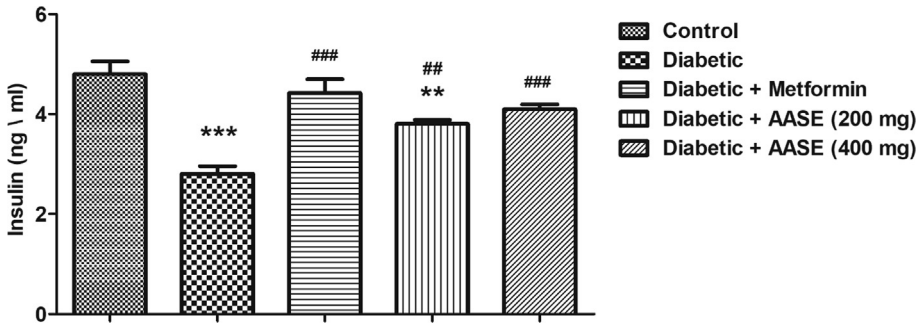

Fig. 7. Effect of AASE and metformin on insulin levels of streptozotocin-nicotinamide-induced diabetic rats. The results are expressed by mean ± SEM (n = 6). One-way ANOVA [$F(4,25) = 16.09$, $p < 0.001$] followed by Tukey-Kramer multiple comparisons test. $^{**}p < 0.01$ and $^{***}p < 0.001$ as compared to the control group; $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ as compared to the diabetic-induced group.

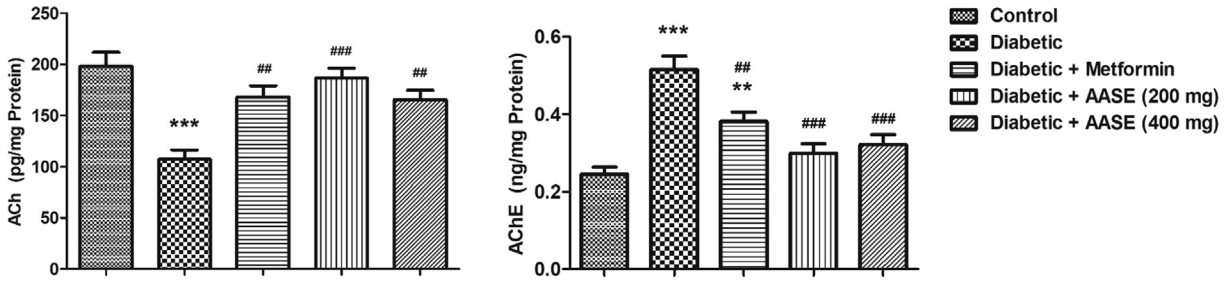

Fig. 8. Effect of AASE and metformin on acetylcholine (ACh) and acetylcholinesterase (AChE) levels of streptozotocin-nicotinamide-induced diabetic rats. The results are expressed by mean ± SEM (n = 6). One-way ANOVA [$F(4,25) = 10.77$, $p < 0.001$ for ACh; $F(4,25) = 15.86$, $p < 0.001$ for AChE] followed by Tukey-Kramer multiple comparisons test. $^{**}p < 0.01$ and $^{***}p < 0.001$ as compared to the control group; $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ as compared to the diabetic-induced group.

the crucial role of prolonged abnormalities in blood glucose levels, lack of insulin activities, and a deficit of cholinergic neuronal functions diabetes-related cognitive and memory dysfunction (Wang et al., 2019). Since DM is linked to the development of neurological comorbidities, efforts were made to probe the influence of DM on the brain. The present study demonstrated that the use of aqueous Ajwa dates seeds extract (AASE) not only reduces the plasma sugar level but also (i) ameliorates the T2DM induced cognitive deficits (ii) recovers the activities of insulin secretion and (iii) neuronal cholinergic transmission in rats.

Administration of nicotinamide followed by STZ is an established procedure to induce T2DM in rats. STZ destroys pancreatic β cells, while prior administration of nicotinamides gives partial protection to insulin-secreting β cells from STZ (Aboonabi et al., 2014). In pancreatic β-cells, STZ induces DNA damage followed by enhancing the action of poly(ADP-ribose) polymerase (PARP-1). Yet, the disproportionate activity of PARP-1 results in a reduction of NAD^+ and ATP concentration intracellularly, resulting in necrosis of insulin-secreting cells. Simultaneous administration of nicotinamide inhibits the elevation of enzyme PARP-1 and prevents over depletion of NAD^+ and ATP in pancreatic β cells (Szkudelski, 2012). As a result, blood glucose levels in diabetic rats might from mild hyperglycemia to severe hyperglycemia depending on the selection of dose and examination time after induction with STZ and nicotinamide. Similarly, blood insulin levels are also reduced slightly or significantly, resulting in hypoinsulinemia (Szkudelski, 2012). As per our results, administration of nicotinamide mg/kg, followed by STZ successfully induced T2DM in groups of rats which was indicated by high glucose and reduced insulin levels in plasma. However, oral administration of two doses. of AASE (200 as well as 400 mg/kg, p.o.) expressively attenuated T2DM by reducing blood glucose levels and enhancing the plasma insulin levels in the rat experimental model.

In addition, insulin and Insulin receptors (IRS) are associated with crucial biological activities in the CNS. IRs are highly expressed in neurons, particularly in the hippocampus. Insulin can reach the brain bypassing the BBB, besides being synthesized by neurons (Soto et al., 2019). Insulin signaling pathways have a major role in neuronal survival in addition to learning and memory processes. Administration of peripheral insulin was reported to improve memory functions in diabetes-induced animal models (Zhao et al., 2004). The same study also emphasized the importance of insulin and its receptors, found in the cerebral cortex and hippocampus for the brain and its cognitive functions. In this study, plasma insulin levels were found to be dropped significantly in the diabetic group. Interestingly, administration of 200 and 400 mg/kg AASE showed not only showed improvements in the regulation of insulin in diabetic rats, but the results were almost identical to the control group for the animals treated with AASE at 400 mg/kg. This effect may be attributed to the possibility, that AASE has a protective effect on pancreatic β-cells against the cytotoxic effect of STZ. Also, previous studies reported that the dates seed extract could protect liver tissue against diabetic cytotoxic damage, which may be involved in preserving of the metabolic homeostasis of glucose and insulin and improving their levels (El Fouhil et al., 2013). The results reveal that AASE is a potential therapeutic candidate for T2DM.

Presently, STZ and nicotinamide-induced diabetic models have numerous pathological outcomes that represent T2DM and can be used as models for pharmacological evaluation, including cognitive abnormalities. Moreover, the induction of hyperglycemia in rats significantly affected the normal cognitive functions observed in various behavioral tests. Results from EPM, NOR, and Y-Maze tests, reveal memory impairment in diabetic rats and the results can line with previously reported studies (Semuyaba et al., 2017; Wang et al., 2019). In addition, AASE was tested for two selected doses (200 as well as 400 mg/kg) to evaluate its influence on memory function. Results reveal that AASE reversed the memory impairment at both dose levels. EPM was used as a tool to study the behavior and cognitive function of rats. EPM is a neutral behavioral model, frequently utilized to assess the memory of rodents. The test showed longer transfer latency (TL) time associated with diabetic animals compared with a control group. Interestingly, this length in the TL time was reduced in the metformin-treated group as well as AASE treated group. Both doses of AASE showed significantly improved results indicating the possibility of a protective effect on the hippocampus neurons. Thus, treatment of AASE also increases the availability of acetylcholine and insulin during the performance of the behavioral tasks as the results showed, which reduces the anxiety behavior of the rats during the performance of the task as previous studies showed and in turn, improves memory performance (Subash et al., 2015).

NOR test is used to distinguish between a novel (NO) and a familiar (FO) object. During the training session, the animals are allowed to attend to two identical objects first to preserve them in working memory (Silvers et al., 2007). The results of the training session considerable reduction in the exploration time for diabetic group rats, as likened to the control group. Treatment of AASE and metformin resulted in an increase of exploration time in diabetic-induced animals and the results were comparable to the control group. It is evidenced that induction of diabetes affects the ability of acquisition of animals. However, no significant difference in exploration time for FO1 and FO2 explained that neither AASE nor diabetic induction altered to recollect the similarity of the identical objects in this study.

During test session (T2), when used a FO and a NO, the animals showed a substantial increase in novel object exploration time as compared to the corresponding group of familiar object exploration time. It indicates that animals preferred to spend a long time with the NO than FO, suggesting the retention capacity and discrimination ability of both objects, as well as the capacity to remember the FOs from T1. Additionally, the diabetic group rats

showed significantly lower exploration time for NO indicating their lower retention as well as the discrimination ability. The administration of both doses of AASE and metformin improved the cognitive functions in T2DM rats. In the NOR test, enhanced cognitive skills are required to recognize novelty, such as exploring a single novel object or performing a task in a novel environment (Silvers et al., 2007). Our results are aligned with the earlier reports, when the animals are allowed to approach between NO and FO and they frequently approach and like to spend more time exploring the NO than the familiar one (Seibenhener and Wooten, 2015; Ennaceur, 2010). Furthermore, the results of DI for treatment groups provided additional proof of the animal's discrimination capacity during a test session. Groups treated with AASE demonstrated a better DI than the diabetic groups. Typically, the DI is used to explain how animals distinguish between familiar and unknown items.

The Y-maze test was utilized in this study to assess spatial working memory. Most brain areas are involved in Y-maze processing, including the hippocampus, prefrontal cortex, and basal forebrain. Principally, the behavior of remembering the arms visited during the training session and showing a tendency to enter a less novel arm are underlined to prefrontal cortical functions (Liet et al., 2015). The results from the Y-maze test results allow examination of the effect of diabetes induction and AASE administration on various cognitive performances in rats, which are connected with several parameters. The differential behavior of animals towards the known arm and novel arm is indicated by the number of entries. Our results show that induction of diabetes in rats resulted in reduced entries to both known and novel arms thus, highlighting the loss of spatial memory of the animals (Tripathi et al., 2017). Interestingly, the treatment of AASE reversed the number of entries to the novel arm is indicating attenuation of diabetic-induced spatial memory impairment.

In the test session, the fraction of time spent in the novel arm and total time was used to calculate the animals' coping behavior in a novel environment. Also, the increase in anxiety behavior of the animals is linked with a decrease in coping behavior (Poimenova et al., 2010). The diabetic-induced group had a lower value of coping behavior than the control group, indicating that the animals were associated with anxiety-like behavior. Oral administration of AASE, on the other hand, reduces the diabetic-induced loss of coping strategy by increasing the amount of time that animals spend in a novel environment. In addition, the total number of entries during the trial and test periods in the Y-maze indicated the animals' curiosity behavior (Kraeuter et al., 2019). Both doses of AASE and metformin significantly increased the curiosity behavior of diabetic-induced rats.

Presently, there is a limited number of evidence found about the influences of dates in memory functions. Recent reports suggest that the 2% and 4% dietary supplementation of date fruits (from Oman) leads to a significant reversal of memory deficit, anxiety-related behavior, and motor coordination in transgenic mice for AD (Subash et al., 2015). Moreover, under *in vitro* conditions, date palm fruits grown in Oman can suppress the production of Aβ fibrils (Essa et al., 2012). In addition, the same treatment significantly lowered $A\beta_{40}$ as well as $A\beta_{42}$ proteins in plasma. Recently, reports showed restoration of learning and memory and impairments improvement obtained through continuous twelve days of treatment of date seed extract (Bamy Mozafaty rutab). The study was conducted by Morris water maze test and diabetic condition was induced with ICV injections of $A\beta_{25\text{-}35}$ (Dehghanian et al., 2017). Our results are parallel to the above-mentioned report.

Acetylcholine (ACh) plays important role in activating nicotinic receptors of the hippocampus and is known for memory formation and converting to long-term memory (Yakel, 2012). Levels of ACh in the hippocampus are linked to memory function. It is also established with a decrease in hippocampus ACh levels is linked to age-related cognitive decline. In addition, it is also reported that ACh release is increased in the hippocampus while performing spatial memory tasks (Stancampiano et al., 1999). Also, damage to the medial septum causes a decrease of ACh levels in the hippocampus and impairs spatial memory performance (Herzog et al., 2000). From our study, diabetes initiated a reduction in the ACh levels in the brain, and these changes were recovered with animals treated with AASE. On the other hand, the enzyme acetylcholinesterase (AChE) found in the synaptic cleft rapidly metabolizes ACh to choline and acetate and inhibits its neuronal signaling. On the other hand, the enzyme acetylcholinesterase (AChE), which is present in the synaptic cleft that quickly converts ACh to choline and acetate and inhibits its neuronal signaling (Ahmad et al., 2014). The induction of diabetic resulted in a higher level of AChE activity in rat's brain, refer to the current study. Earlier, the diabetic-induced group showed the elevation of AChE levels in different brain areas including hippocampus, cortex, and striatum as 58%, 46%, and 30%, respectively as compared to control rats (Schetinger, 2014). Nevertheless, thirty days of treatment with AASE improved the central cholinergic transmission by declining AChE action in the diabetic-induced rats' brain.

Neurotransmitter concentrations in specific brain regions are altered in individuals with diabetes mellitus and animals with experimental diabetes, and these alterations are connected to CNS diseases. In terms of the relationship between insulin and ACh levels in the CNS, our research reveals that insulin-resistant rats had lower ACh levels in the brain. The biosynthesis of ACh in neurons requires sufficient availability of choline and acetyl-Co-A and is aided by insulin stimulation (Rivera et al., 2005). Furthermore, earlier research demonstrated that reduction of insulin transmission in brain results in decreased ACh production in AD. As a result, changes in insulin signaling in the brain connected to cognitive dysfunction may be linked further to AD. Moreover, increasing uptake of glucose uptake and level of insulin in the brain and hence, higher ACh concentration with ACh agonist treatment also support the relationship between the levels of insulin and ACh (Dubey et al., 2020). Administration of AASE considerably increased plasma insulin and brain ACh levels in type-2 diabetic-induced rats, which confirm this relationship.

The present findings support AASE's potential efficacy in reversing T2DM-related cognitive impairments in the STZ-nicotinamide-induced rat model. The present study has some limitations, it is a preliminary pre-clinical evaluation using an animal model with focused limited mechanisms. However, these results will initiate the researchers to explore the additional supportive evidence related to the potential effects of Ajwa seeds on different causes of dementia including diabetes and cognitive issues to support its further clinical uses. In the future, the study can be extended to explore the effect of Ajwa seeds on the specific areas of the brain such as the hippocampus, cortex, along with focusing on other major neurotransmitters like serotonin, norepinephrine, and dopamine. it will be more informative to target brain-related disorders including memory dysfunctions.

5. Conclusions

Overall, the findings revealed that AASE had a promising neuroprotective effect against diabetic-induced cognitive impairment in rats. In maze models, continuous thirty-day oral therapy with AASE (200 and 400 mg/kg) improved cognitive functions. The shorter transfer latency in the EPM, improved the exploration time of a NO and DI between a FO and NO in the NOR, and reversal in the number of novel arm entries, coping behavior, and curiosity behavior in the Y-Maze test were recorded. Further exploration of mechanisms reveals that the AASE treatment reduces blood glucose

levels, elevates plasma insulin levels, improves the CNS cholinergic activity by elevating the ACh and reducing AChE levels in the diabetic-induced rat model. In conclusion, AASE was also identified as a possible therapeutic target for decreasing diabetic-induced cognitive deficits in this investigation; however, further extensive mechanistic explorations are needed to validate present preliminary preclinical findings.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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