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Rosa canina L. improves learning and memory-associated cognitive impairment by regulating glucose levels and reducing hippocampal insulin resistance in high-fat diet/streptozotocin-induced diabetic rats

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ABSTRACT

Ethnopharmacological relevance: Recent studies claim that Type-2 diabetes mellitus (T2DM) and Alzheimer's disease (AD) overlap in several common pathological pathways which from neuronal damage to impaired memory performance. It is known that the use of *Rosa canina* L. (*R. canina*) as medicine in folk medicine dates back to ancient times and is used in the treatment of nervous diseases in Persian medicine. However, the effect of *R. canina* on diabetes-related cognitive decline and memory impairment has not yet been studied.

Aim of the study: We evaluated the impact of T2DM on AD-like alterations and examined the molecular mechanism of a possible effect of *R. canina* on cognitive alterations in diabetic rats.

Materials&methods: R. canina ethanol extract was obtained by maceration method. This study was performed with male Sprague-Dawley rats fed with a high-fat diet (HFD) for 8 weeks, low-dose streptozotocin (STZ; 35 mg/kg IP) injection for 4 weeks, and *R. canina* (250 mg/kg; per oral) and metformin (400 mg/kg; per oral) administration for 4 weeks. The weight and blood glucose of rats were measured weekly. To evaluate glucose tolerance area under the curve (AUC) was calculated by performing an oral glucose tolerance test. Then the rats were subjected to behavioural tests, and their hippocampus and cortex tissues were obtained for biochemical and morphological analyses.

Results: R. canina could manage glucose responsiveness by reducing post-prandial blood glucose levels, preventing weight loss, and raising serum insulin levels in T2DM-induced rats. Behavioural tests showed that *R. canina* significantly improves diabetes-related cognitive decline in recall and long-term memory. Treatment with *R. canina* significantly reversed HFD/STZ-induced increases in insulin, amyloid-β, amyloid precursor protein levels, and acetylcholinesterase activity in the prefrontal cortex and hippocampus. Furthermore, histological analyzes revealed the protection of *R. canina* against neuronal disruption in the cortical and hippocampal CA3 region caused by chronic hyperglycemia.

Conclusion: Analyzed collectively, these results suggest that *R. canina* can correct T2DM-related cognitive decline may be attributed to insulin pathway modulation, prevention of amyloid deposition, and increased cholinergic transmission.

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1. Introduction

Type-2 Diabetes Mellitus (T2DM) is a complex disease related to insulin resistance (IR) and hyperglycemia (Pugazhenthi et al., 2017). Clinical and preclinical studies suggest that memory impairment characterized by AD in T2DM may be caused by progressive insulin insensitivity with the contribution of IR status and glucose toxicity (Tumminia et al., 2018). Generally, common mechanisms such as vascular injury, decreased cerebral flow, altered insulin signaling, hyperglycemia, advanced glycation, and abnormal inflammatory response are overlapping points in the pathogenesis of both conditions (Bedse et al., 2015; Srikanth et al., 2020). Although there are mixed results regarding the contribution of diabetes to amyloid-β (Aβ) accumulation in the brain, the fact that it promotes cognitive deficits and affects insulin sensitivity is considered to be a remarkable factor (Moran et al., 2015). Induction of IR in diabetic obese rodents (through a high-fat diet) results in increased levels of secretase enzymes and amyloid precursor protein (APP) that produces brain Aβ levels (Tumminia et al., 2018). Chronic hyperglycemia-induced amyloid deposition causes cognitive impairments by promoting several mechanisms including a reduction in cholinergic transmission and damage to the antioxidant defense (Moreira, 2013). Increasing evidence is that up-regulated Aβ in DM increases inflammatory responses in the brain (De Felice and Ferreira, 2014). The deterioration of homeostasis is caused by the increase in inflammatory factors in the brain and the activation of the neuroinflammation pathway causes damage to the synaptic connections and activates the pathway to cognitive damage (Plata-Salamán and Ffrench-Mullen, 1994). Various clinical studies provide a wealth of evidence associated with cognitive and reasoning dysfunctions in the T2DM (You et al., 2021). According to the data, the dramatic escalation of the patient with diabetes leads to an increase in the incidence of dementia and shows that it will bring many socio-economic difficulties that society and the patient's relatives will have difficulty overcoming (McNay and Recknagel, 2011). Currently, drug development studies that offer effective solutions for the treatment of AD are increasing exponentially (Zhao et al., 2021). Research proves that medicinal plants are beneficial in managing the course of diseases, thanks to their active ingredients. Moreover, natural products are the main advantages in terms of effectiveness, reduced side effects, and low cost, compared to chemical drugs (Hasanein and Shahidi, 2012). Modern studies commonly concluded that many natural products and their bioactive components can exhibit neuroprotective effects (Kamdi et al., 2021).

Recent studies have shown that *Rosa canina* L. (*R. canina;* Nastaran or Nasrin in Persian), a plant widely used in traditional medicine, has anti-inflammatory, anti-oxidant, hypoglycemic, and hypolipidemic actions. From a biochemical point of view, *R. canina* fruit contains bioactive compounds (e.g., phenolic acids, proanthocyanidins, polyphenols, minerals, vitamin C, and carotenoids) which have multiple functions (Golsorkhi et al., 2022). Thanks to the rich content of the fruit of the *R. canina* plant and its strong pharmacological activity, its use as a medicinal plant in the treatment of diseases dates back to past history. Even so that it was included in the diet for scurvy due to its ascorbic acid content in England during World War II (Lattanzio et al., 2011). The first information about its use in alleviating forgetfulness by traditional Persian medicine has been transferred from the History of Medicine book written by Ibn Sina (Avicenna, 2005). It is stated that it is widely used especially in Persian medicine with its strengthening effect on some neural diseases and memory (Khazaei and Pazhouhi, 2020; Shirbeigi et al., 2018; Taghizadeh et al., 2016; Yousefsani et al., 2021). Its use as a brain tonic is recommended in The Storehouse of Medicaments (Shoara et al., 2015). Several experimental *in vivo* studies, based on its use in traditional folk medicine, have provided evidence that it alleviates cognitive impairment (Daneshmand et al., 2016). Attenuation in oxidative stress and neuroinflammation and increased expression of synaptic proteins in the hippocampus are indicated as the underlying mechanism of improved cognitive function in behavioural tests of rats exposed to heat stress treated with *R. canina* treatment (Erfani et al., 2019). It has been shown in previous studies that the methanol extract of *R. canina* improves memory impairment due to heat stress in rats (Erfani et al., 2019) and has antioxidant and alpha-amylase inhibitory effects. It is also known that *R. canina* methanol extract is richer in antioxidant polyphenol content compared to distilled water and ethanol extracts (İlbay et al., 2013). The aforementioned reports (Daneshmand et al., 2016; Yousefsani et al., 2021) point to the potential of *R. canina* to exert efficacy against AD-like pathology and cognitive decline associated with T2DM. However, according to the literature, it is known that there is a lack of experimental studies on this subject (Erfani et al., 2019). We hypothesized that *R. canina* would affect pathological mechanisms involved in insulin resistance, AD-like amyloid-β (Aβ), cholinergic transmission, and cognitive impairment in T2DM rats. Rats fed a high-fat diet experimentally cause T2DM, aggravated cognitive impairment, and glucose intolerance. Therefore, this model was used to enlighten the possible mechanism of *R. canina* (Ko et al., 2021).

2. Materials & Methods

2.1. Chemicals

High-fat diet (HFD) comprises 34.5% carbohydrate, 20.5% protein, 45% fat, and 3266 kcal/kg diet energy content. Normal-fat diet (NFD) comprises 77.3% carbohydrate, 20% protein, 2.7% fat, and 3266 kcal/kg diet energy content. Streptozotocin (STZ) was purchased from Sigma-Aldrich. Metformin was provided by Merck Serono (Shanghai, China). Enzyme-linked immunosorbent assay (ELISA) kits of rat insulin, amyloid-β, APP, and AChE were purchased by Shanghai Enzyme-linked Biotechnology Co. Ltd. (Shanghai, China).

2.2. Plant collection and preparation of the Rosa canina methanol extract

Rosa canina fruits were purchased from the market in Turkey and authenticated by Dr. Zeki Severoğlu from the Biology Department of Marmara University with the number 5107 for future reference. The fruits of the plant were processed with a mechanical grinder (Renas, RBT1250) to a fine powder. Methanol extracts were prepared from the powdered samples using the maceration method. A rotary evaporator was used to evaporate the solvents under low pressure and temperature. The refrigerator was set to keep the raw extracts at +4 °C.

2.3. Animals

Male Sprague–Dawley rats were used, each weighing 250 ± 20 g at Marmara University the Experimental Animal Implementation and Research Center (DEHAMER) of Marmara University. All procedures were approved by the Marmara University Animal Experiments Local Ethics Committee (permission no: 77.2018.mar) and were performed following the Guidance for the Care and Use of Laboratory Animals. Animals were housed in conditions suitable for experimental and ethical conditions (22 ± 2 °C room temperature, a 12 h light/12 h dark cycle, relative humidity (50 ± 5%) and provided with water and fed a standard diet ad libitum before dietary change).

2.4. Experimental design

Immediately after a one-week laboratory acclimation period, the rats were randomly assigned to diet patterns: (1) Normal fat diet: The control rats were fed with standard laboratory chow throughout the 8-week; (2) High-fat diet (HFD) and an injection of a low dose of STZ: In the HFD group was fed with HFD containing 45% energy from fat. At the end of week 4, diabetes was induced in HFD-fed rats with STZ after 24 h of fasting (i.p 35 mg/kg; pH = 4.5 in 0.1 M citrate buffer). The control group was injected with saline. HFD-fed rats received a 20% glucose solution overnight to prevent the formation of a hypoglycaemic profile.

To confirm diabetes status blood glucose level was measured with a glucometer (ContourTM TS, Bayer Diagnostics) after 72 h of STZ injected. Blood glucose level above 200 mg/dL was considered for diabetic conditions (Cam et al., 2019). Body weight and food intake were monitored weekly.

HFD diet-fed and STZ-injected animals were randomly assigned to one of three groups (n = 8): (n = 8): HFD/STZ, HFD/STZ + *R. canina,* and HFD/STZ + Metformin (MET) groups. For four weeks, these experimental groups were given R. *canina* at 250 mg/kg (Taghizadeh et al., 2018) and MET at 400 mg/kg (Cam et al., 2019) intragastrically per day, whereas the control group received only normal saline. In this study, MET was preferred as the control group due to its effects on indirectly lowering blood glucose levels and improving memory performance with its central effect (Oliveira et al., 2016). After examination of behavioural tests, rats were sacrificed and the blood, hippocampal, and cortex samples were collected for analysis of biochemicals. The operating schedule is given in Fig. 1.

2.5. The oral glucose tolerance test (OGTT) and the insulin resistance index

It is well known that the OGTT offers the opportunity to assess glucose homeostasis and insulin resistance in rodents. Briefly, after fasting for 24 h, rats were given a 2 g/kg dose of 50% glucose solution orally, in the last week of the experiment. Blood glucose (Bg) was measured in samples collected from a small incision made at the tip of the tail at baseline, 30, 60, 90, and 120 min after administration of glucose, and the area under the curve (AUC) describing blood glucose levels in each rat during OGTT was calculated as reported previously (Cao et al., 2016). ELISA kit was used for plasma insulin level determination. As the insulin resistance index, homeostasis model assessment of insulin resistance (HOMA-IR) = Fasting plasma insulin level (mIU/L) * Fasting plasma glucose (mmol/L)/22.5 (Bowe et al., 2014).

2.6. Behavioural tests

2.6.1. Open field test

The open field test (OFT) was performed to determine general locomotor activity capacities and exploratory behaviours. Briefly, the animals were placed in the center of the box with a size of 50 × 50 × 30 cm^3 and the bottom of which was divided into four identical squares. The distance traveled was recorded for 5 min with a video camera.

2.6.2. Novel object recognition test

The test consists of 3 phases: familiarization, training, and testing phases as previously described by (Cevikelli-Yakut et al., 2020). One day before the training test, during the acclimatization phase, the rats were kept in this semi-dark box for 3 min and allowed to adapt to the environment. It was ensured that the rats were left facing the wall at an equal distance from the objects. Then, conditions were set up in which the rat was allowed to explore (time spent sniffing or touching the object) two objects that were the same in appearance, but different in the training phase. After 1 h, one of the familiar objects was replaced with a new object in the test phase. The time the rat spent exploring each object was recorded in the previous stage (Cevikelli-Yakut et al., 2020). Afterward, the indexes of discrimination and preference were calculated as follows: *Discrimination Index (DI) = (tN1-tF1)/(tN1 + tF1)*

Preferential Index (PI) = tN1/(tN1 + tF1)

tF and tN are exploration times for familiar and new objects, respectively.

2.6.3. Passive avoidance test

The passive avoidance test (PAT) was carried out in the apparatus, which consists of 2 sections-one dark and the other light- and these are separated from each other by a door. During the acquisition experiment, the rats were individually placed in the light compartment and then the rat entered the dark compartment, with automatic door closure, and supplied an electric shock (40 V, 0.5 A, 3 s) to the bottom of the chamber. After 24 h from this stage, the time delay for animals to pass into the dark room as the step-through latency without electric shock was recorded in the retention test. The cut-off time was considered as a step-through latency of 300 s.

2.6.4. Morris water maze test

The MWM test, which has a remarkable importance in evaluating hippocampus-dependent spatial memory, was performed in the 8th week of our study. A pool with a circular shape and dimensions of 150 cm in diameter and 40 cm in height was used for the test (Topal et al., 2022). The tank filled with water (26 °C ± 1 °C), which is painted with non-toxic black paint, is divided into 4 equal parts N, S, W, and E for ease of direction determination. Then, we placed a platform in the WS (target quadrant) part so that it was 1 cm below the water. In the acquisition phase, rats were subjected to daily training trials in each quadrant for four consecutive days (once a day), each limited to 75 s. The trial in each direction was ended by allowing the rat to stand on the platform for 20 s. At this stage, shortening the time to find the submerged platform is generally accepted as a normal memory indicator. In the acquisition phases, the escape latencies to the hidden platform and total swimming distance during the learning phase were recorded for each group for four consecutive days. On the 5th day, the probe trial was performed in absence of the platform to examine reference memory. During the probe trial, parameters such as the time to first pass to the target quadrant, the time spent in the target quadrant, and the trajectory in the tank were recorded As in our previous study, the specified data were recorded with

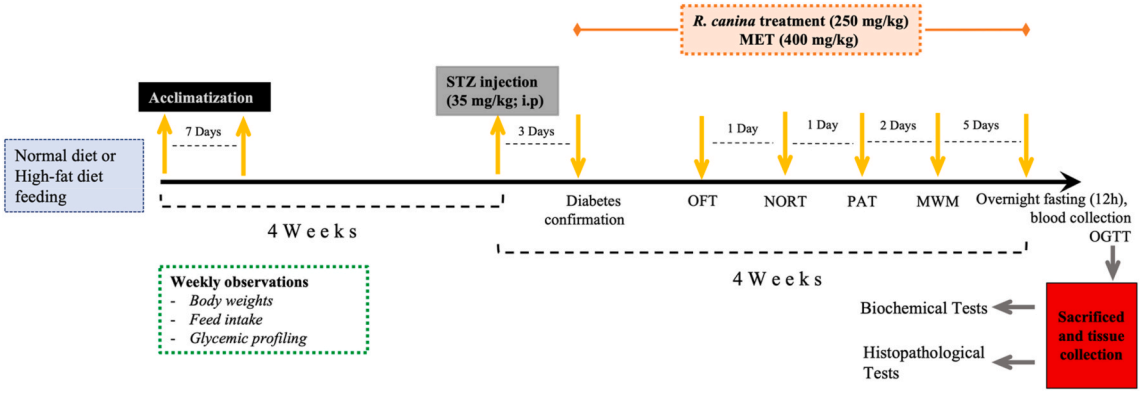

Fig. 1. The experimental timeline. STZ: Streptozotocin; MET: Metformin; OFT: Open field test; NORT: Novel object recognition test; PAT: Passive Avoidance test; MWM: Morris's Water maze.

the automatic video-camera processing system we developed (Topal et al., 2022).

2.7. Specimen collection and storage

After the behavioural tests were completed, euthanasia was performed by anaesthetizing with 1% pentobarbital sodium (40 mg/kg, i.p.). The brains were quickly removed, paying attention to their suitability for each test: (1) The hippocampus and prefrontal cortex sections were kept separately from each other at −80 °C for the commercial kit. (2) Brain tissue samples from each of the three rats were kept in 4% paraformaldehyde for hematoxylin and eosin (H&E).

2.8. Enzyme-linked immunosorbent assay

Isolated tissue samples were prepared in ice-cold phosphate buffer (pH 7.4, 0.1 M) to be 10% w/v homogenates using IKA brand Ultra-Turrax T25 (USA) homogenizer. Then, samples were centrifuged at 3000 g for 10 min and obtained supernatant. Insulin, Aβ, APP, and AChE levels of the serum, hippocampus, and prefrontal cortex were estimated using rat ELISA kits. The manufacturer's instructions were followed during all procedures.

2.9. Histopathologically analysis

Prefrontal cortex and hippocampus tissues were fixed for 24 h for histological analysis. For histological analysis, prefrontal cortex and hippocampus tissues were fixed and cleaned in a gradient alcohol dryer and then dried. The dehydrated tissues were cleared by keeping them in xylene for 2 × 10 min and were then embedded in paraffin in a 60 °C oven overnight. The samples were sliced into 4-μm thick slices with a paraffin slicer. The pathological changes of the hippocampal CA3 and cerebral cortex regions were stained with H&E. Images were observed and analyzed under a florescent microscope (LEICA DM 1000) at a magnification of 200 × .

2.10. Statistical analysis

The grading and scoring of behavioural tests were done with the support of an independent researcher. Data were collected in GraphPad 8.0 software (GraphPad Software Inc., San Diego CA, USA) and expressed as mean ± SEM. Behavioural tests such as OFT, NORT, PAT, probe trial and biochemical test results were analyzed with one-way ANOVA followed by Tukey multiple comparison tests. Escape latency and path length results of MWM were evaluated with two-way ANOVA. The p-value of <0.05 in the changes was an indication of significance.

3. Results

3.1. Effects of R. canina and metformin on the metabolic outcomes in HFD/STZ-treated rats

As shown in Fig. 2a, an increase in body weights was observed in all groups in the first four weeks of the study. Body weights did not differ significantly in the first four weeks between the HFD/STZ, *R. canina*, and MET treatment groups. The reason for that can be interpreted as the low-dose STZ administration after the first four weeks of HFD feeding and the initiation of the treatments (Zhou et al., 2017). The weights of the

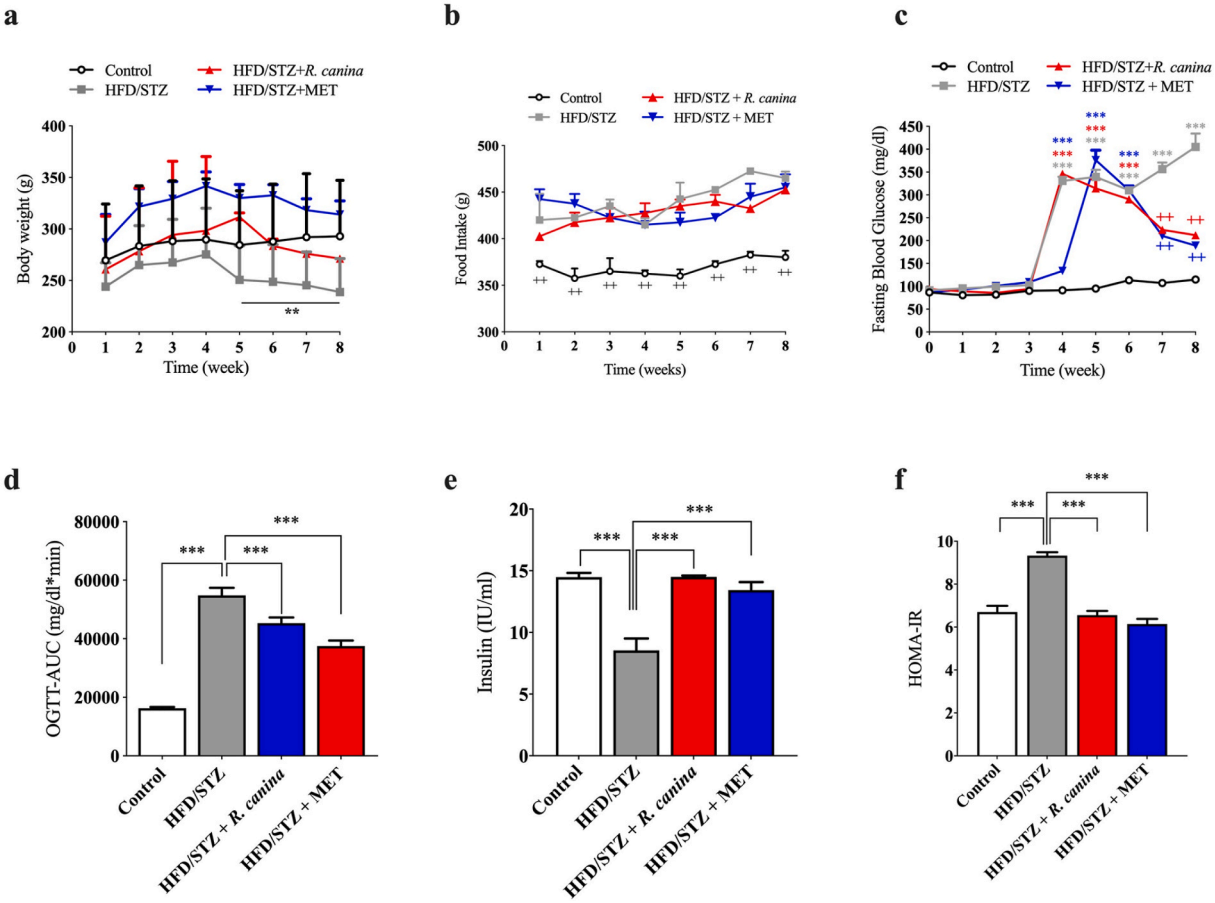

Fig. 2. Effects of *R. canina* on the level of a) Body weight, b) Food intake, c) Fasting blood glucose, d) Oral glucose tolerance test (OGTT)-Area under the curve (AUC), e) Serum insulin (ELISA), and (f) HOMA-IR. **p < 0.01, ***p < 0.001 vs. Control group; ++ p < 0.01 vs. HFD/STZ group.

animals in the HFD/STZ and HFD/STZ + MET groups had decreased by the fifth week. The body weight of the HFD/STZ rats significantly decreased ($p < 0.01$) in comparison to the control group between 5 and 8 weeks. The weight of *R. canina*-treated HFD/STZ rats was slightly compared to HFD/STZ group. However, HFD/STZ rats that received HFD and low-dose STZ showed a significant ($p < 0.001$) 3.5-fold elevation of blood glucose level in the 5th week compared with the control rats. However, *R. canina* treatment caused a significant drop in the blood glucose level compared to HFD/STZ rats between 7 and 8 weeks ($p < 0.05$). *R. canina* treatment produced a significant 1.5-fold decrease in blood glucose compared with the diabetic rats in the 7th week. At the end of the experiment, the *R. canina*-treated group had lower ($p < 0.05$, 1.8 fold) blood glucose levels than the HFD/STZ group and showed a similar glucose level to the HFD/STZ + MET group. Serum insulin levels in the MET ($p < 0.01$) and *R. canina* ($p < 0.01$) groups were significantly higher than in the HFD/STZ group (Fig. 2e). HOMA-IR has been widely used as an insulin resistance index (El-Sayed et al., 2022). A comparison of groups in HOMA-IR (Fig. 2f) showed that the HFD/STZ group had significantly higher level of HOMA-IR than the control groups ($p < 0.001$). These results confirm that T2DM rats fed with the HFD present insulin resistance and glucose intolerance. However, compared to the HFD/STZ group, HOMA-IR was significantly lower in the *R. canina* ($p < 0.001$) and MET ($p < 0.001$) groups.

3.2. Effects of R. canina on locomotor activity

After 4 weeks of treatment, anxiety state and locomotor activity performance in rats were assessed by OFT. As shown in Fig. 3a–c, the results showed that there was no significant difference in latency to enter the center, the time spent in the central zone, and the number of square crossings between all groups. Furthermore, the HFD/STZ rats subjected to rearing and grooming of activity scores had no alteration compared to the control group. In this regard, there was no significant change between the *R. canina* and MET groups.

3.3. Effects of R. canina on short-term memory

When the time to recognize and discover the new object and the old object is compared, there is a visible difference ($p < 0.05$) in the control group, but not in the HFD/STZ group (Fig. 3f). In addition, the fact that DI ($p < 0.01$) and PI ($p < 0.001$) were dramatically reduced in the HFD/STZ group compared to the control group confirms this result (Fig. 3f and g). Likewise, *R. canina* showed a considerably increased ($p < 0.01$) compared to HFD/STZ group in DI. PI was significantly increased in the *R. canina* and MET treatment group compared to the HFD/STZ group ($p < 0.05$).

3.4. Effects of R. canina on passive avoidance performance of HFD/STZ rats

In contrast to the control group, HFD/STZ rats displayed a significant ($p < 0.05$) impairment in retention and memory during the passive avoidance test, as shown in Fig. 4a. Additionally, rats treated with *R. canina* had higher retention and memory than the control group ($p < 0.05$).

3.5. Effects of R. canina on cognitive dysfunction of HFD/STZ rats

It is well known that there is a correlation between T2DM and reduced memory performance. In this context, we evaluated hippocampal spatial memory in HFD/STZ-induced rats with the MWM test. The results illustrated that the HFD/STZ group attempted to reach the platform later than the control group between days 2–4, as seen in Fig. 4b. Remarkable differences were noticed between the HFD/STZ and

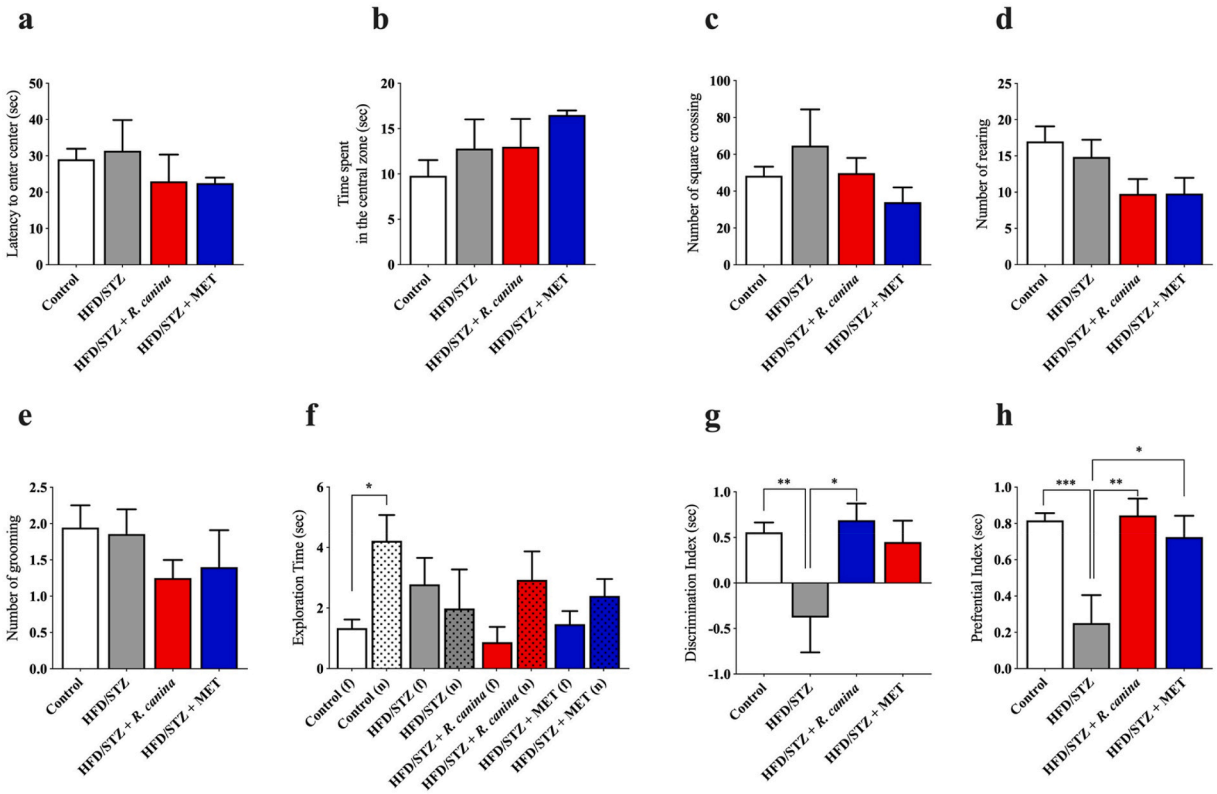

Fig. 3. Behavioural performance in all groups' open field test (OFT) and novel object recognition test (NORT). a) Latency to enter the center, b) Time spent in the central zone, c) Number of square crossing, d) Number of rearing, e) Number of grooming, f) Exploration time, g) Discrimination index, and h) Preferential index. *p < 0.05, **p < 0.01, ***p < 0.001 vs. Control group.

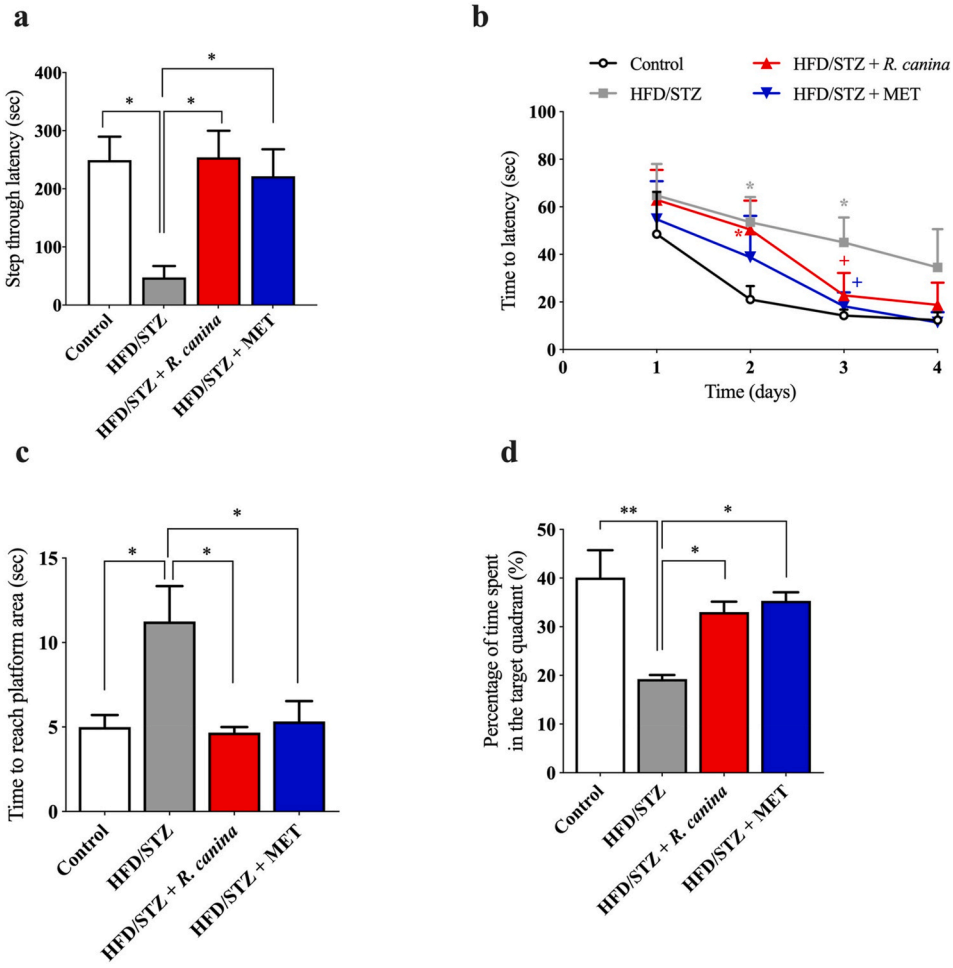

Fig. 4. Effects of *R. canina* on the a) Step-through latency, b) Time to latency, c) The percentage of time spent in the target quadrant, and d) Time to reach platform area. *p < 0.05, **p < 0.01, ***p < 0.001 vs. Control group;+p <0.05 vs. HFD/STZ group.

HFD/STZ + *R. canina*-treated groups on days 2 and 3 ($p < 0.05$). *R. canina* and MET groups had a decreased time to reach the platform on 3rd day, suggesting *R. canina* groups displayed recovery in memory recall. At the end of the acquisition sessions on day 5, no marked differences between all groups appeared. In the other part of the test, the memory retention performance was tested. Therefore, on the 5th day, the hidden platform was removed from the tank. Considering the first time to enter the target quadrant where the platform is located, the HFD/STZ group was noticeably ($p < 0.05$) delayed compared to the control group, while this delay was less ($p < 0.05$) in the *R. canina* group compared to the HFD/STZ group (Fig. 4c). As shown in Fig. 4d, HFD/STZ rats spent considerably ($p < 0.01$) less time in the target quadrant than control rats. However, the R. *canina* group spent more time in the target quadrant than the HFD/STZ group, which may indicate a protective activity in T2DM-related memory loss. The ability to treat memory impairment of HFD/STZ-induced rats with *R. canina* treatment was significantly similar to that of the MET and control groups. (Fig. 4c and d). In addition, decreased movement between quadrants in the tank during probe testing of HFD/STZ rats indicates memory retention impairment.

3.6. Biochemical alterations

The extracellular amount of insulin rises when it is unable to bind to the receptor during its resistance. When compared to the control group in our study, insulin levels increased in the prefrontal cortex and hippocampus of the HFD/STZ group ($p < 0.001$) (Fig. 5a). *R. canina* treatment showed a significant reduction of insulin levels in the prefrontal cortex ($p < 0.01$) and hippocampus ($p < 0.001$) of HFD/STZ animals prefrontal cortex and hippocampal tissue samples from the HFD/STZ-induced T2DM rats had considerably higher levels of Aβ than samples from the animals in the control group ($p < 0.05$ and $p < 0.001$, respectively) (Fig. 5b). However, the level of Aβ in the hippocampal tissue samples of HFD/STZ animals treated with the *R. canina* significantly decreased similar to MET treatment compared to the HFD/STZ group but *R. canina* treatment did not show a noticeable change on Aβ level in the prefrontal cortex compared to the HFD/STZ group. HFD/STZ group showed a considerable increase in the prefrontal cortex ($p < 0.05$) and hippocampus ($p < 0.05$) levels of APP relative to the control group (Fig. 5c). Although the cortical level of APP in the *R. canina*-treated HFD/STZ rats was no change, the hippocampal level of APP was significantly ($p < 0.01$) reduced relative to HFD/STZ group by *R. canina* treatment. As shown in Fig. 5d, cortical ($p < 0.01$) and hippocampal ($p < 0.001$) AChE activity of the HFD/STZ group was higher than the control group. The cortical activity of AChE of *R. canina*-treated had no discernible alteration in HFD/STZ animals, whereas *R. canina* showed AChE inhibitory effects ($p < 0.01$) in the hippocampus of HFD/STZ-induced diabetic rats. Compared to the HFD/STZ group, both cortical ($p < 0.01$) and hippocampal ($p < 0.001$) AChE activities were significantly reduced with MET treatment.

3.7. Effect of R. canina on brain histomorphological changes

In the cortical and hippocampal CA3 sections of the HFD/STZ group,

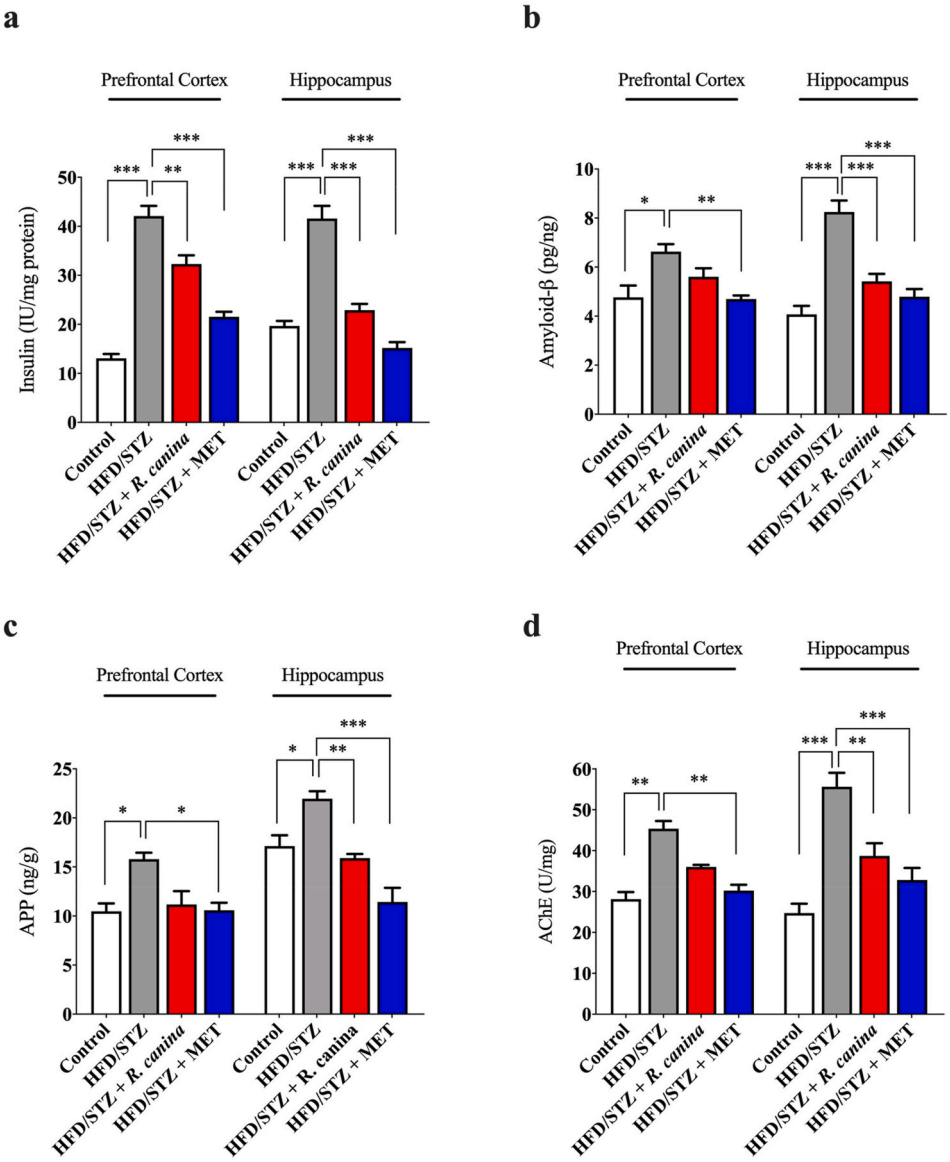

Fig. 5. Effects of *R. canina* on the level of a) insulin, b) amyloid-β, c) APP, and d) AChE on the prefrontal cortex and hippocampus. Significance differences were found at × $p < 0.05$, **$p < 0.01$, ***$p < 0.001$ between groups.

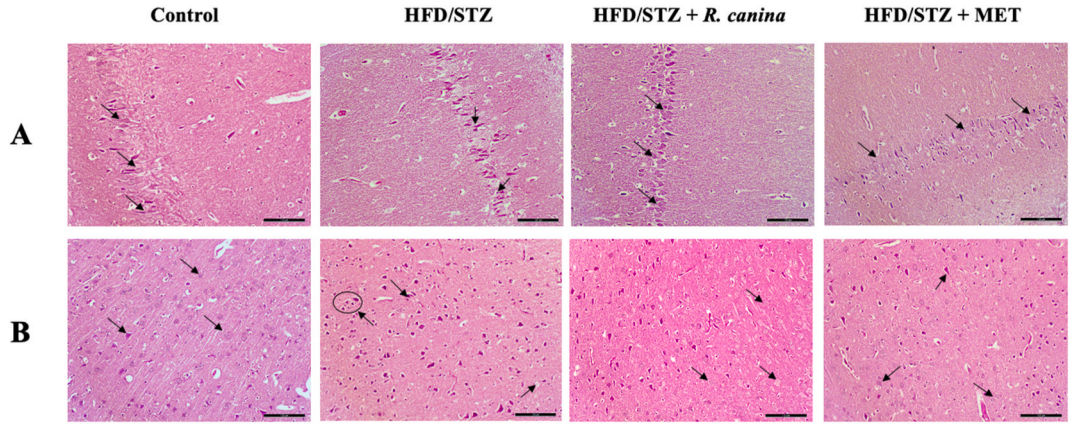

Fig. 6. Effects of *R. canina* on brain injury and neuronal viability of HFD/STZ rats. H&E-stained sections of A) hippocampal CA3 and B) prefrontal cortex regions of the rats (× 200), Scale bar: 100 μm.

severe oedema in the perivascular tissue, pycnotic nucleus, and scattered cell was revealed. Studies show that HFD/STZ disrupts synaptic function by causing neuronal deterioration. Moreover, *R. canina* treatment for 4 weeks resulted in fewer eosin-stained cells, regular cell structure, and less vacuolization in the same tissues, indicating reduced neuronal damage (Fig. 6A and B).

4. Discussion

Epidemiological evidence suggests that AD significantly overlaps with T2DM in terms of risk factors and pathophysiological mechanisms (Luchsinger et al., 2004). Hyperglycemia and insulin resistance activate many pathways that can ultimately cause neuronal damage and cognitive impairment (Ohara et al., 2011). Our first observation was that insulin resistance induced by HFD/STZ enhanced Aβ deposition on the brains of HFD/STZ-induced rats. Secondly, *R. canina* extract could have therapeutic effects on memory functions and pathological deficits associated with AD in T2DM rats. Behavioural test results showed that *R. canina* treatment partially alleviated diabetes-related cognitive impairment. Data from animal studies indicated that induction of hyperglycemia requires high doses of STZ (Ventura-Sobrevilla et al., 2011). High doses of STZ (>50 mg/kg) increase the mortality rate, whereas low-dose STZ (25 mg/kg) is not sufficient for hyperglycemia (Hu et al., 2013). In addition, numerous studies are presenting the pathophysiological resemblance of the HFD/STZ rat model to the human late-stage T2DM (Chao et al., 2018). Because low-dose STZ causes mild β-cell dysfunction resulting in moderate impairment of insulin secretion and HFD-inducing insulin resistance. The combination of these two models is often preferred to reflect T2DM-related complications such as neurodegenerative diseases and used for observing the possible effect of new therapeutic agents (Skovsø, 2014). In the study of Zheng et al. (2021), HFD and low-dose STZ model deteriorated learning memory performance by hippocampal neuronal damage, up-regulation of neuroinflammation pathways, and apoptosis in rats. Evidence is presented that the 7-week HFD diet is exacerbated by STZ injection, with increased TLR4 and JNK content in the neuroinflammation pathway resulting decreased BDNF and deteriorated neuronal structure in hippocampus (Hussein et al., 2022). Moreover, the relationship between this neuropathology and the reduction of learning-memory performances is accepted in different ways (Hao et al., 2022; Solas et al., 2017; Zhang et al., 2021). Therefore, in this study, we preferred a diabetes model with HFD and low-dose STZ to induce hyperglycemia-induced AD.

In the present study, HFD/STZ-induced diabetic rats were observed to increase post-prandial glucose levels and insulin resistance compared to rats fed a normal diet. From this point of view, it is possible to say that our results are consistent with previous reports (Das et al., 2019; Kellar and Craft, 2020). Increased insulin resistance and decreased insulin sensitivity in tissues are the fundamental factors in the development of T2DM. HFD-fed induces insulin resistance and stimulates mechanisms that reduce insulin sensitivity such as reducing PI3K activation, IRS-1, and Akt phosphorylation and activity (Gheibi et al., 2017). Regulation of CNS insulin signaling is considered to be an important factor in preventing the development of AD pathogenesis (Yamamoto et al., 2018). Significant loss of insulin signaling in the brain in T2DM is the main factor in its association with the pathogenesis of AD. Also, the loss of hippocampal insulin receptors causes insulin resistance in the AD neuropathology (Craft, 2012). Accumulating evidence proved that insulin resistance in the CNS, which is seen as a result of continuous increase in glucose concentration in the brain, induces neuronal loss with interconnected conditions such as oxidative damage (Takeishi et al., 2021), increased intracellular calcium level, mitochondrial dysfunction, neuroinflammation (Kellar and Craft, 2020), and neurochemical changes (De Felice, 2013). Recent studies have shown that serum insulin levels increase and brain insulin levels decrease in AD rats (Das et al., 2019; Hu et al., 2013; Yamamoto et al., 2018). HOMA-IR is a parameter that indicates the degree of insulin resistance (Janchevska et al., 2018), and an increase in HOMA-IR indicates an increase in insulin resistance. In this study, *R. canina* decreased fasting blood glucose levels and HOMA-IR, as well as increased insulin levels in rats. It indicates that *R. canina* can alleviate the degree of insulin resistance in rats and it may be mediated by mechanisms that weaken insulin resistance. It is known that persistent hyperglycemia and insulin resistance caused by HFD and low-dose STZ exhibit AD-like neuropathology by causing synaptic loss and neuronal damage (Chen et al., 2021). The remarkable result here is *R. canina* treatment improved peripheral insulin resistance by reducing HOMA-IR. Disruptions in the insulin signaling pathway in peripheral tissues affect insulin metabolism in the brain. By looking at this intertwined relationship, *R. canina* demonstrated decreased memory performance and beneficial effects on AD-like pathologies by regulating glucose and insulin metabolism.

The remarkable difference between HFD/STZ group and the control group in behavioural test findings in our study supports that diabetes-induced long-term hyperglycemia may be associated with learning memory performance. Diabetes-associated cognitive dysfunction may involve inflammation, oxidative stress, cholinergic neurotransmission, and microvascular dysfunction. After determining that locomotor functions and anxiety levels tested with OFT were similar for all groups, NORT which is based on the integrity of the hippocampus was the first of the behavioural tests applied. It is possible to evaluate an animal's memory performance in terms of its ability to discover and distinguish a new object compared to a familiar object (Kadıoğlu Yaman et al., 2020). In the test phase, the length of time taken to identify the new object indicates that memory performance has not deteriorated. Also, another behavioural test, the PAT, assesses the amygdala-dependent emotional memory (Ennaceur, 2010). In this test, the tendency to stay on the side without electric shock for a long time indicates improvement in memory performance (Aykac et al., 2019). In this study, the difference between the time to explore new and familiar objects in the control group rats fed the normal diet showed intact memory. However, in HFD/STZ-induced diabetic rats, the time spent in recognizing the new object was close to that of the familiar object, indicating impaired cognitive performance. The DIs and PIs of the *R. canina* group expressing the new object discovery times were similar to the control group, indicating that they may have the ability to preserve memory. The reduction in step-through latency of the HFD/STZ group compared to the control group fed a normal diet also confirms cognitive impairment. *R. canina* treatment improved the HFD/STZ-induced regression memory by increasing the latency. The curative effect of *R. canina* in NORT and PAT, in which short-term memory was assessed, was similar to that of the positive control MET. Consistent with previous studies, animals with HFD/STZ exhibited increased latency to find the platform in acquisition trials, decreased exploratory time in the target quadrant, and increased reach platform area in the probe trial, reflecting deterioration in spatial memory functions. Furthermore, we observed that the decrease in the duration of the escape latency, short transition time to the platform area in the probe test, and increase of time in the target quadrant of *R. canina* may indicate that it can effectively improve memory disorders. According to all these, it can be assumed that the effective recovery of the impairments in the procedural learning and consolidation process caused by the combination of HFD and STZ may be due to the regulation of the insulin resistance mechanism and neurochemical changes provided by *R. canina*. There is a need for more comprehensive studies with the major components that mediate the effects of medicinal plants and our results may guide new studies in a wider range. According to our findings, cognitive improvement with *R. canina* was noticeable and similar to MET. It is known that MET (positive control), an antidiabetic drug, improves spatial learning in the MWM test due to its beneficial effects on the improve of neuronal functions and neurogenesis (Asadbegi et al., 2016).

The hallmark pathology of AD is Aβ peptides that accumulate excessively in the brain. Aβ, a product of APP cleavage, is normally cleared from the neuronal extracellular space by various potential mechanisms. T2DM accompanied by insulin resistance causes increased

Aβ production and disrupted neurotransmitter transmission, one of the underlying mechanisms of cognitive impairment in the AD (Willette et al., 2015). Numerous studies have provided evidence that increased Aβ levels in the brains of HFD-fed diabetic rodents are associated with insulin resistance (Vandal et al., 2014). Plum et al. (2005) showed that insulin resistance causes neurodegenerative diseases by affecting APP metabolism and causing Aβ accumulation. Long et al. (2019) confirmed that insulin disrupts Aβ aggregation and can prevent Aβ-induced cellular events such as tau phosphorylation that may occur due to this. We found that HFD/STZ increases the deposition of hippocampal β-amyloid plaques in T2DM-induced rats. This result is associated with insulin resistance, in line with experimental evidence showing that glucose intolerance is related with impaired energy metabolism in the brain (Dubey et al., 2022). In our study, *R. canina* significantly reversed the increase in β-amyloid accumulation in the hippocampus in the HFD/STZ rats. There was no effect of *R. canina* treatment on the significant increase in Aβ level in the prefrontal cortex tissue. On the other hand, MET is neuroprotective against the effects of Aβ (Wang et al., 2012) and reduced amyloid levels in brain tissues compared to the HFD/STZ group. Concurrently, our findings reveal that *R. canina* significantly reduced the hippocampal APP level. Recent accumulating evidence suggests that activation of insulin-mediated pathways modulates the expression of secretase enzymes (α, β, γ) on APP and controls Aβ level (Wang et al., 2014). Increasing evidence indicates that when whole-body insulin sensitivity is improved, hippocampal and cortical amyloid deposition is reduced as a result of attenuated mRNA expression of tau and APP in rats induced by HFD/STZ (Park et al., 2017). Besides, in HFD/STZ-induced T2DM, disruptions in cholinergic transmission leading to cognitive impairments are associated with increased AChE activity (Singh and Bodakhe, 2022). According to Wang et al. the decrease in acetylcholine level causes Aβ plaque formation in various regions of the brain with the effect of impaired insulin pathways (Wang et al., 2019). Given the above evidence, we confirmed that HFD/STZ increases hippocampal and cortical AChE activity. Moreover, *R. canina* treatment reversed the increase in AChE activity of HFD/STZ-induction, which might be an important mechanism related to T2DM-induced memory deficits. Interesting features of MET in the treatment of neurodegenerative diseases are presented experimentally in the studies (El-Mir et al., 2008; Pintana et al., 2012). MET has been shown to increase insulin sensitivity and improve spatial memory by regulating the Akt pathway in a sporadic rodent model of AD (Kazkayasi et al., 2022). MET provides the regulation of insulin signaling and hence, decreases p-tau and amyloid levels, and improves memory (Nassar et al., 2020). The reduction in amyloid load, APP level, and AChE activity in both brain tissues was more effective with the MET-treated group compared to *R. canina*. In our current results, *R. canina* reduced insulin levels in different brain tissues similar to MET, which may indicate its effectiveness in AD-like pathologies. Considering these results, the molecular action of this process resulting in improved memory and judgment can be ascribed to decreased insulin level, Aβ accumulation, APP level, and AChE activity by *R. canina* treatment.

Chronic hyperglycemia causes damage to nerve cells and neurotoxicity by causing Aβ deposition in brain microvascular cells and triggering oxidative damage mechanisms (Huang et al., 2007). In this context, it is aimed to prevent cognitive disorders by modulating insulin sensitivity and protecting against AD-like pathologies by treatments that can prevent synaptic pathology accompanied by diabetes (Durairajan et al., 2012). Histopathological examination illustrated that the HFD/STZ group brain tissues had morphological changes and irregular neurons in cells indicating neuronal damage. Treatment with *R. canina* for 4 weeks was able to prevent HFD/STZ-induced neuronal damage in the region CA3 of the hippocampal tissue and prefrontal cortex. As previous reports reported (Carvalho et al., 2014), excess glucose and insulin resistance disrupted the neuronal structure, contributing to the gradual and progressive pathology of AD. In addition, since *R. canina* contributes to the preservation of the neuronal structure at this stage, it may have improved judgment and memory performances by preventing AD-like neuronal damage due to diabetes.

5. Conclusion

In conclusion, our data indicate that *R. canina* can be a good therapeutic option to prevent the progression of cognitive decline associated with diabetes via attenuating neurochemical challenges. Moreover, our results provide that insulin signaling pathway inhibition plays a key role in the development of AD by delaying amyloid degradation. The mechanism by which the extract of *R. canina* enhanced learning and memory in diabetic rats can be related to its impact on the insulin signaling pathway and AD-like mechanism which includes amyloid deposition and cholinergic transmission. Complementary studies will be needed to consolidate the results of this study and to purify and apply the components of the extract to determine the major component that provides cognitive improvement. To assist these studies, we would like to mention a few limitations of this study: We performed an 8-week HFD feeding and induction with low-dose STZ at week 4. Our treatment started in the 4th week and lasted for 4 weeks. A longer-term treatment protocol can be applied to examine the inclusion of AD-like pathologies in the evaluation of diabetes-related cognitive functions and the effects of *R. canina* on these pathologies. To assess the effect of *R. canina* on hyperglycemia and insulin resistance-induced neurodegeneration, BrdU staining and DAPI staining can be performed to evaluate cell proliferation and morphology in brain tissues.

CRediT authorship contribution statement

Busra Ertas: Investigation, Data curation, Visualization, Writing – original draft. **Ayse Nur Hazar-Yavuz:** Methodology, Validation, Data curation. **Fadime Topal:** Methodology, Validation. **Rumeysa Keles-Kaya:** Investigation, Data curation. **Özge Karakus:** Investigation, Data curation, Formal analysis. **Gul Sinemcan Ozcan:** Investigation, Formal analysis. **Turgut Taskin:** Investigation, Resources, Formal analysis. **Muhammet Emin Cam:** Resources, Supervision, Supervising, Writing – review & editing.

Declaration of competing interest

The authors all declare that there is no existing conflict of interest.

Data availability

The authors do not have permission to share data.

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