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Original article

Exercise and *Urtica dioica* extract ameliorate hippocampal insulin signaling, oxidative stress, neuroinflammation, and cognitive function in STZ-induced diabetic rats

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ABSTRACT

Introduction: Diabetes mellitus is related to cognitive impairments and molecular abnormalities of the hippocampus. A growing body of evidence suggests that *Urtica dioica* (Ud) and exercise training (ET) have potential therapeutic effects on diabetes and its related complications. Therefore, we hypothesized that the combined effect of exercise training (ET) and Ud might play an important role in insulin signaling pathway, oxidative stress, neuroinflammation, and cognitive impairment in diabetic rats.

Methods: Forty animals were divided into five groups (N = 8): healthy-sedentary (H-sed), diabetes-sedentary (D-sed), diabetes-exercise training (D-ET), diabetes-*Urtica dioica* (D-Ud), diabetes-exercise training-*Urtica dioica* (D-ET-Ud). Streptozotocin (STZ) (Single dosage; 45 mg/kg, i.p.) was used to induce diabetes. Then, ET (moderate intensity/5day/week) and Ud extract (50 mg/kg, oral/daily) were administered for six weeks. We also investigated the effects of ET and Ud on cognitive performance (assessed through Morris Water Maze tests), antioxidant capacity, and lipid peroxidation markers in hippocampus. Furthermore, we measured levels of insulin sensitivity and signaling factors (insulin-Ins, insulin receptor-IR and insulin-like growth factor-1 receptor-IGF-1R), and neuroinflammatory markers (IL-1 β, TNF-α). This was followed by TUNEL assessment of the apoptosis rate in all regions of the hippocampus.

Results: D-sed rats compared to H-sed animals showed significant impairments ($P < 0.001$) in hippocampal insulin sensitivity and signaling, oxidative stress, neuroinflammation, and apoptosis, which resulted in cognitive dysfunction. Ud extract and ET treatment effectively improved these impairments in D-ET ($P < 0.001$), D-Ud ($P < 0.05$), and D-ET-Ud ($P < 0.001$) groups compared to D-sed rats. Moreover, diabetes mediated hippocampal oxidative stress, neuroinflammation, insulin signaling deficits, apoptosis, and cognitive dysfunction was further reversed by chronic Ud+ET administration in D-ET-Ud rats ($P < 0.001$) compared to D-sed animals.

Conclusions: Ud extract and ET ameliorate cognitive dysfunction via improvement in hippocampal oxidative stress, neuroinflammation, insulin signaling pathway, and apoptosis in STZ-induced diabetic rats. The results of this study provide new experimental evidence for using Ud+ET in the treatment of hippocampal complications and cognitive dysfunction caused by diabetes.

1. Introduction

Consuming *Urtica dioica* has been shown to minimize diabetic markers, blood glucose, and can increase insulin sensitivity [1]. Diabetes disease is a metabolic disorder that adversely affects many brain regions, including the hippocampus, and increases the risk for cognitive decline [2]. Physiological factors such as insulin resistance are among the major causes of central neurological complications in diabetic patients [3]. Diabetes disease is linked with impairments in insulin signaling transduction pathway components, including insulin (Ins), insulin receptor

Abbreviations: BW, Body weight; ET, Endurance training; FIns, Fasting insulin; GPx, Glutathione peroxidase; GSH, Glutathione; Ins, Insulin; IR, Insulin receptor; IGF-1R, Insulin-like growth factor-1 receptor; MDA, Malondialdehyde; MWM, Morris Water Maze test; STZ, Streptozotocin; SOD, Superoxide dismutase; Ud, *Urtica dioica*.

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(IR) and insulin-like growth factor-1 receptor (IGF-1R) in insulin signaling transduction pathway [3,4]. Furthermore, poor cognitive performance in diabetes is associated with IR and/or decreased insulin resistance signaling [3]. In addition to impaired insulin signaling, increased oxidative stress also impairs the function of various organs [4]. A sedentary lifestyle and bad dietary habits are non-physiological factors in the development of diabetes [2]. The beneficial effect of ET on the hippocampus function and structure are well-known. Increasing the activity of antioxidant enzymes, reducing the level of neuroinflammation, reducing the levels of lipid peroxidation, and improving insulin signaling are several cellular and molecular processes to explain the beneficial effects that occur as a result of exercise. These beneficial effects are involved in preventing the neurological complications of diabetes, reducing cognitive functions, and tissue damage caused by oxidative stress [5,6].

In addition to activating endogenous antioxidant systems through exercise, natural compounds normally supplied within the diet can act as exogenous antioxidants [7]. Biologically active substances of medicinal plants can improve the neurological disorders caused by diabetes via several pathways [8]. *Urtica dioica* (Ud) (from the plants' family of Urticaceae) is a perennial plant that has long been used as a medicinal plant in many parts of the world. Ud has been shown to have antioxidant, anti-inflammatory and immunosuppressive properties [9]. Chemical analysis shows that Ud extract contains thymol, quercetin, flavonoids, carotenoids, carvacrol, and salicylic acid. Other ingredients in Ud extract include acetylcholine, serotonin, and vitamins such as A, B and B12 [10,11]. Various studies have linked Ud extract with maintenance and improvement of cognitive performances. It has been shown that Ud extract attenuates associative memory dysfunction in diabetic animals [11–13]. UD administration may also decrease the expression of TNF-α in hippocampal regions of diabetic mice [14]. Some other studies have shown that natural components in UD extract like carvacrol can regulate dopamine and serotonin levels in the hippocampus and prefrontal cortex, and provide neuronal protection against focal cerebral ischemia/reperfusion damage [15]. Other compounds in Ud extract like scopoletin and 5-HT have also been shown to enhance long-term hippocampal potentiation and improve memory impairment by increasing acetylcholine and insulin secretion, respectively [16].

Taken together, the above-mentioned studies suggest that Ud is the right candidate for the treatment of diabetes and its related complications, such as impaired memory and central neuropathy. However, it has not been established whether ET plus Ud extract supplementation could improve diabetes-associated cognitive dysfunction by regulating insulin signaling pathway. The present study was aimed to investigate the effect of ET and Ud in STZ-induced diabetic rats and its associated complications such as insulin signaling deficits, oxidative stress, neuroinflammation, and cognitive impairment.

2. Material and methods

2.1. Animals

Male Wistar rats weighing, 200–220 g (6-weeks-old), were purchased from Lorestan University of Medical Sciences. After transferring to the laboratory, animals were kept in a standard environment in a room with a 12-h cycle of light and dark, 25 ± 1 °C with fed pellet diet (Pars Khorakdam Co, Tehran, Iran) and water ad libitum. Initially, the animals were familiarized with new environmental conditions in the first week and treadmill activity (10–15 min, 5–10 m/s, 5 days) in the second week. To stimulate running and to avoid the possible effect of electric shock on the findings, animals were trained on the treadmill by sound conditioning method to avoid approaching and resting on the treadmill end. Forty animals were randomly divided into five equal groups of eight each: healthy-sedentary (H-sed), diabetes-sedentary (D-sed), diabetes-exercise training (D-ET), diabetes-*Urtica dioica* (D-Ud), diabetes-exercise training-*Urtica dioica* (D-ET-Ud). This study was conducted according to the ethical principles of the Lorestan University Animal Ethics Committee (Reference Number: LU. ECRA. 2018.16) and according to the NIH guidelines for the care and use of laboratory animals. Fig. 1 shows a schematic of the study implementation process over 10 weeks.

2.2. Induction of diabetes

After 12 h of fasting, the type 1 diabetic model was induced by STZ solution (Sigma, St. Louis, MO; 45 mg/kg dissolved in fresh citrate buffer 0.5 mol/L, PH 4.0) in the experimental group animals. After STZ-injection, no signs of abdominal swelling and gastrointestinal problems were observed in animals. Forty-eight hours after inducing diabetes, blood samples were collected from the tail of the remaining rats and analyzed using the glucometer (Emperor, South Korea Isotech). Animals with fasting blood glucose (FBG) level 300 mg/dl and above were considered as diabetic. Two weeks after the STZ injection, the rats were kept in the laboratory without intervention [17]. FBG levels, serum fasting insulin (FIns) (Mercodia Rat Insulin ELISA Kit, Uppsala, Sweden), and body weight (BW) using a digital scale with the accuracy of 0.001 kg (Seca, Hamburg, Germany) of all animals in each group was measured before STZ injection, 48 h after STZ injection, and at the beginning of the first, third, fifth weeks, and 48 h after the end of the study.

2.3. Plant gathering, preparation of extract and dosage

Ud leaves were prepared in the mountains of Lorestan province and then were confirmed by an expert from the Agricultural Organization of Lorestan (Sample No. 13776). The aerial parts of the collected samples were dried in the shade and then powdered. To prepare the hydro alcoholic extract, 500 g of dried powder was added in 70% aqueous ethanol and incubated for 18 h. Then, the obtained materials were filtered, and were placed in a rotary machine at 60 °C to evaporate the extra ethanol. To obtain the dry extract and without ethanol, the extract was placed in the incubator for 24 h at 60 °C and kept at − 20 °C in the dark until use. According to our previous study, the GC/MS method was used to determine the amount of the active compounds in the extract [11]. GC-MS spectrum of hydro-alcoholic extract of Ud leaves extract showed peak at *m/z* 53 (kaempferol), *m/z* 55.1 (apigenin), *m/z* 61 (catechin), *m/z* 67.1 (myricetin), *m/z* 68 (carvacrol), *m/z* 69.1 (ursolic), *m/z* 81.1 (scopoletin), *m/z* 83 (chlorogenic acid), *m/z* 93.1 (β-sitosterol), *m/z* 96.1 (quercetin), *m/z* 103.0 (rutin), *m/z* 108.1 (esculetin), *m/z* 109 (vanillic acid), *m/z* 110 (isorhamnetin), and *m/z* 152.1 (gentisic acid). Chromatogram of UD extract is presented in Supplementary material S1. All animals in D-Ud and D-ET-Ud groups were treated daily for six weeks by oral gavage (50 mg/kg body weight Ud hydro alcoholic extract) in the 0.0166 w/v concentration (500 mg of UD extract per 30 ml of distilled water).

2.4. Treadmill exercise

Two weeks after diabetes induction, the treadmill exercise protocol was performed for 5 days a week for 6 weeks at moderate intensity. The training sessions consisted of three parts: warm-up (3 min), main training (10–30 min), and cool-down (3 min). The speed and duration of the main exercise gradually increased from the first to the sixth week, so that the speed and duration of the training in the first week was 10 m/min for 10 min, the second week 10 m/min for 20 min, the third week 15–14 m/min for 20 min, and in weeks 4 and 5 respectively, 14–15 m/min and 17–18 m/min for 30 min. To achieve consistency, speed and the time of treadmill exercise were kept constant during the 6th week [17].

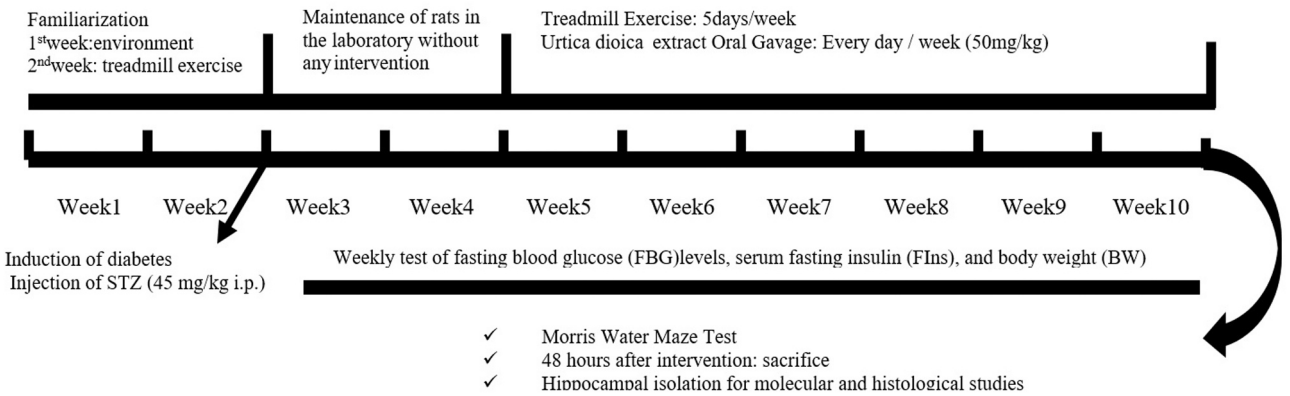

Fig. 1. Summary of experimental design: before starting the study, the steps of *Urtica dioica* (Ud) extract preparation were performed. After familiarizing the animals with the laboratory environment and treadmill, diabetes was induced by STZ-injection (45 mg/kg, IP) in diabetic groups. Then, all rats were kept in the laboratory for two weeks (without any intervention). Treatment with treadmill exercise training (ET) and Ud began for six weeks. Thereafter, in the final week, animals were subjected to behavioral studies and then they were sacrificed for histological and molecular studies.

2.5. Experiments of behavioral test

In the sixth week of ET and Ud interventions, the Morris Water Maze (MWM) test was performed to test hippocampal dependence, including spatial learning and memory. MWM consisted of a black circular pool filled with water (22 °C ± 2 °C, 200 cm diameter, walls 76 cm depth), located in a room with visual signs on the walls. A platform with a diameter of 10 cm and a height of 35 cm was designed, which was placed in the middle of the S and E quartile during the spatial learning test. The platform was 2 cm below the water's surface. The pool was divided into four quadrants (compass locations: N, S, W, and E) by a computerized tracking/image analyzer system. The animal spatial learning test was performed on animals for four consecutive days. Each day of the spatial learning test, each rat was randomly abandoned from the N, S, W, and E points in the water. To find the platform by rats, it was necessary sixty seconds. If the rat found the platform, they were placed on the platform for 10 s, and otherwise, the rat was guided by hand and allowed to remain on the platform for 10 s, and their escape latency was accepted as 60 s. Twenty-four hours after the last day of the spatial learning test, rats were tested in a spatial memory test (probe trial). During the probe trial, the platform was removed, and the time spent in the target quadrant was recorded for a 60 s [18,19].

2.6. Tissue samples

Forty-eight hour after the last session of ET and Ud consumption, rats were anaesthetized using inhalation of 2% halothane in a mixture of 70% N^2O and 30% O^2; and perfused with 100 ml of saline followed by 250 ml 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). In each group, four rats were used for molecular studies, and four rats for histological studies and the average value was used for statistical analysis.

2.7. Western blot analysis

Western blot analysis was performed as described previously [20], with polyclonal antibodies for Ins (ab181547), IR (ab60946), IGF-1R (ab182408), IL-1β (ab205924), TNF-α (ab205587) and GAPDH (ab181602). For testing the proteins in a similar size, we used primary antibodies raised in different species, or the blot was stripped and reprobed. Data were normalized to GAPDH in the same membrane and expressed as a percentage of control values.

2.8. Biochemical assays for SOD, CAT, GPx, GSH, and MDA

For biochemical experiments, 100 mg of hippocampal tissue was cut, and a certain amount of PBS (100 mmHg, 4.7 pH) was added for homogenization. The sample was thawed and kept at 2–8 °C and a certain amount of PBS (pH 7.4) was added. Then, the sample (~100 mg tissue per 1 ml PBS buffer) homogenized entirely with a homogenizer. Tissue homogenate fluid was prepared by centrifuging for 20 min at 4000–6000 RPM. After preparation of tissue hemogenic fluid, they were placed in separate micro-tubes and were placed into − 80 °C freezer.

The activities of Superoxide dismutase (SOD), Catalase activity, Glutathione peroxidase (GPx), glutathione peroxidase (GSH), and as well as malondialdehyde (MDA) levels in the homogenate of the hippocampus were measured by the specific ELISA kits developed for rats according to their manuals (ZellBio GmbH, Germany). Kits measured quantity assay samples based on colorimetric methods that should be read by ELISA reader (for SOD: 420 nm, CAT: 405 nm, GPx: 412 nm, GSH: 412 nm, and MDA: 535 nm).

2.9. TUNEL staining, immunohistochemistry assay

Terminal deoxynucleotidyl Transferase dUTP nick end labeling (TUNEL) reaction was used to observe DNA fragments after apoptosis. The tissue sections prepared from different groups were evaluated by in Situ Cell Death Detection Kit (Roche) and the apoptosis rate of the hippocampus areas (CA1, CA2, CA3, CA4, dentate gyrus (DG)) was examined in different groups. The TUNEL reaction steps were performed as follows: shortly, after deparaffinization, the sections were preserved with 20 mg/ml proteinase K for 15 min. After action with 0.3% H2O2 in methanol for 15 min, the sections were incubated with the TUNEL reaction mixture for 65 min at 37 °C. Farther incubation with peroxidase-conjugated antibody was done for 35 min at 37 °C. The sections were stained with Diaminobenzidine solution for 15 min at room temperature and then counterstained with hematoxylin. The nuclei of apoptotic cells were categorized dark brown under DAB staining. After that, using 10 randomly selected fields of five sections per animal (n = 4 hippocampus/groups), the number of neurons and TUNEL-positive nuclei were counted using coded blind slices in each section. The number of TUNEL positive cells and the total number of cells in the hippocampus were expressed as the mean rate and counted under 400× magnifications.

2.10. Statistical analysis

Data were analyzed using SPSS 22 for Windows (SPSS Inc., Chicago, IL) and all results were presented as mean ± SD. The normality and homogeneity of data were analyzed using the Shapiro-Wilk and Levene's test, respectively. Besides, a one-way analysis of variance (ANOVA) was used to compare the levels of research variables, and Kruskal-Wallis analysis was used to compare groups in the histological analysis of the

hippocampus in all groups. Tukey's post-hoc test was used to compare the difference between groups. The probability level of statistical significance was set at $P < 0.05$.

3. Results

3.1. ET+Ud treatment ameliorate diabetes-induced BW loss, decrease FBG, and increase serum FIns in STZ-induced diabetic rats

To determine the effect of STZ-induced diabetes on BW, FBG, and FIns, these variables were measured intermittently. At the beginning of the study, the mean BW in all groups was 211.37 ± 4.46 g ($F = 0.251$, $P = 0.907$). BW decreased in the diabetic groups two weeks after STZ-injection compared with the H-sed group ($F = 17.043$, $P = 0.001$), and this decline continued until the sixth week ($F = 167.40$, $P = 0.001$). Mean BW values of the D-sed group were lower than the mean BW values of the D-ET, D-Ud, and D-ET-Ud groups throughout the six-week post-STZ period, particularly in weeks 3, 5, and posttest of the study ($P < 0.01$). Besides, there was no difference in the BW of D-Ud, and D-sed-Ud groups throughout the study ($P > 0.05$). In the sixth week, the weight of the rats in the D-ET group was less than that of the rats in D-Ud and D-ET-Ud groups ($P < 0.05$) (Fig. 2a). These results indicate that ET and Ud did not impact the body weight of diabetic rats, but ET and Ud consumption is likely to ameliorate the process of weight loss due to diabetes.

Before the STZ-injection, the mean FBG levels in all groups was 106.82 ± 2.99 mg/dl ($F = 0.183$, $P = 0.946$). FBG significantly increased 48 h after STZ-injection in rats of D-sed, D-ET, D-Ud, and D-ET-Ud groups compared to H-sed group ($F = 133.11$, $P = 0.001$), and mean FBG in diabetic groups was 498.07 ± 42.06 mg/dl, demonstrating that diabetes induction was successful. Following 6 weeks of the experiment, a significant difference in FBG levels was observed between the study groups ($F = 710.516$, $P = 0.001$), so that FBG levels were significantly lower in the D-ET, D-Ud, and D-ET-Ud groups than in the D-sed group ($P < 0.001$). In addition, the lower levels of FBG were observed in the D-ET-Ud ($P < 0.001$), and D-ET ($P < 0.01$) groups as compared to the D-Ud groups, and there was no significant difference between D-ET and D-ET-Ud groups in the sixth week ($P > 0.05$) (Fig. 2b). Mean serum FIns levels in all groups before STZ-injection, were 10.67 ± 0.77 μU/ml ($F = 0.645$, $P = 0.643$). Serum FIns significantly decreased 48 h after STZ-injection in rats of D-sed, D-ET, D-Ud, and D-ET-Ud groups compared to H-sed group ($F = 86.101$, $P = 0.001$), and mean serum FIns in diabetic groups was 6.09 ± 0.26 μU/ml. During the six weeks of ET and Ud extract consumption, serum FIns levels were significantly higher in the D-ET, D-Ud, and D-ET-Ud groups than in the D-sed group ($F = 212.471$, $P = 0.001$). In addition, the higher levels of serum FIns were observed in the D-ET-Ud as compared to the D-Ud and D-ET groups ($P < 0.001$), and there was no significant difference between D-ET and D-Ud groups ($P > 0.05$) (Fig. 2c). These results suggested that diabetes could lead to abnormal blood glucose and insulin levels, while ET and Ud played a positive role in improving these abnormalities.

3.2. ET+Ud improve cognitive impairment in STZ-induced diabetic rats

Cognitive dysfunction is a consequence of diabetes, leading to memory and learning impairments. We examined the effects of diabetes on cognitive function with the MWM test in STZ-induced diabetic rats. As shown in (Fig. 3a), in the MWM test, significant differences in escape latency of different groups were observed on the 1st ($F = 12.504$, $P = 0.001$), 2nd ($F = 39.520$, $P = 0.001$), 3rd ($F = 21.590$, $P = 0.001$), and 4th ($F = 28.851$, $P = 0.001$) days, so that STZ-induced diabetic rats in D-sed group showed significantly higher escape latency on the 1st, 2nd, 3rd and 4th days compared to the H-sed group rats ($P < 0.001$). ET or Ud treated animals showed improvement in learning between trials from day 1–4 and decreased in the escape latency compared to the D-sed group rats ($P < 0.001$). But ET+Ud treated animals in D-ET-Ud group showed a significantly higher decrease in learning day 3 and 4 trials than H-sed, D-Ud, and D-ET groups in escape latency ($P < 0.01$). As shown in (Fig. 3b and c), a significant difference was observed in the time spent in target quadrant ($F = 41.522$, $P = 0.001$) and the number of crossings ($F = 47.019$, $P = 0.001$) across the platform area (probe trial) of different groups. The time spent in target quadrant and the number of crossings were significantly decreased in D-sed group rats compared to H-sed group rats ($P < 0.001$). ET or Ud treatment improved the time spent in the target quadrant, and the number of crossings across the platform area compared to D-sed group rats ($P < 0.001$). The time spent in the target quadrant, and the number of crossings the platform area in D-ET-Ud group was longer than that of H-sed, D-ET, and D-Ud groups ($P < 0.001$). There was no significant difference between H-sed, D-ET and D-Ud groups ($P > 0.05$).

In general, these results implicated that diabetes could lead to cognitive impairment and ET along with the consumption of Ud in the D-ET-Ud group, had the best effect on cognitive improvement in the STZ-induced diabetic rats.

3.3. ET+Ud mitigate pro-inflammatory cytokines in STZ-Induced diabetic rats

Diabetes has been reported to contribute to inflammation. To examine whether STZ-induced diabetes is associated with neuroinflammation and if ET and Ud consumption are linked to the prevention of neuroinflammation in the hippocampus, we examined a key pro-inflammatory cytokines (TNF-α and IL-1β) in the hippocampus. As shown from (Fig. 4), a significant difference was observed in levels of TNF-α ($F = 33.517$, $P = 0.001$) and IL-1β ($F = 53.973$, $P = 0.001$) of

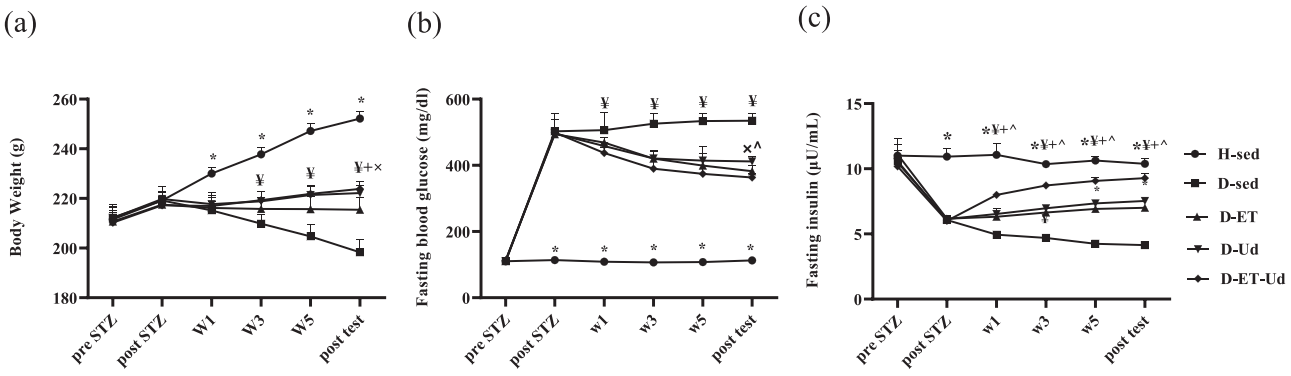

Fig. 2. ET+Ud treatment ameliorate diabetes-induced body weight loss (a), decrease fasting blood glucose level (b), and increase serum fasting insulin level (c) in STZ-induced diabetic rats. *$P < 0.001$ for H-sed group vs. other groups, ¥ $P < 0.001$ for D-sed group vs. D-ET, D-Ud, and D-ET-Ud groups, + $P < 0.01$ for D-ET-Ud group vs. D-ET group, ^ $P < 0.01$ for D-ET-Ud group vs. D-Ud group, and × $P < 0.05$ for D-Ud group vs. D-ET group.

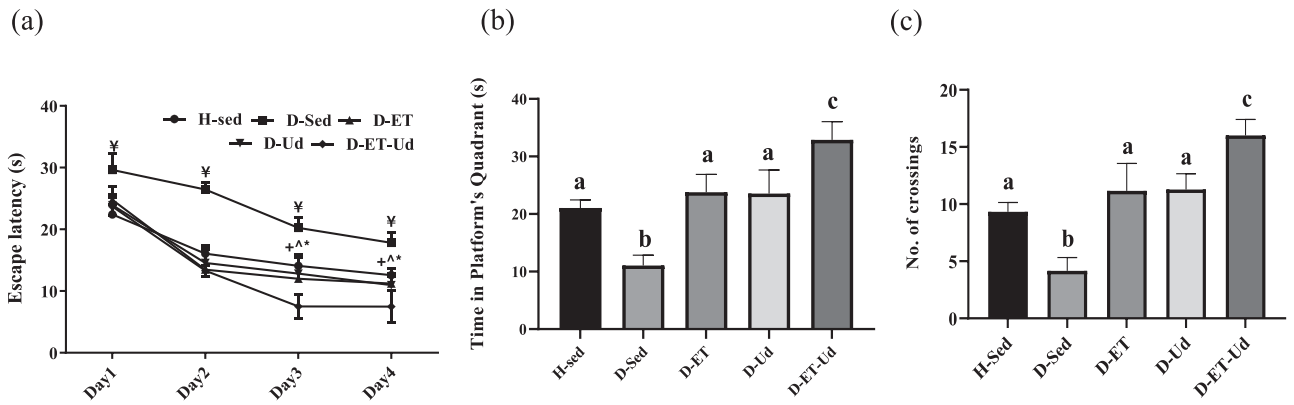

Fig. 3. ET+Ud improve cognitive impairment in STZ-induced diabetic rats. Results extracted from Morris Water Maze (MWM) test in spatial learning (Morris water maze task) (a), spatial memory (probe trial) (b), and number of crossing across platform (probe trial) (c). In a: *P < 0.001 for H-sed group vs. other groups, ¥ P < 0.001 for D-sed group vs. D-ET, D-Ud, and D-ET-Ud groups, + P < 0.01 for D-ET-Ud group vs. D-ET group, ^ P < 0.01 for D-ET-Ud group vs. D-Ud group, × P < 0.05 for D-Ud group vs. D-ET group. In Fig. 3b and c: the different letters indicate a significant difference (ANOVA and subsequent Tukey's HSD, P < 0.05).

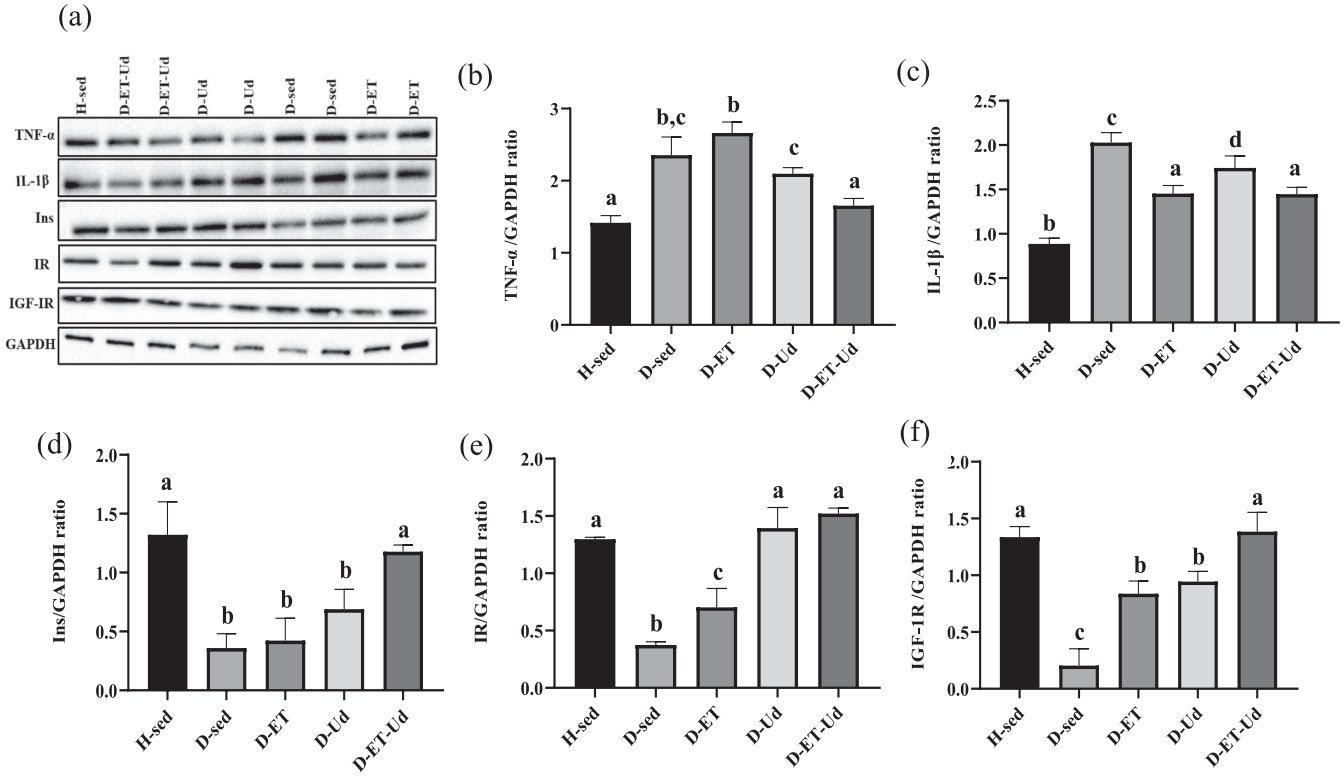

Fig. 4. ET+Ud mitigate pro-inflammatory cytokines, and improve insulin sensitivity and insulin signaling deficits in the hippocampus of STZ-induced diabetic rats. Western blot analysis of TNF-α, IL-1β, Ins, IR, and IGF-1R proteins to GAPDH ratio in the total the hippocampal tissue (a). The ratio of TNF-α (b), IL-1β (c), Ins (d), IR (e), and IGF-1R (f) proteins to GAPDH. The different letters indicate a significant difference (ANOVA and subsequent Tukey's HSD, P < 0.05).

different groups, so that, rats in D-sed group showed significantly higher levels of TNF-α and IL-1β in the hippocampus than H-sed rats (P < 0.001). Rats treated with ET and Ud significantly reduced IL-1β expression in the hippocampus in D-ET (P < 0.001) and D-Ud (P < 0.05) rats compared to D-sed group. However, the reduction in TNF-α expression in the D-Ud group compared to the D-sed group was not statistically significant (P > 0.05). In contrast, ET in the D-ET group increased the TNF-α level compared to the D-sed group, but this increase was not significant (P > 0.05). A comparison of D-ET and D-Ud groups showed that ET caused a more significant reduction in IL-1β (P < 0.05). Reduced of TNF-α was observed in the hippocampus of D-ET-Ud rats compared with D-sed (P < 0.01), D-ET (P < 0.001), D-Ud (P < 0.05) rats, and there were no significant differences in the levels of TNF-α hippocampus between H-sed and D-ET-Ud rats (P > 0.05). Level of IL-1β was decreased in D-ET-Ud rats compared with D-sed (P < 0.001), and D-Ud (P < 0.05) rats.

3.4. ET+Ud ameliorate insulin sensitivity, and insulin signaling deficits in the hippocampus of STZ-induced diabetic rats

Insulin signaling deficiency in the hippocampus is known to modulate cognitive function. We examined whether ET and Ud prevent STZ-induced diabetes insulin sensitivity and signaling deficiency in the hippocampus. As shown in (Fig. 4), a significant difference was observed in proteins expression levels of Ins (F = 17.617, P = 0.001), IR (F = 58.218, P = 0.001), and IGF-1R (F = 42.736, P = 0.001) of

different groups, the expression of Ins, IR, and IGF-1R proteins in the hippocampus of STZ-induced diabetic rats (D-sed) was significantly decreased compared to the H-sed group ($P < 0.001$). Rats treated with ET or Ud significantly increased the expression of IR and IGF-1R, but not Ins, in the hippocampus of D-ET and D-Ud groups rats compared to the D-sed group ($P < 0.05$), and rats in D-ET-Ud group revealed a significant higher Ins, IR, and IGF-1R protein expression in the hippocampus than D-ET and D-Ud groups ($P < 0.05$). No significant difference of Ins, IR, and IGF-1R protein expression was observed between D-ET-Ud and H-sed rats ($P > 0.05$). Our results in this part implicate that diabetes could lead to insulin sensitivity and signaling deficits and ET or Ud are capable of improving insulin sensitivity without changing the amount of insulin in the hippocampus of STZ-induced diabetic rats. In contrast, a combination of ET along with the consumption of Ud not only can improve insulin sensitivity, but also modify insulin level to normal values in the hippocampus of STZ-induced diabetic rats.

3.5. ET+Ud abolish lipid peroxidation and oxidative stress in STZ-induced diabetic rats

Given that neuroinflammation in the hippocampus is highly associated with oxidative stress and lipid peroxidation, we investigated if ET and Ud-induced anti-pro-inflammatory effects play a role in preventing STZ-induced diabetes oxidative stress and lipid peroxidation. We first examined malondialdehyde (MDA) levels to estimate the probable amount of lipid peroxidation in hippocampal tissue. As shown in (Fig. 5a), a significant difference was observed in level of MDA ($F = 125.915$, $P = 0.001$) of different groups. STZ-induced diabetes significantly increased hippocampal MDA level in the D-sed group in comparison with the H-sed group ($P < 0.001$). Administration of Ud or/and ET in the D-ET, D-Ud, and D-ET-Ud groups decreased the hippocampal MDA level compared with D-sed group ($P < 0.001$), but MDA levels in the hippocampus of D-ET-Ud and D-ET groups was significantly lower than the D-Ud group ($P < 0.01$), and no significant difference of MDA levels was observed between D-Ud and H-sed rats ($P > 0.05$).

We further examined whether ET and Ud-mediated suppression of lipid peroxidation are linked to the modulation in antioxidant capacity in the hippocampus of STZ-induced diabetic rats. Consistent with MDA results, a significant difference was observed in level of GPx ($F = 62.320$, $P = 0.001$), SOD ($F = 65.604$, $P = 0.001$), CAT ($F = 83.050$, $P = 0.001$), and GSH ($F = 59.177$, $P = 0.001$) activities of different groups, so that GPx, SOD, CAT, and GSH activities of the hippocampus in D-sed rats were significantly lower than H-sed group rats ($P < 0.001$), Rats treated with ET or Ud significantly increased GPx, SOD, CAT, and GSH activities in the hippocampus of D-ET and D-Ud groups rats compared to the D-sed group ($P < 0.001$). No difference in GPx, SOD, CAT, and GSH activities were observed between D-ET and D-Ud groups rats ($P > 0.05$). D-ET-Ud group rats revealed a significantly higher GPx, SOD, CAT and GSH activities in the hippocampus than D-ET and D-Ud groups ($P < 0.01$). The activity of GPx, CAT, and GSH in the hippocampus of D-ET-Ud group was significantly higher than the H-sed group ($P < 0.01$), but no significant difference in SOD activities was observed between D-ET-Ud and H-sed rats ($P > 0.05$) (Fig. 5). In general, these results suggest that diabetes could lead to abnormal levels of lipid peroxidation and markers of antioxidant defense system in the hippocampus and ET and Ud played a positive role in improving these abnormalities. At the same time, ET+Ud had the best effect on their improvement in the hippocampus of STZ-induced diabetic rats.

3.6. ET+Ud treatments attenuate diabetes-induced apoptosis in the hippocampus

Oxidative stress, insulin sensitivity and signaling and neuroinflammation strongly induce apoptosis in the hippocampus, which is contributed to cognitive dysfunction. To examine whether STZ-induced diabetes insulin sensitivity and signaling deficiency, inflammation and oxidative stress promote apoptosis, we finally assessed neuronal apoptosis in the hippocampus. Diabetes-induced apoptotic cells were

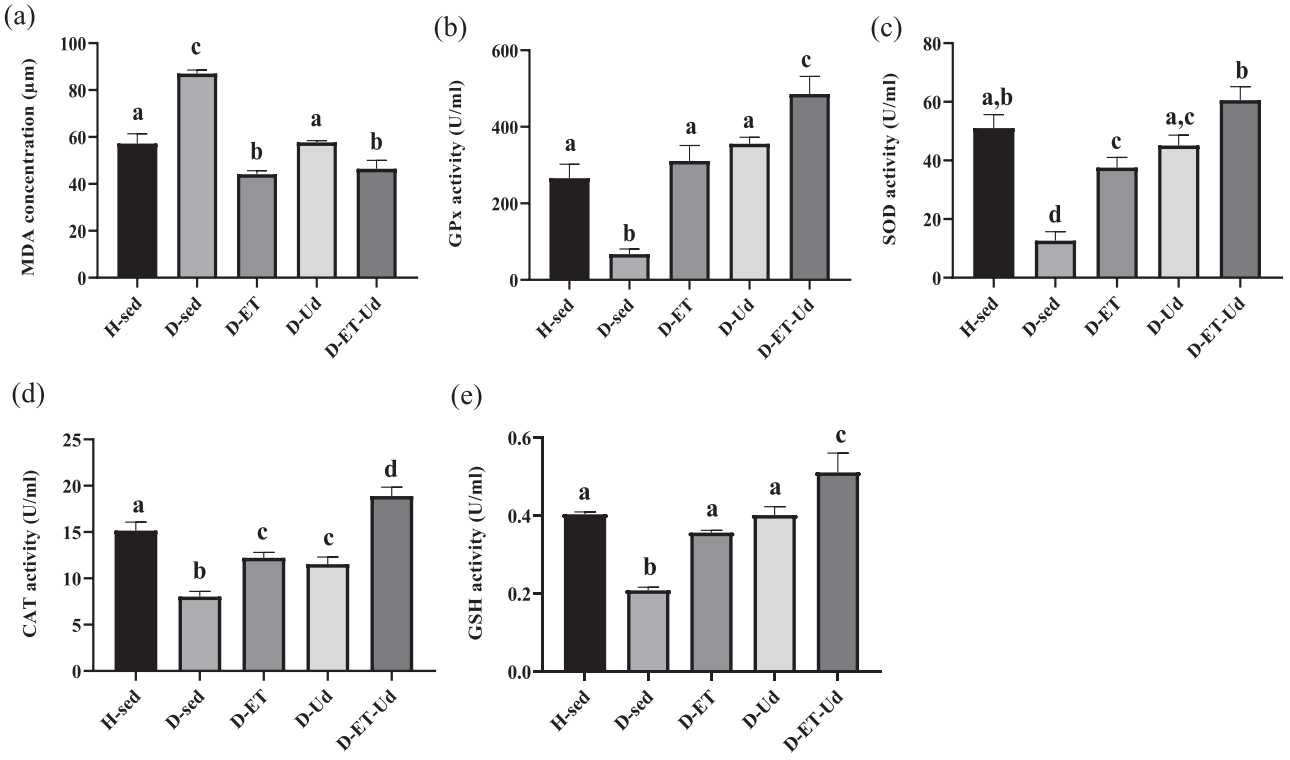

Fig. 5. ET+Ud abolish lipid peroxidation and oxidative stress in STZ-induced diabetic rats. ELISA quantitative analysis of malondialdehyde (MDA) (a), Glutathione peroxidase (GPx) (b), Superoxide dismutase (SOD) (c), Catalase (CAT) (d), and glutathione peroxidase (GSH) (e) in the total the hippocampal tissue. The different letters indicate a significant difference (ANOVA and subsequent Tukey's HSD, $P < 0.05$).

characterized by TUNEL staining in the hippocampus slices (Fig. 6). We investigated 10 vision fields in each region of the hippocampus, and counted all the apoptotic cells numbers in these fields. In H-sed group, TUNEL positive neuron was absent in the hippocampal areas.

In contrast, significant amounts of TUNEL-positive neurons appeared in the hippocampal DG, CA4, and CA3 regions in diabetic rats in D-sed group compared to H-sed group ($p < 0.01$). Positive TUNEL cells were also found in the D-ET, D-Ud, and D-ET-Ud groups. However, when compared with the D-sed group, in the DG region all three groups showed an equal decrease in apoptotic cells, but, in the CA4, CA3, CA2, and CA1 regions, combination treatment with ET and Ud in the D-ET-Ud group caused a more significant decrease in apoptotic cells compared to the D-ET and D-Ud groups. ET in regions of CA1 and CA2 increased apoptotic cells more than D-sed group. Also, the effect of Ud in CA1 region was neutral, and in CA2 region it increased apoptotic cells compared to D-sed group. Figures DG, CA4, and CA2 are shown in Supplementary material S1, Supplementary material S2.

4. Discussion

The present study is the first to demonstrate that Ud extract and ET treatment ameliorate diabetes-associated cognitive decline by reducing hippocampal oxidative stress, neuroinflammation, insulin signaling pathway, and apoptosis in STZ-induced diabetic rats. Following high FBG and decreased serum FIns in STZ-induced diabetic rats, our molecular results showed that diabetes reduced the expression of Ins, IR, and IGF-1R proteins in the hippocampus. Moreover, we observed a significant decrease in the levels of antioxidant enzymes (GPx, SOD, CAT and GSH), a significant increase in the expression of pro-inflammatory cytokines (TNF-α and IL-1β) and lipid peroxidation (MDA) in the hippocampus of STZ–induced diabetic rats. Further, TUNEL staining results showed an increase in apoptotic cells in the hippocampus of STZ-induced diabetic rats. These results are in agreement with the previous findings indicating that diabetes impairs cognitive function [21,22] and insulin signaling [23] and increases oxidative stress, inflammatory responses [16], and apoptosis in the hippocampus [6]. In this study, in addition to investigating the effect of diabetes on hippocampal tissue and cognitive impairment, the therapeutic goals of ET and Ud extract were evaluated alone and together. To our knowledge, this is the first study that has examined the interaction between ET and Ud extract on impairments in insulin signaling, neuroinflammation, oxidative stress, and cognitive function in STZ-induced diabetic rats. The results of the present study showed that ET and Ud extract reduced insulin signaling deficits, oxidative stress, apoptosis and neuroinflammation in the hippocampus of diabetic rats. In addition, we found that ET and Ud extract improve cognitive function in STZ-induced diabetic rats. Furthermore, when we combined ET with UD extract consumption, we found greater improvements in hippocampal insulin signaling, oxidative stress, apoptosis, neuroinflammation, and cognitive function.

Glucose metabolism disorder in the hippocampus of diabetic rats is mainly associated with inhibition of the tricarboxylic acid (TCA) cycle and activation of several pathways including polyol, glycolysis, and pentose phosphate [5]. It has shown that glucose metabolism in the hippocampus of diabetic rats tends to shift from the oxidative phosphorylation pathway to glycolytic pathway [24]. However, the association between the metabolic pathways and cognitive function is not yet fully understood. Additionally, it has been demonstrated that mitochondrial dysfunction and overproduction of reactive oxygen species are considered to be the main mechanisms for the progression of hippocampal insulin resistance [25]. Exercise has been shown to regulate glucose homeostasis and improve diabetes-related cognitive impairment by reducing energy metabolism and insulin resistance [26]. Hippocampal neuroinflammation leads to oxidative stress, which in turn, becomes a source of inflammation. In fact, this deleterious chain reaction

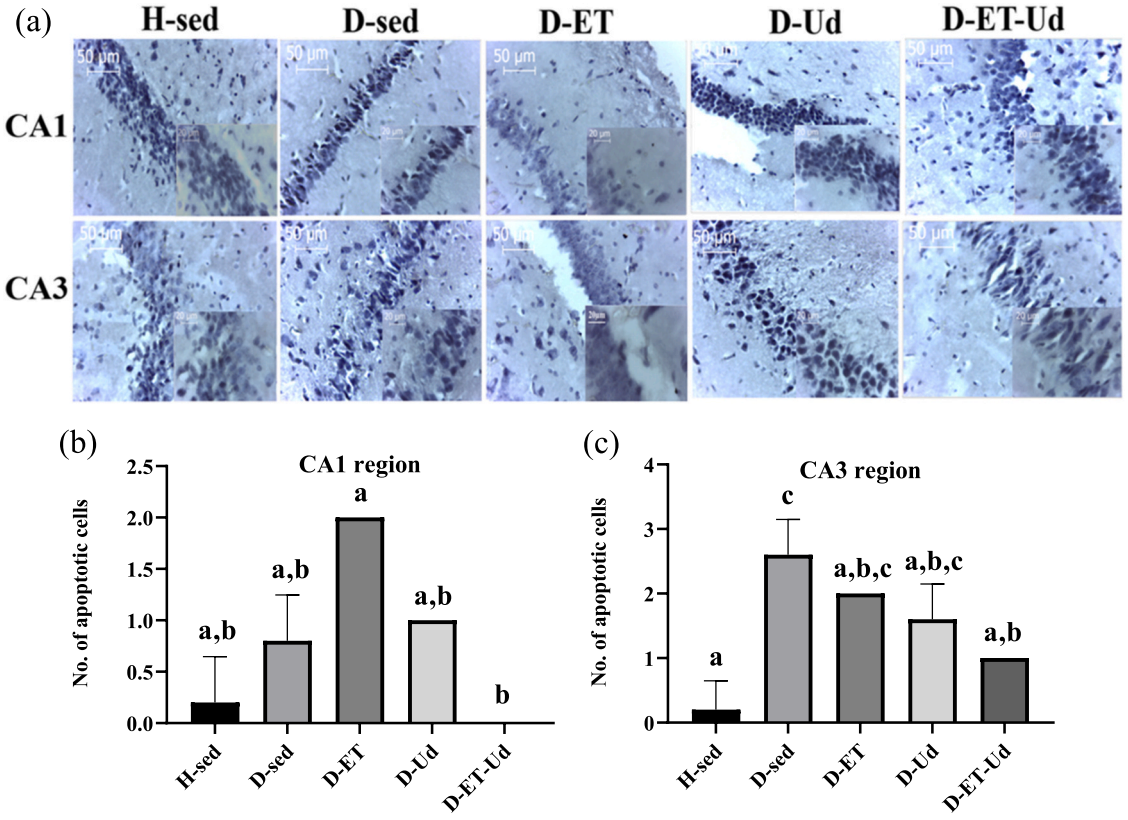

Fig. 6. Diabetes induced apoptosis of cells in the hippocampus of rats. Apoptosis was examined by the TUNEL method. (a) Photomicrographs of TUNEL-positive cells in CA1 and CA3 in each groups. Scale bar 50 & 20 µm. Number of TUNEL-positive cells in CA1 (b) and CA3 (c) in each groups. The different letters indicate a significant difference (Kruskal-Wallis, Bonferroni error correction, $P < 0.05$).

has played an important role in diabetes-related cognitive impairment [16]. In addition, endurance exercise by reducing the levels of inflammatory cytokines such as IL-1β and TNF-α, increases the expression of neuroplasticity-related proteins and can also promote learning performance in the hippocampus [5,27,28]. It has been reported that exercise training up-regulates hippocampal antioxidant defenses system [29]. The effect of exercise on central nervous system is very complex and not fully understood yet, but it is probably due to the function of neurogenesis through increased neurotrophic factors and angiogenesis, and reduced oxidative damage in the brain [30]. Studies have demonstrated various neuroprotective possible mechanisms of aerobic exercise-related upregulation of synaptic plasticity-associated proteins including activation of insulin signaling pathway, improvement of energy metabolism, and inhibition of inflammation [5]. However, the regulatory effects of endurance exercise and its related mechanism in diabetic conditions are not yet fully understood. In the present study, we observed that endurance exercise ameliorates insulin signaling and reduces oxidative stress, apoptosis and neuroinflammation in the hippocampus of STZ-induced diabetic rats, which eventually leads to improved cognitive function. Moreover, we found that Ud leaves extract supplementation improves the adverse effect of diabetes in the hippocampus of STZ-induced diabetic rats. Similarly, several studies have shown the beneficial effects of Ud leaves extract on cognitive improvement in STZ-induced diabetic animals [14,18,23]. In the present study we found the following compounds in Ud leaves extract: kaempferol, apigenin, catechin, myricetin, carvacrol, ursolic, scopoletin, chlorogenic acid, β-sitosterol, quercetin, rutin, esculetin, vanillic acid, isorhamnetin, and gentisic acid. Among these compounds, scopoletin [31], rutin [32], esculetin [33], and quercetin [34] are known to play role in hyperglycemia prevention. The results of the present study confirm and extend previous observations based on the anti-diabetic properties of Ud extract [35,36]. In a study by Kumar et al. the protective effects of quercetin against cognitive dysfunction have confirmed in rats with Alzheimer disease [37]. Quercetin, as a bioflavonoid in Ud has been shown to have anti-inflammatory, anti-oxidative, and anti-apoptotic effects [37]. Quercetin activates GPx and inhibits hydrogen peroxide (H2O2) from oxidative damage. Furthermore, quercetin weakened oxygen–glucose deprivation, free radical, and excitotoxin-induced neurotoxicity, and hindered breaking DNA strands and cytotoxicity. The cytoprotection capacity of quercetin has confirmed in brain neurons [37]. Increased hippocampal cyclooxygenase-2 (COX-2) expression due to diabetes has been shown to play an important role in neuronal apoptosis [38]. Anti-apoptotic effects of quercetin have been documented by suppression of the c-Jun N-terminal kinase (JNK)/activator protein 1 (AP-1) and the extracellular signal-regulated protein kinase (ERK)/c-jun/AP-1 pathways [39]. It has been shown that quercetin is one of the most effective compounds of Ud leaves extract reducing inflammation, increasing antioxidants, preventing apoptosis, and improving insulin signaling in hippocampal neurons [23,37]. As another effective compound of Ud leaves extract, scopoletin also ameliorates long-term potentiation by increasing neurotransmitters' release of nicotinic acetylcholine receptor (nAChR) and promoting neural plasticity in the hippocampus, and ultimately improves cognitive impairments [10]. Furthermore, rutin is another effective compound of Ud leaves extract in controlling oxidative stress, neuroinflammation, cognitive impairment, and neuronal loss in the hippocampus [40]. However, the exact underlying cellular and molecular mechanisms responsible for the rutin-induced neuroprotection have not been fully elucidated. Similarly, studies have shown that Ud leaves extract reduces diabetes-induced neurological impairment [13]. However, a limitation of our study is that it is unclear how ET and Ud extract impact hippocampal insulin signaling, oxidative stress, apoptosis, neuroinflammation and cognitive function in STZ-induced diabetic rats. Examining these parameters and identifying signaling pathways associated with ET and Ud extract are important next steps for determining their potentials as metabolically favorable supplements for diabetes induced cognitive dysfunction. However, further research is required to better understand the effects of ET and Ud extract on hippocampal plasticity and elucidate various aspects of their biological effects.

5. Conclusion

In summary, our study for the first time demonstrates that ET and Ud extract can ameliorate insulin signaling deficits, strengthen antioxidant defense system, reduce neuroinflammation and apoptosis, and protect the ability of learning and memory from STZ-induced diabetes cognitive impairment. Thus, the results of the present study provide evidence to explore new therapeutic options in patients with diabetes-associated cognitive decline.

Ethical approval

This study was conducted according to the ethical principles of the Lorestan University Animal Ethics Committee (Reference Number: LU. ECRA. 2018.16) and according to the NIH guidelines for the care and use of laboratory animals.

Author contributions

M.R. and M.K. designed this study. F.C. performed research and wrote the paper. M.R., M.K., F.C. and R.M. analyzed the data. The final approval of the published version was the responsibility of all authors.

Conflict of interest statement

There is no conflict of interest, and all authors support the submission to this journal.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2021.111577.

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