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Terpenoid-rich *Elettaria cardamomum* extract prevents Alzheimer-like alterations induced in diabetic rats via inhibition of GSK3β activity, oxidative stress and pro-inflammatory cytokines

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ABSTRACT

Purpose: Recent studies suggested that the non-familiar form of Alzheimer's disease (AD) could be consequence of metabolic syndrome and neuroinflammation. *Elettaria cardamomum* extract (EC) has exhibited antidiabetic, antioxidant and anti-inflammatory properties. This research was conducted to evaluate the effects of EC on AD-like alterations in rats induced by high fructose and high fat diet coupled with a single small dose of STZ (25 mg/kg) (T2DM rats).

Methods: Phytochemical analysis was carried out. Behavioral tests, immunohistochemical examination, biochemical analysis and gene expression determination were performed in treated and controls rats.

Results: The majority of EC compounds were terpenoids. EC extract administration for 8 weeks attenuated AD-like alterations. It reversed a T2DM-induced decline in cognitive functions in passive avoidance task and Morris water maze test. It significantly lowered the elevated hippocampal level of AChE activity and caspase-3 activity, an indicator of degeneration in T2DM rats Also, it reduced the accumulation of Aβ and p-tau in the brain of T2DM rats. Furthermore, it elevated the suppressed glutamate receptor expression (AMPA GluR1 subunit and NMDA receptor subunits NR1, NR2A, NR2B). EC treatment reduced hippocampal lipid peroxidation marker malondialdehyde (MDA) and augmented antioxidant defensive system, including superoxide dismutase (SOD) and reduced glutathione (GSH). Meanwhile, it lowered hippocampal TNFα, IL β1but not IL6 and reduced GSK-3β in brainT2D rats.

Conclusion: EC treatment could ameliorate AD-like alterations in T2DM rats through activation of blunted insulin signal transduction in the brain, attenuation of associated oxidative stress and neuroinflammation.

1. Introduction

Alzheimer's disease (AD) is a degenerative brain disease and the most common cause of dementia. AD is characterized by extracellular amyloid beta (Aβ) plaques and intraneuronal deposits of neurofibrillary tangles (NFTs) [1]. Interestingly, obesity, T2DM, and AD are reported to be related to each other according to the Mayo Clinic Alzheimer Disease Patient Registry, 80% of AD patients show impairment in glucose tolerance or have diabetes [2]. Epidemiological studies have demonstrated that T2DM induces cognitive impairment and that patients with T2DM are more likely to be diagnosed with dementia [3]. Previous studies in high fat diet-induced AD animal models have shown that brain insulin resistance in these animals leads to the accumulation of amyloid beta (Aβ) and the reduction in GSK-3b phosphorylation, which promotes tau phosphorylation to cause AD [4]. Metabolic derangements cause inflammation, insulin resistance, endoplasmic reticulum stress in brain and impairment of cognitive functions [5]. Insulin resistance plays an important role in AD pathogenesis, possibly

Abbreviations: AD, Alzheimer's disease; Aβ, Amyloid beta; STZ, Streptozotocin; HF/Hfr, High-fat/High-fructose; T2D, Type2 diabetes; EC, *Elettaria cardamomum*; veh., Vehicle; GSK-3β, glycogen synthase kinase-3 beta; AChE, Acetylcholiesterase; TNF-α, Tumor necrosis factor alpha; IL-1β, Interleukin one beta; IL-6, Interleukin-6; GSH, Glutathione; SOD, Superoxide dismutase; MDA, Malondialdehyde; NMDA, N-methyl-D-aspartate; AMPAR, α-amino-3-hydroxy-5-isoxazole propionic acid receptor

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due to abnormal GSK3β activation, causing intra- and extracellular amyloid-beta (Aβ) accumulation [6]. Persons suffering from metabolic diseases show higher levels of oxidative stress. Increased ROS causes brain metabolism alterations. Oxidative stress induces brain metabolism alterations, resulting in Alzheimer's disease [7].

Although both cholinesterase inhibitors and NMDA receptor blockade have well-proven efficacy levels, the clinical outcomes of patients receiving such treatments are rather limited [8]. An adequate therapeutic treatment that targets AD has not yet been established. Recent suggestions about disease-modifying“ therapeutic options included anti-amyloid and anti-Tau or agents block the synthesis and aggregation of amyloid β42 or the formation of neurofibrillary tangles as specific Y-secretase inhibitors are still needed to prove its efficiency in treating AD [8]. Therefore the new targeted therapies for AD could potentially decrease neuroinflammation and oxidative stress have recently gained attention [9].

Elettaria cardamomum (EC) possesses various pharmacological activities. EC administration in rats down-regulated carrageen induced increase in cytokines such as COX-2, IL-6, and TNF-a and inhibited i-NOS-mediated NO generation. EC also exhibited antioxidant effects by restoring SOD, catalase, GSH levels [10]. Rhman et al. [11] suggested that EC powder supplementation prevents obesity, improves glucose intolerance, inflammation and oxidative stress in liver of high carbohydrate, high-fat diet induced obese rats. In addition, Bhat et al. [12] reported that EC has comparable efficacy to pioglitazone in preventing dexamethasone induced hepatomegaly, dyslipidemia, and fasting hyperglycemia. Moreover, Kazemi et al. [13] demonstrated that EC could improve some parameters of inflammation and oxidative stress in prediabetic subjects. Therefore, they suggested that EC may be useful in reducing complications associated with inflammation and oxidative stress in these patients. Interestingly, Kunwar et al. [14] suggested that EC may improve learning and memory of amnesic mice induced by scopolamine and this effect can be attributed to decreased oxidative stress and reduction in brain cholinesterase levels.

An adequate therapeutic treatment that targets AD in T2DM patients has not yet been established. Meanwhile, we believe that EC could be a therapeutic option for treating AD resulted in T2DM patients. The possible therapeutic effect of cardamom on AD-like alterations in T2DM rats characterized by brain insulin resistance has not yet been investigated. Considering all above reports, the current investigation was undertaken to evaluate the potential benefit and possible mechanism of cardamom extract in high fat/high fructose (HF/HFr) diet and STZ induced T2DM and AD-like literations rats.

2. Materials and methods

2.1. Extraction and phytochemical analysis of cardamom

2.1.1. Preparation of cardamom extract

The powdered fruits of Elettaria cardamomum (EC) were conveniently charged in a percolator and extracted thrice with petroleum ether at room temperature using 2 L of the solvent each time. The marcs were air-dried and then extracted thrice with alcohol (3 × 2 L). Subsequently, the extracts were subjected to complete dryness under vacuum.

2.1.2. Estimation of total phenolic content

Total phenols in the extract were determined spectrophotometrically according to the method of Lee et al. [15] using gallic acid as a standard. Briefly, EC extract was thoroughly mixed with Folin-Ciocalteu reagent, followed by the addition of Na_2CO_3 solution and the mixture was incubated in dark at room temperature for 60 min. The absorbance was then recorded at 730 nm and results were expressed in μg gallic acid equivalent (GAE)/mg dry weight (DW).

2.1.3. Estimation of total flavonoid content

Determination of total flavonoid content of EC extract was carried out by the method of Chang et al. [16], where the methanolic solution of the crude extract was mixed with 5% $NaNO_2$, 10% $AlCl_3$ and 1 M NaOH solution; and absorbance was measured at 510 NM using rating as a standard. Results were expressed in μg rutin equivalent (RE)/mg DW.

2.1.4. Gas chromatography/mass spectrophotometry

Analysis of EC extract was carried out by Gas Chromatography/Mass Spectrophotometry (GC/MS) (7890A-5975B) [17] at the Analytical Chemistry Unit, Faculty of Science, Assiut University. The analytical column used was a DB-5 ms (30 m × 0.25 mm × 025 μm), with a temperature profile between 40 °C and 280 °C, a total run time of 47.5 min and a flow rate ranging from 0.5 to 1 ml/min.

2.2. Materials

Streptozotocin was purchased from Sigma Aldrich (St. Louis, MO, USA). Rat TNF-α, IL-1β, IL-6, GSK-3β and caspase-3 ELISA kits were purchased from Wkea (China). Rat serum insulin ELISA kit was purchased from Calbiotech (USA). Serum glucose and total cholesterol detection kits were purchased from Spectrum (Egypt). Spectrophotometric kits for superoxide dismutase, reduced glutathione, malondialdehyde were purchased from Biodiagnostic (Egypt) and that for cholinesterase from Chronolab (Spain). Beta-amyloid (Aβ 1-42) antibody and p-tau (Ser202, Thr205) antibody (AT8) were purchased from Abcam (Cambridge, UK) and Thermo Fisher (USA); respectively. Species-specific biotinylated secondary antibody (Goat anti-mouse IgG) was purchased from ScyTek (USA). Direct-zol™ RNA MiniPrep kit was purchased from Zymo research corporation (USA). SensiFAST™ cDNA Synthesis and SensiFAST™ SYBR Hi-ROX Kits were purchased from Bioline reagents Ltd (London, UK). Gene-specific primers were purchased from Invitrogen (UK).

2.3. Animals

Male Wistar rats (6 months old) were used for the induction of T2DM. For the acute toxicity study, male Swiss albino mice, weighing 25–30 g, were employed. Animals were purchased and housed in the Assiut University animal care facility until sacrificed. The animal was acclimatized to controlled room temperature (25 °C) and humidity (65–75%) under a 12 h: 12 h light–dark cycle. The rats had free access to tap water and diet. All experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee guidelines (Medical ethics committee, Assiut University).

2.4. Experimental design

2.4.1. Acute oral toxicity study

An acute toxicity study was performed on 5 different groups of mice (n = 5). Each group administered 10, 15, 20, 25 and 30 folds of the largest tested dose of EC extract (400 mg/kg; i.e., 4, 6, 8, 10 and 12 g/kg; respectively) orally by gavage. Animals were observed periodically over the next 24 hrs for any ultimate mortality [18]. LD50 was calculated

2.4.2. Induction of type 2 diabetes and Alzheimer's disease like alterations

After one week of adaptation to laboratory conditions, T2DM with the insulin resistant state was induced in rats by the method of Anderson et al. [19] and Niu et al. [20] with slight modifications. Rats were fed with either conventional chow for the normal control group (NC) or high fat/high fructose (HF/Hfr) diet. The composition of the HF/HFr diet was as follows: 20% fructose, 5% sucrose, 15% starch, 30% lard, 3.5% soybean oil, 5% fiber, 15% casein, 3.5% mineral mix, 1% vitamin mix, 0.3% dl-methionine and 0.2% choline bitartrate, added to

1.5% normal pellets. The HF/HFr-fed animals were injected at the end of week 4 with a single low dose of streptozotocin (STZ; 25 mg/kg/IP, dissolved in 0.1 M citrate buffer, pH 4.4) and received 10% w/v sucrose solution in their drinking water for the first 24 hrs to prevent hypoglycemia.

Single tail tip pricks were performed 72 hrs following STZ injection to HF/HFr/L-STZ animals and blood glucose levels were measured, using glucose test strips and a glucometer (Smart Test, Taiwan). To confirm hyperglycemia, additional blood samples were collected 4 days later from overnight fasted animals for the measurement of blood glucose. Rats with fasting blood glucose levels ≥ 250 mg/dl were considered diabetic and included in the study. Rats were then constantly fed with the same HF/HFr-diets for an additional 8 weeks. This model of induction of some Alzheimer's disease -like alterations was characterized by Niu et al. [20].

2.4.3. Animal grouping

After confirmation of hyperglycemia following STZ injection, T2DM rats were randomly divided into 4 groups of 6 rats each and started receiving drugs or vehicle while they were constantly fed with the same diets. Group II was fed with HF/HFr diet, received oral Tween 80 (1% v/v) and served as T2DM control (T2DM + vehivle). Groups III, IV and V were fed with HF/HFr diet, and received EC extract, orally by gavage, emulsified in 1% Tween 80 at doses of 100, 200 and 400 mg/kg; respectively (T2DM + EC100, T2DM + EC200 and T2DM + EC400). However,The group I was non diabetic rats and fed with conventional chow, once injected i.p. with 0.1 ml of 0.1 M citrate buffer (pH 4.4), and served as normal control (NC + vehicle). All groups received the tested drugs once daily for the rest of the 8 weeks of the experimental period.

At the end of the treatment period, the effect of treatments on behavioral tests for learning and memory was investigated by passive avoidance and Morris water maze tests.

2.5. Behavioral tests

2.5.1. Passive avoidance task

This test relies on the rats' natural preference of darkness. The passive avoidance apparatus (Ugo Basile, Italy) consists of an electric grid floor and is divided into two equally sized compartments, light and dark, by a partition with a sliding (guillotine) door. The test (retention) trial was conducted 24 hrs after the acquisition trail, where rats were again placed in the light compartment, and the latency time was measured, with a cut-off period of 300 s [21].

2.5.2. Morris water maze (MWM)

Spatial learning was assessed using the Morris water maze according to method of Shin and Lee [21]. Rats were required to escape onto a platform submerged in a circular pool of 1.4-m-diameter.. Animals of all groups underwent 3 trials/day for 6 consecutive days, and the latency to reach the hidden platform was recorded for each trial. On the 7th day, animals received a probe trial (retention test), in which the platform was removed from the tank, and animals were allowed to swim freely for 60 sec. The latency to locate the position of the platform and the time spent on the target quadrant were recorded [22].

2.6. Specimens preparation

After finishing the behavioral tests and after overnight fasting, all animals were sacrificed after anesthetization with ether 2% and blood samples were withdrawn from posterior vena cava for separation of serum, and serum was stored at −20 °C until further use. Brain tissue from each rat was isolated and bisected into hemispheres; the left ones were fixed for 48 hrs in 10% neutral-buffered-formalin to be used in immunohistochemical staining. The hippocampi of the right hemispheres were immediately dissected on dry ice, dried, weighed, stored at −80 °C, and upon use, they were homogenized in PBS (pH 7.4) and homogenates were centrifuged for 10 min to remove debris. The supernatants of all samples were snap-frozen in liquid nitrogen and kept at −20 °C until ELISA, spectrophotometric and quantitative real-time polymerase chain reaction (qRT-PCR) analyses were performed. Protein concentration was measured by the Lowry's method [23].

2.7. Experimental measurements

2.7.1. Immunohistochemistry (IHC):

Immunohistochemical analysis was conducted in order to investigate the impact of EC on the accumulation of Aβ and hyperphosphorylated tau, the major constituents of senile plaques and the neurofibrillary tangles in the diabetic rats. In brief, formalin-fixed left hemispheres were embedded in paraffin, and sections (3–4 μm) were dewaxed and rehydrated. Antigen retrieval was achieved by microwave heating of tissue sections in citrate buffer for 8 min. To assess patterns of development of Aβ 1-42 and p-tau proteins in rat hippocampus, sections were incubated with anti-Aβ 1-42 antibody (1:100) for 2 hrs and anti- p-tau (Ser202, Thr205) antibody (AT8, 1:100) overnight at room temperature; respectively. Then, all sections were incubated with corresponding biotinylated secondary antibody, visualized by chromogen diaminobenzidine and counterstained by hematoxylin stain. Negative control slides were included in the study, where the primary antibody was omitted from the immunobuffer. All immunostained brain sections were blind-coded and examined under the same standardized conditions with light microscopes (Olympus) [24].

2.7.2. Marker of neurodegeneration

Caspase-3 activity was measured in hippocampal homogenate using an ELISA kit and assay was performed according to the manufacturer's protocol [25].

2.7.3. Determination of hippocampal acetylcholinesterase (AChE) activity

AChE inhibitory activity in hippocampal homogenate was detected spectrophotometrically at 405 NM based on Ellman's method [26]. The method depends on the reaction of thiocholine, produced by hydrolysis of acetylthiocholine by cholinesterase, with dithiobis-nitrobenzoic acid (DTNB), forming a product whose rate of formation is proportional to the enzymatic activity of cholinesterase in the sample.

2.7.4. Measuring the serum levels of glucose, insulin and total cholesterol

Serum glucose levels were determined using commercially available glucose detection kits. The estimation of serum insulin levels was performed using a rat insulin ELISA kit based on the manufacturer's protocol. Briefly, standards and samples were loaded into precoated microplate wells with antibodies specific for rat insulin and incubated with the enzyme conjugate. After washing and substrate incubation, the absorbance was recorded on ELISA microplate reader at 450 nm.

Serum total cholesterol levels were measured using commercially available colorimetric diagnostic kits according to the manufacturer's instruction.

2.7.5. Assessment of insulin resistance

A. HOMA-IR index for assessment of peripheral insulin resistance

The homeostatic model assessment of insulin resistance (HOMA-IR) index was used to estimate insulin resistance, as reported previously by Wang et al. [27], and Gomaa et al.[28] using fasting serum insulin and glucose concentrations. The HOMA-IR was calculated using the following formula:

HOMA-IR = [fasting insulin (μU/ml) × fasting glucose (mmol/l)]/ 22.5

B. Brain insulin resistance

To determine the effect of treatments on insulin receptor signaling in the brain, GSK-3β levels were quantified in the hippocampal homogenates using ELISA kits and assays were performed according to the manufacturer's protocol [29]. Standard and samples (1:4 diluted with sample diluent) were added to a precoated 96-well plate. After incubation with enzyme conjugate and substrates, absorbance was determined at 450 nm.

2.7.6. Biomarkers of hippocampal inflammation

These experiments were performed to determine the effect of treatments on the hippocampal levels of proinflammatory cytokines TNF-α, IL-1β, and IL-6 using ELISA kits and assays were performed following the manufacturer's instructions [30]. This experiment was carried out to evaluate the role of inflammatory cytokines in the impairment of cognitive function in HF/HFr/L-STZ rats and to assess the effect of EC on brain levels of proinflammatory cytokines (TNF-α, IL-1β, and IL-6).

2.7.7. Biomarkers of hippocampal oxidative stress

A. Reduced glutathione (GSH)

In order to assess the effect of treatments on the impaired antioxidant status in the hippocampus of T2DM rats, GSH concentration was determined in rat hippocampus by the use of a glutathione detection spectrophotometric kit, assay was performed according to the manufacturer's protocol [31].

B. Superoxide dismutase (SOD)

The activity of SOD was evaluated colorimetrically according to the manufacturer's protocol. The increase in absorbance was measured at 560 nm every min for 5 min at 25 °C [32].

C. Malondialdehyde (MDA)

Malondialdehyde, a lipid peroxidation marker, was estimated colorimetrically at 534 nm according according to the manufacturer's protocol [33].

2.7.8. Quantitative real-time polymerase chain reaction (qRT-PCR) for quantifying the gene expression of glutamate receptor subunits in hippocampus of T2D rats

In oreder to evaluate the effect of treatment on the hippocampal gene levels of glutamate receptors, qRT-PCR analysis was conducted. Gene expression of AMPAR subunit GluR1 and the NMDA receptor subunits NR1, NR2A, NR2B, NR2C, and NR2D was quantified. Total RNA was extracted from hippocampal specimens dissected from the right hemispheres of rat brain using Direct-zol™ RNA MiniPrep kit in accordance with the manufacturer's instructions. Samples were treated with DNase to avoid DNA contamination. RNA concentrations were determined using a NanoDrop® (Epoch Microplate Spectrophotometer, Biotek, VA, USA). Complementary DNA (cDNA) needed for qRT-PCR was obtained using the SensiFAST™ cDNA Synthesis Kit (Bioline reagents Ltd London, UK, catalog no. Bio-65053). Real-time polymerase chain reactions were carried out using sybrgreen dye (SensiFAST™ SYBR Hi-ROX Kit). Gene-specific primer sequences are presented in Table1.Analysis of results was performed by the aid of 7500 fast biosystem software using the comparative cycle threshold method (comparative ct method) [34].

2.8. Statistical analysis

Data are expressed as the mean ± standard error (SE). Statistical analysis was performed by a one-way analysis of variance (ANOVA), followed by Tukey's post hoc test, using GraphPad Prism 5.03 (GraphPad Software, Inc.). For all statistical comparisons, a P-value < 0.05 was considered statistically significant.

3. Results

3.1. Phytochemical profile of EC extract

3.1.1. Total phenolic and total flavonoid contents

The total phenolic content of the fruit extract of EC was 26.17 ± 2.88 μg GAE/mg DW whereas the flavonoid content was 9.69 ± 0.23 μg RE/mg DW.

3.1.2. Chemical composition of EC extract by GC/MS

The GC/MS analysis showed that the majority of the content of EC was terpenoid compounds. The GC/MS analysis of EC extract demonstrated the presence of monoterpenes, as α-terpinyl acetate (38.3%), α-terpineol (1.6%), β-terpineol (0.16%) and 1,8-cineole (0.21), and sesquiterpenes including nerolidol (3%) and iso spathulenol (0.04%) (Fig. 1A).

3.2. Acute oral toxicity of EC

The acute toxicity of EC extract on mice revealed that there were no mortality or adverse effects on the behavior of the tested animals at doses of 4 and 6 g/kg. In contrast, doses of 8, 10 and 12 g/kg resulted in 20, 60 and 80% death in the tested animals; respectively. The LD50 of orally administered EC extract in mice was estimated to be 9.034 g/kg, with 95% confidence limits 7.784–10.67 g/kg. These results could suggest that the oral EC extract has no acute toxic effects at doses up to 6 g/kg.

3.3. Effect of EC extract on learning and memory deficits in diabetic Wistar rats

3.3.1. Passive avoidance task

The initial latency (IL) didn't differ significantly among animal groups during the acquisition phase. On the contrary, in the retention trial, either control or diabetic rats that received EC displayed significant increases in step-through latency (STL), compared to diabetic control rats that received vehicle (288 ± 3.3, 68.5 ± 6.8, 277 ± 4.7 and 285 ± 4.5 s for NC, T2DM + EC100, T2DM + EC200 and T2DM + EC400 respectively vs. 13.2 ± 1.2 s for T2DM control; $p < 0.01$). The diabetic treated rats but not control diabetic rats have memorized that their presence in the dark room was accompanied by an aversive stimulus (Fig. 1B).

3.3.2. Morris water maze test

During the acquisition phase, there was a gradual improvement in escape latency to the hidden platform over the 6 days of training. Diabetic control rats showed significantly higher latencies from the 2nd day onwards ($p < 0.01$) (Fig. 1C). Consistent with these findings, in the probe trial, the latency to locate the position of the platform for the T2DM control group was obviously longer than that of NC (34.38 ± 3.59 vs 6.50 ± 0.87 s; $p < 0.01$). Treatment of diabetic rats with EC extract significantly ($p < 0.01$) and dose-dependently reduced the prolonged escape latency to the position of the formerly submerged platform (8.75 ± 1.57, 8.13 ± 1.32 and 7.63 ± 1.44 s for T2DM + EC100, T2DM + EC200 and T2DM + EC400 respectively, Fig. 1D). Similarly, T2DM control rats spent markedly less time on the quadrant where the platform was located than NC group, whereas this time was significantly longer in EC diabetic rats, compared to T2DM control (31.25 ± 2.63, 26.88 ± 2.97, 27.50 ± 2.84 and 30.00 ± 2.9 s for NC, T2DM + EC100, T2DM + EC200 and T2DM + EC400 vs. T2DM + vehicle: 10.88 ± 1.69 s; $p < 0.01$) (Fig. 1E).

Overall, the results of the behavioral tests demonstrated that HF/

Table 1
Primer sequences for qRT-PCR reaction.

Gene	Sense	Antisense	Reference
β-actin	TGACAGGATGCAGAAGGAGA	TAGAGCCACCAATCCACA	Zhou et al. [35]
GAPDH	CCATCCCAGACCCCATAAC	GCAGCGAACTTTATTGATG	Xi et al. [36]
GluR1	GCTTCATGGACATTGACTTA	ATCTCAAGTCGGTAGGAGTA	Lin and Lee [37]
NR1	CTTCCTCCAGCCACTACCC	AGAAAGCACCCCTGAAGCAC	Xi et al. [36]
NR2A	AGGACAGCAAGAGGAGCAAG	ACCTCAAGGATGACCGAAGA	Xi et al. [36]
NR2B	TGAGTGAGGGAAGAGAGAGAGG	ATGGAAACAGGAATGGTGGA	Xi et al. [36]
NR2C	GGGCTCCTCTGGCTTCTATT	GACAACAGGACAGGGACACA	Xi et al. [36]
NR2D	CCCAAATCTCACCCATCCT	GAGAGGTGTGTCTGGGGCTA	Xi et al. [36]

HFr/STZ induced T2DM in rats can cause learning and memory deterioration, and administration of EC extract can reverse or cause memory conservation against T2DM-induced alterations in cognitive functions.

3.4. Effect of EC treatment on amyloid load and tauopathy in insulin resistant brain of T2D rats

A remarkable increase in the mean count of both Aβ 1-42 and p-tau (Ser202, Thr205, AT8) immunoreactivity was observed in the hippocampal slices of vehicle-treated T2DM rats compared to NC (Aβ 1-42: 5.93 ± 1.2 vs. 0.11 ± 0.11; p-tau: 5.28 ± 1.33 vs. 0.0 ± 0.0; $p < 0.01$). Compared with T2DM control group, diabetic rats that received 100, 200 and 400 mg/kg of the EC extract for 8 weeks showed significant decrease in Aβ 1-42 deposits and p-tau-positive cells count in the total brain area analyzed (Aβ 1-42: 1.28 ± 0.47, 0.94 ± 0.25 and 0.33 ± 0.15; $p < 0.01$ and p-tau: 1.72 ± 0.16 for T2DM + EC100; $p < 0.05$, 1.11 ± 0.55 and 1.0 ± 0.56 for T2DM + EC200 and T2DM + EC400 respectively; $p < 0.01$). These findings allow us to suggest that EC may act both extra- and intracellularly to reduce Aβ and tau burden(Fig. 2A–D).

3.5. Effect of EC extract on caspase-3 activity in the hippocampus of T2DM rats

In order to investigate the effect of EC extract on neurodegeneration and apoptosis in diabetic rats, caspase-3 activity was measured in the hippocampus (Fig. 2E). A significant increase in caspase-3 level was found in the hippocampus of T2DM control group compared to NC (37.83 ± 0.82 vs. 10.25 ± 0.99 ng/mg protein; $p < 0.01$). Interestingly, EC extract significantly and dose-dependently decreased the hippocampal level (activity) of caspase-3 in diabetic rats compared to T2DM control group (23.5 ± 0.95, 21.0 ± 1.44 and 18.33 ± 1.55 ng/mg protein in T2DM + EC100, T2DM + EC200 and T2DM + EC400 respectively; $p < 0.01$).

3.6. Effect of EC on acetylcholinesterase (AChE) activity in the hippocampus of the T2DM rats

The present study showed a significant increase in the activity of AChE in the hippocampal homogenates of diabetic control animals compared to their normal surrogate (0.007 ± 0.0005 vs. 0.003 ± 0.0002 U/mg protein; $p < 0.01$). Administration of EC for 8 weeks significantly lowered AChE activity, indicating that EC extract exhibited AChE inhibitory activity at all the tested dose levels (0.0048 ± 0.0001, 0.0047 ± 0.0001 and 0.0046 ± 0.0002 U/mg protein in T2DM + EC100, T2DM + EC200 and T2DM + EC400 group respectively vs. 0.007 ± 0.0005 U/mg protein in T2DM + vehicle; $p < 0.01$) (Fig. 2F).

3.7. Effect of EC extract on fasting serum glucose, insulin and cholesterol levels in diabetic rats

HF/HFr diet coupled with a single STZ injection markedly increased fasting serum glucose (307.3 ± 21.5 vs. 92.86 ± 4 mg/dl; $p < 0.01$) and insulin (24.59 ± 0.78 vs. 19.77 ± 0.47 μU/ml $p < 0.01$) levels compared to normal diet control. Eight weeks of treatment with EC extract in diabetic rats significantly reduced fasting serum glucose levels compared to T2DM control group (253.3 ± 8.74 mg/dl for EC100; $p < 0.05$, 146.4 ± 3.71 and 112.3 ± 4.96 mg/dl for EC200 and 400; $p < 0.01$). Serum insulin levels were significantly lower in diabetic rats treated with 200 and 400 mg/kg of EC compared to T2DM control group (21.84 ± 0.6 μU/ml; $p < 0.05$ and 21.33 ± 0.49 μU/ml; $p < 0.01$ vs. 24.59 ± 0.78 μU/ml; Fig. 3A and B).

Moreover, significant increase in serum levels of cholesterol was established in HF/HFr-fed-L-STZ-injected rats compared with rats fed with normal diet (119.6 ± 6.84 vs. 60.47 ± 4.62 mg/dl; $p < 0.01$) (Fig. 3C). EC extract at the 3 doses (100, 200 and 400 mg/kg) significantly reduced the elevated cholesterol level in diabetic rats compared to T2DM control rats (80.38 ± 2.21, 78.89 ± 1.62 and 71.93 ± 1.25 mg/dl; $p < 0.01$).

3.8. The influence of EC extract on insulin resistance in diabetic rats

3.8.1. Effect of EC on peripheral insulin resistance index in diabetic rats

In order to assess insulin resistance, the HOMA-IR index was subsequently calculated for each animal group. HF/HFr diet -STZ regimen resulted in a significant increase in HOMA-IR scores in the T2DM control group compared with NC (18.43 ± 1.44 vs 4.52 ± 0.19 $p < 0.01$), whereas EC treated rats showed significantly lower HOMA-IR values than T2DM control rats. (13.94 ± 0.72,7.98 ± 0.25 and 5.94 ± 0.25 for T2DM + EC100, T2DM + EC200 and T2DM + EC400 respectively; $p < 0.01$; Fig. 3D).

3.8.2. Impact of EC treatment on hippocampal level of GSK-3β, (central insulin resistance indicator) in type 2 diabetes rats

Glycogen synthase kinase-3 beta (GSK-3β) is an important kinase involved in insulin signaling pathway and in tau hyperphosphorylation in the brain. Activation of insulin receptors leads to inactivation of GSK-3β. The resuls of this study revealed that hippocampal levels of GSK-3β in vehicle-treated diabetic rats were notably increased compared to normal rats (26.12 ± 2.22 vs. 10.77 ± 1.29 pg/mg protein; $p < 0.01$). This may point to reduction in insulin sensitivity and brain insulin signaling with the development of central insulin resistance in HF/HFr/L-STZ T2D rats that received the vehicle. In contrast, a significant reduction in GSK-3β was found in the hippocampus of EC treated T2DM rats compared to control T2DM rats (11.92 ± 1.49, 11.58 ± 1.38 and 11.33 ± 0.88 pg/mg protein for the 3 doses respectively; $p < 0.01$) (Fig. 3E). Improvement of cognitive functions by treatment with EC could be due to attenuation of central insulin resistance.

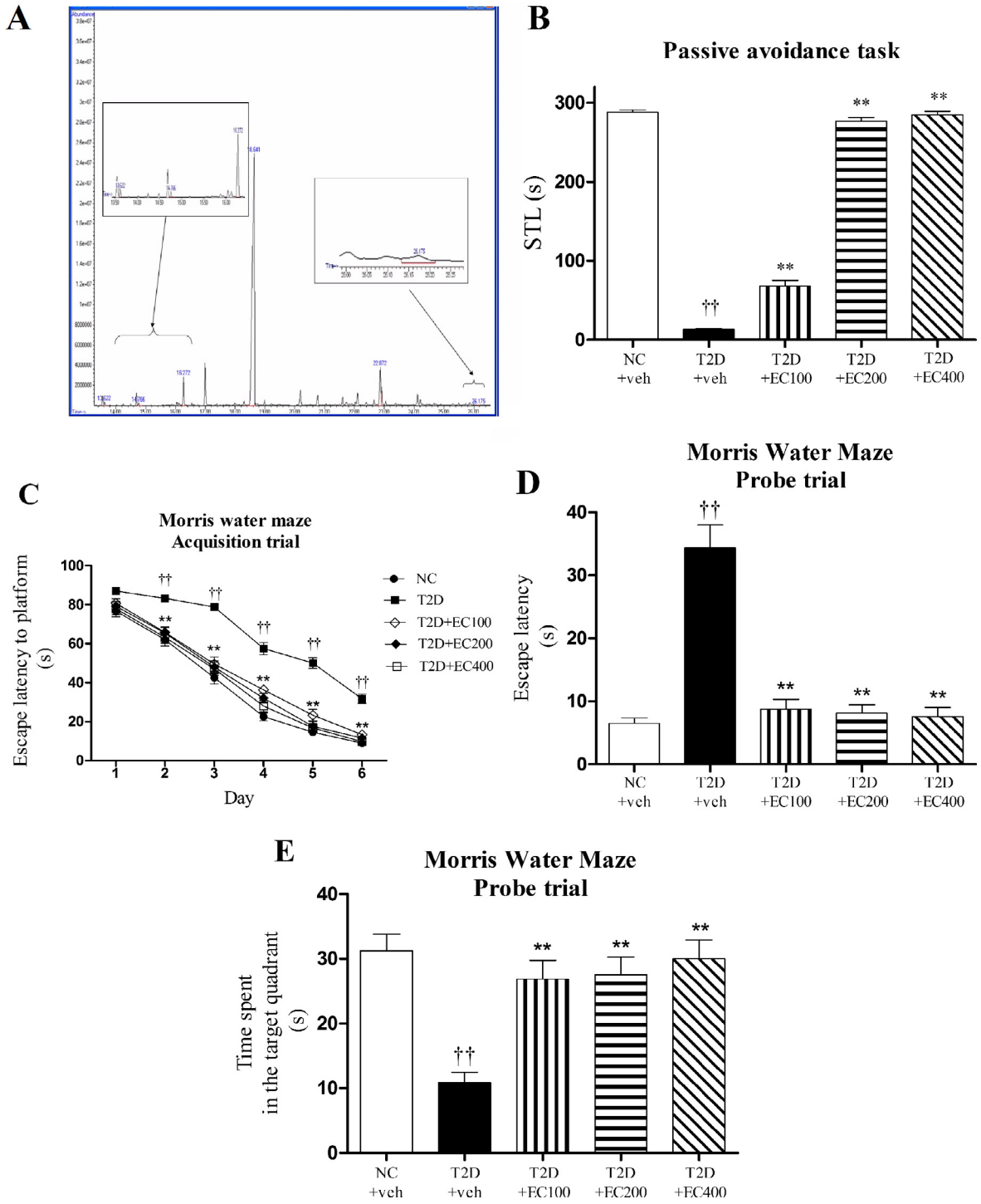

Fig. 1. GC/MS chromatogram of EC extract (A). Effect of EC administration on learning and memory deficits in HF/HFr/L-STZ Wistar rats. (B) Step-through latency (STL) to enter the dark compartment during the retention trial, as determined in the passive avoidance task. (C) Escape latency to the hidden platform along the 6 days of the acquisition trial in the Morris water maze (MWM) test. Escape latency to reach the position of the previously immersed platform (D) and time spent on the target quadrant where the platform was located (E) during MWM probe trial; $^{\dagger\dagger}p < 0.01$ vs NC + veh, $^{**}p < 0.01$ and $^{*}p < 0.05$ vs T2D + veh. NC: normal control, T2D: type 2 diabetic control, T2D + EC100, T2D + EC200 and T2D + EC400.

3.9. Effect of EC on the hippocampal level of proinflammatory cytokines in diabetic rats

A significant increase in proinflammatory cytokines levels was found in the hippocampus of T2DM control rats compared to NC. The hippocampal level of TNF-α in T2DM control group was remarkably higher than that of the normal control group (4.78 ± 0.15 vs. 3.69 ± 0.09 pg/mg protein; $p < 0.01$). Marked reduction in the elevated TNF-α levels was observed following 8 weeks of treatment with 100, 200 and 400 mg/kg of EC extract (4.19 ± 0.22 pg/mg protein for EC100; $p < 0.05$; 3.86 ± 0.1 and 3.69 ± 0.06 pg/mg protein for EC200 and 400; $p < 0.01$) (Fig. 4A). Similarly, significant elevations in the levels of IL-1β (2.71 ± 0.19 vs. 1.42 ± 0.13 pg/mg protein; $p < 0.01$) and IL-6 (3.07 ± 0.11 vs. 2.66 ± 0.07 pg/mg protein;

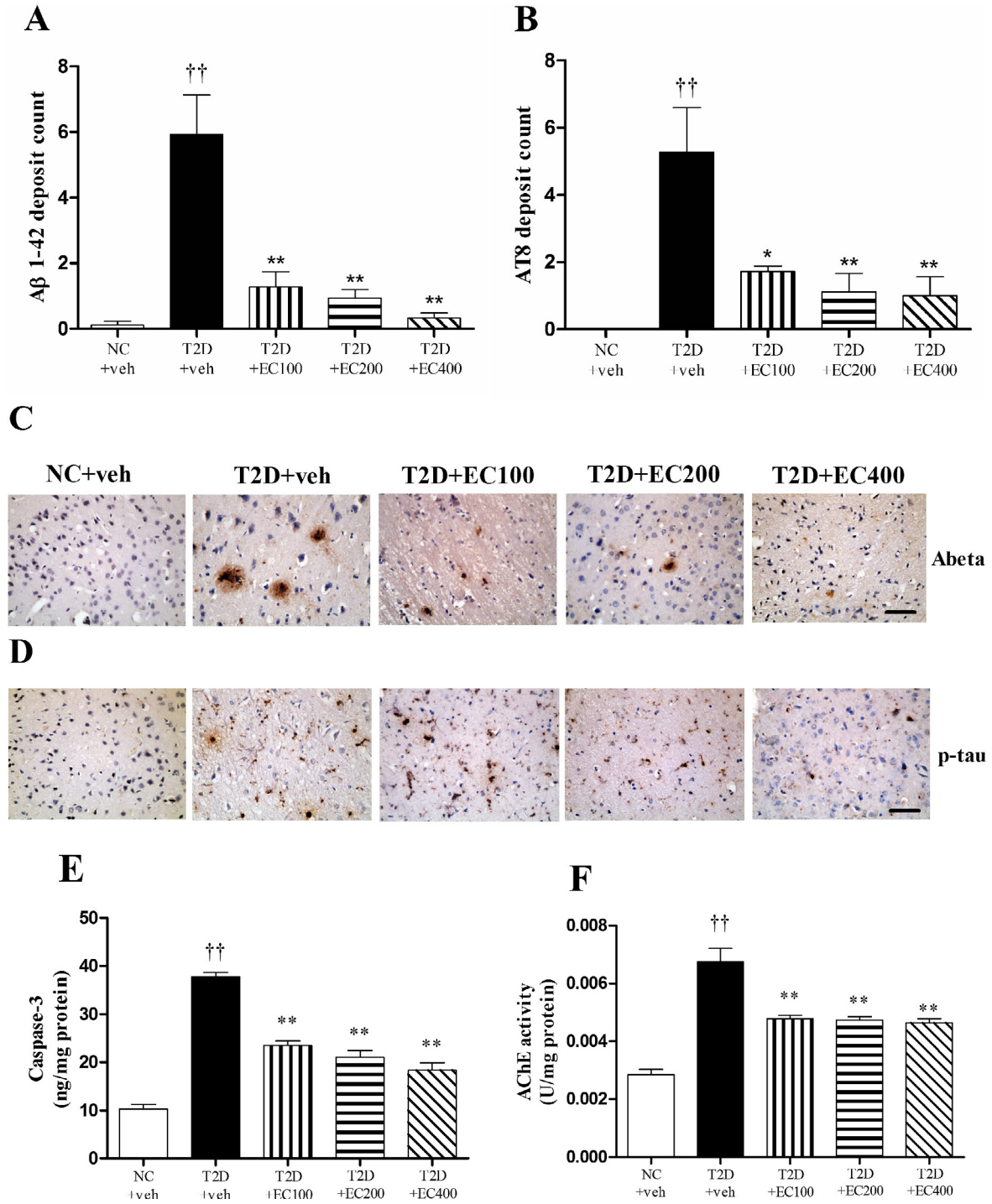

Fig. 2. Immunohistochemical quantification of Aβ 1-42 plaques (A) and AT8-labeled neurofibrillary tangles (B) in the brain of EC treated T2D rats. Photomicrographs of hematoxylin-stained brain sections from different animal groups showing Aβ 1-42 plaques (C) and AT8-labeled neurofibrillary tangles (D). Effect of EC extract on hippocampal caspase-3 (E) and AChE activities (F) in T2D rats. $^{\dagger\dagger}p < 0.01$ vs NC + veh, $^{**}p < 0.01$ and $^{*}p < 0.05$ vs T2D + veh (ANOVA). Aβ: amyloid-β, AT8: phospho-tau epitope, AChE: acetylcholinesterase, ☐ NC: normal control, ■ T2D: type 2 diabetic control, ▥ T2D + EC100, ▤ T2D + EC200; and ▨ T2D + EC400.

$p < 0.05$) were detected in the hippocampus of T2DM control rats compared to NC rats. Likewise, IL-1β levels were notably declined in the hippocampus of animals treated with 400 mg/kg of EC (1.86 ± 0.11 vs. 2.71 ± 0.19 pg/mg protein; $p < 0.01$) (Fig. 4B). Interestingly, there was a slight and non-significant decrease in hippocampal IL-6 in EC treated rats at all dose levels (Fig. 4C). These results may also emphasize the potential anti-inflammatory and cytokine suppressant effects of EC in T2DM rat brain, which may underlie its beneficial effects on cognitive decline induced by T2DM.

3.10. Influence of EC on the oxidant/antioxidant status in the hippocampus of T2DM rats

As shown in Fig. 4D–F, HF/HFr diet along with STZ injection resulted in disruption in the oxidant/antioxidant status in the brain of T2DM control group, with significant lowering, in the hippocampal

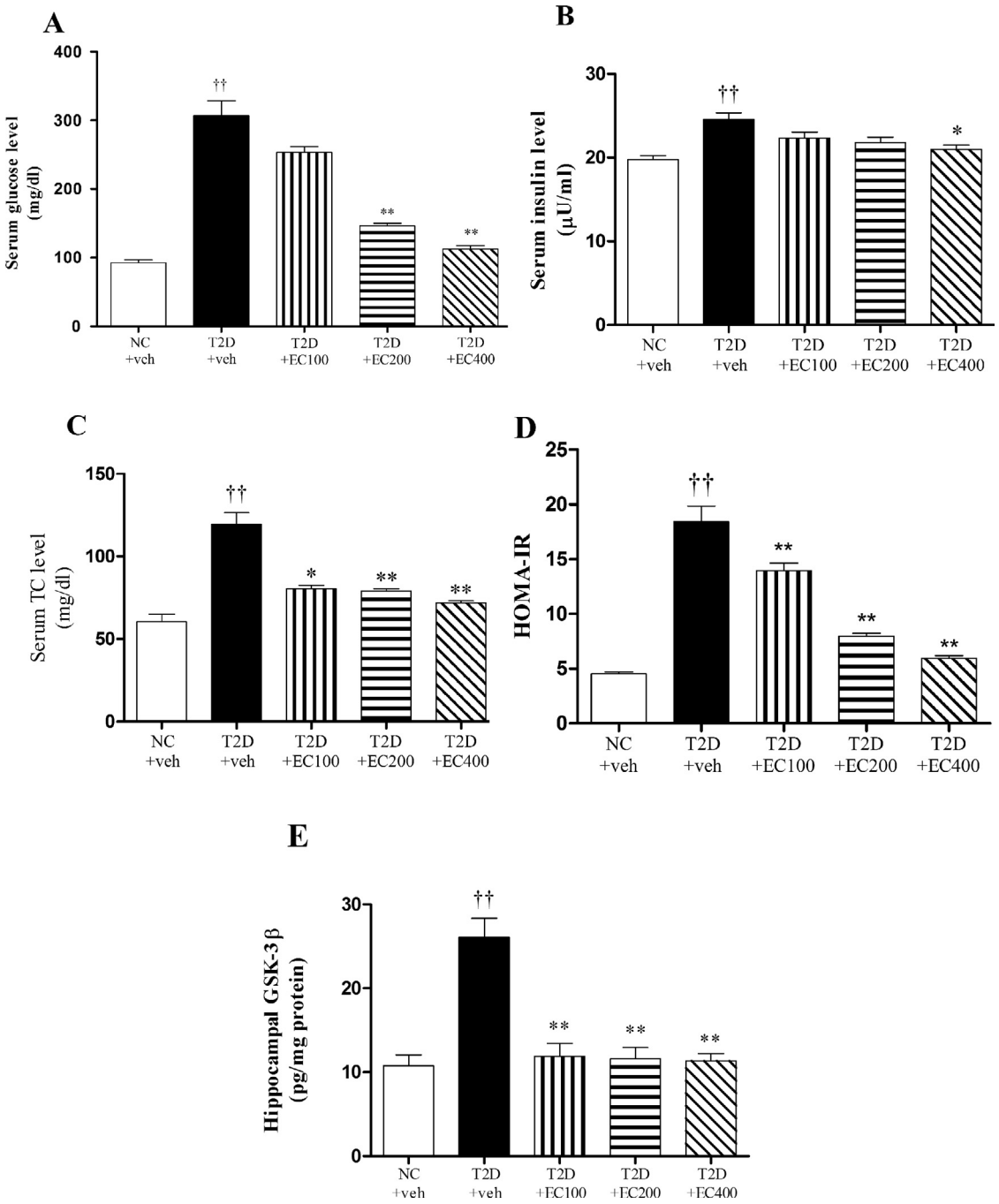

Fig. 3. Effect of EC administration on fasting serum glucose (A), insulin (B), TC levels (C), HOMA-IR) scores (D) and hippocampal GSK-3β level (E) in T2D rats. $^{††}p < 0.01$ vs NC + veh, $^{**}p < 0.01$ and $^{*}p < 0.05$ vs T2D + veh (ANOVA). TC: total cholesterol, HOMA-IR: homeostatic model assessment of insulin resistance, GSK-3β: glycogen synthase kinase-3 beta, NC: normal control, T2D: type 2 diabetic control, T2D + EC100, T2D + EC200 and T2D + EC400.

levels of GSH (3.01 ± 0.31 vs. 6.49 ± 0.39 nmol/mg protein; $p < 0.01$) and SOD (425.8 ± 44.9 vs 755.9 ± 17.63 U/mg protein; $p < 0.01$) (Fig. 4D and E), whereas MDA level was significantly higher than that of NC group (49.05 ± 0.39 vs. 14.3 ± 0.75 nmol/mg protein; $p < 0.01$) (Fig. 4F). Eight weeks of EC administration at all dose levels restored oxidant/antioxidant balance with significant and dose-dependent elevation in the antioxidant status. The GSH hippocampal tissue levels were 5.99 ± 0.33, 6.28 ± 0.39 and 6.39 ± 0.4 nmol/mg protein and SOD levels were 785.3 ± 20.35, 797.8 ± 21.19 and 839.5 ± 20.16 U/mg protein for T2DM + EC100, T2DM + EC200 and T2DM + EC400; respectively ($p < 0.01$). Also, EC treatment produced a significant reduction in the oxidative damage marker MDA in the hippocampus of diabetic rats (32.08 ± 1.76, 28.17 ± 1.46 and 23.58 ± 2.12 nmol/mg protein for T2DM + EC100, 200 and 400, respectively; $p < 0.01$). These findings illustrate that the observed antioxidant effect of EC extract could repair the oxidative imbalance in the brain of T2DM rats with central insulin resistance. This may have an impact on understanding the potential mechanisms involved in ameliorating cognitive dysfunction by EC treatment.

3.11. Effect of EC on hippocampal gene expression of AMPA and NMDA glutamate receptor subunits in T2DM rats

The hippocampal gene expression of AMPA GluR1 subunit and NMDA receptor subunits NR1, NR2A, NR2B, NR2C, and NR2D were measured using qRT-PCR. As shown in Fig. 5, a detectable decrease in

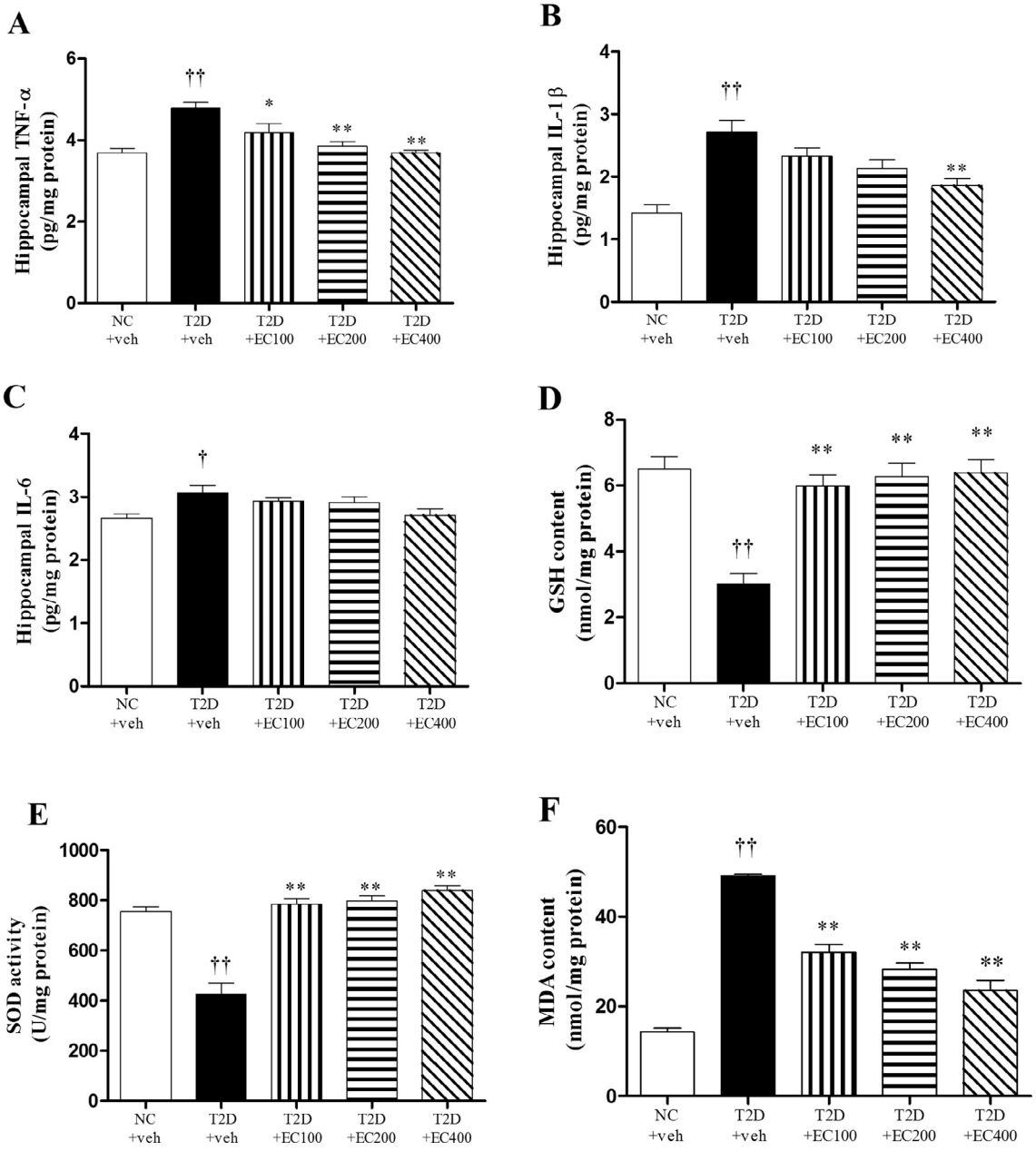

Fig. 4. Effect of 8 weeks of treatment with EC on the level of proinflammatory cytokines and the disrupted oxidant/antioxidant balance in the hippocampus of T2D rats. hippocampal TNF-α (A), IL-1β (B), IL-6 (C), GSH (D) levels, SOD activity (E) and MDA content (F). $^{\dagger\dagger}p < 0.01$, $^{\dagger}p < 0.05$ vs NC + veh and $^{**}p < 0.01$ vs T2D + veh (ANOVA). ☐ NC: normal control, ■ T2D: type 2 diabetic control, ▥ T2D + EC100, ▤ T2D + EC200 and ▨ T2D + EC400.

fold change (% NC) of the mRNA levels of GluR1, NR1, NR2A, and NR2B, but not NR2C and NR2D, was observed in vehicle-treated T2DM group (57.8 ± 3%, 62.6 ± 5.6%, 58.4 ± 4.41% and 57.42 ± 3.89%; respectively). Indeed, EC treatment was able to correct the reduced expression found in T2DM rat hippocampus. Significant elevation was observed with EC extract at doses of 200 and 400 mg/kg, compared with T2DM control group, in the mRNA levels of GluR1 (79.78 ± 4.51% and 85.36 ± 4.5% vs. 57.8 ± 3%; $p < 0.01$) and NR2B (78.6 ± 3.17% and 90.84 ± 4.99% vs. 57.42 ± 3.89%; $p < 0.01$), while only treatment with 400 mg/kg of EC produced a considerable increase in both NR1 and NR2A mRNA levels, compared to diabetic control rats (NR1: 84.5 ± 9.5% vs. 62.6 ± 5.6% and NR2A: 91.03 ± 5.77% vs. 58.4 ± 4.41%; $p < 0.05$).

4. Discussion

The non-familiar form of AD could be a consequence of metabolic syndrome, characterized by obesity and the development of a brain-specific insulin resistance known as type III diabetes [4]. Insulin resistance plays an important role in AD pathogenesis, possibly due to abnormal GSK3β activation, causing intra- and extracellular amyloid-beta (Aβ) accumulation [5]. The present study demonstrated that high fructose and high-fat diet coupled with a single small dose of STZ lead to the development of brain-specific insulin resistance with abnormal GSK3β activation favor the formation of βA deposition and Tau accumulation in the brain and enhancing of oxidative stress. Cardamom extract administration, in the present study, for 8 weeks reduced peripheral insulin resistance, improved serum glucose and cholesterol levels and improved the reduction in central insulin signaling where it reduced the GSK-3β in the hippocampus which in turn reduced the

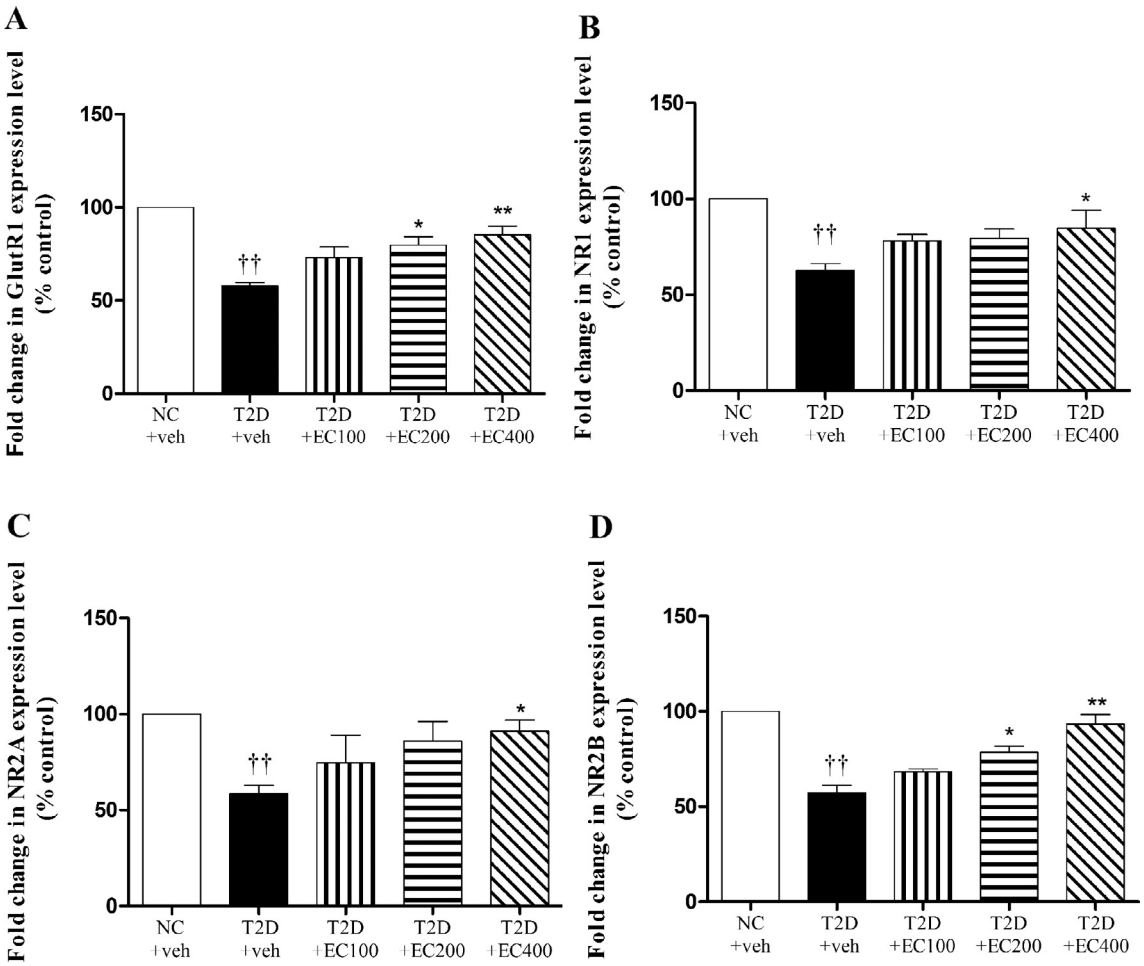

Fig. 5. Quantification of glutamate receptors subunits gene levels in the hippocampus of treated T2D rats using qRT-PCR. Effect of E. cardamomum treatment on gene expression of AMPA GluR1 (A), NMDA NR1 (B), NR2A (C) and NR2B (D) in the hippocampus of T2D rats. $^{††}p < 0.01$ vs NC, $^{**}p < 0.01$ and $^{*}p < 0.05$ vs T2D. NC: normal control, T2D: type 2 diabetic control, T2D + EC100, T2D + EC200 and T2D + EC400.

accumulation of Aβ and AT8 in the brain (Fig. 6). Winarsi et al. [38] reported that ethanolic cardamom extract was capable to reduce blood glucose level as well as total cholesterol, and also repair body weight of alloxan-induced diabetic rats.. Therefore, the present investigation suggests that EC prevent AD in diabetic rats primarily due to the improvement of insulin signaling in brain or alleviation of central insulin resistance

The accumulation of free radical damage, alterations in the activities or expression of antioxidant enzymes such as superoxide dismutase and catalase are characteristic of AD brains. Oxidative stress may augment amyloid β production and aggregation as well facilitate tau phosphorylation and polymerization, forming a vicious cycle that promotes the initiation and progression of the AD [7,39,40]. EC extract administration, in this study, for 8 weeks inhibited the enhanced oxidative stress and concomitant weakening of antioxidant defensive system in the brain of diabetic rats. It restored oxidant/antioxidant balance. It elevated the reduced GSH and SOD brain tissue levels and reduced the rise in MDA brain level of T2DM rats. These findings may explain the underlying mechanism of improvement of the pathogenesis of AD.

Kunwar et al. [14] suggested that *Elettaria cardamomum* shows promise as a natural memory booster in scopolamine-induced amnesia. Also, They reported that the effect of EC extract increases in a dose-dependent manner with a medium dose of EC extract (500 mg/kg) producing most beneficial effects on memory and learning. They concluded that The beneficial effect of EC extract may be attributed to its anti-cholinesterase and anti-oxidant activity with a significant rise in the levels of endogenous antioxidants like glutathione and Superoxide dismutase. Moreover, Kazemi et al. [13] reported that cardamom could improve some parameters of inflammation and oxidative stress in pre-diabetic subjects. Thus, it may be useful in reducing complications associated with inflammation and oxidative stress in these patients.

Many studies have shown a role for direct and indirect TNF-α inhibition in prevention and treatment of chronic neuroinflammation of neurodegenerative diseases such as Alzheimer' disease [41]. In the present study, EC treated T2DM animals showed a significant reduction in neuroinflammation and decreased neuronal degeneration. This was evident by the decreased production of pro-inflammatory TNF-α and IL1β. A recent study by Kandikattu et al. [10] reported that Cardamom extract is rich in flavonoids, and terpenoids. It showed potent anti-inflammatory effects and down-regulated cytokines such as COX-2, IL-6, and TNF-a and inhibited i-NOS-mediated NO generation. They also demonstrated that cardamom exhibited antioxidant effects by restoring SOD, catalase, GSH levels and inhibited lipid peroxidation in carrageenan challenged rats.

Glutamatergic system, and in particular GluN2A-c GluR1, NR1, NR2A, and NR2B, containing NMDA but not NR2C and NR2D have an important role in the processes of learning and memory [42]. EC treatment was able to correct the reduced expression of glutamate receptors found in T2DM rat hippocampus. Significant elevation was observed with EC extract at doses of 200 and 400 mg/kg in GluR1 mRNA and in NR2B, while only treatment with 400 mg/kg of EC, produced a considerable increase in both expressions of NR1 and NR2A mRNA. Because the cholinergic and glutamatergic systems significantly

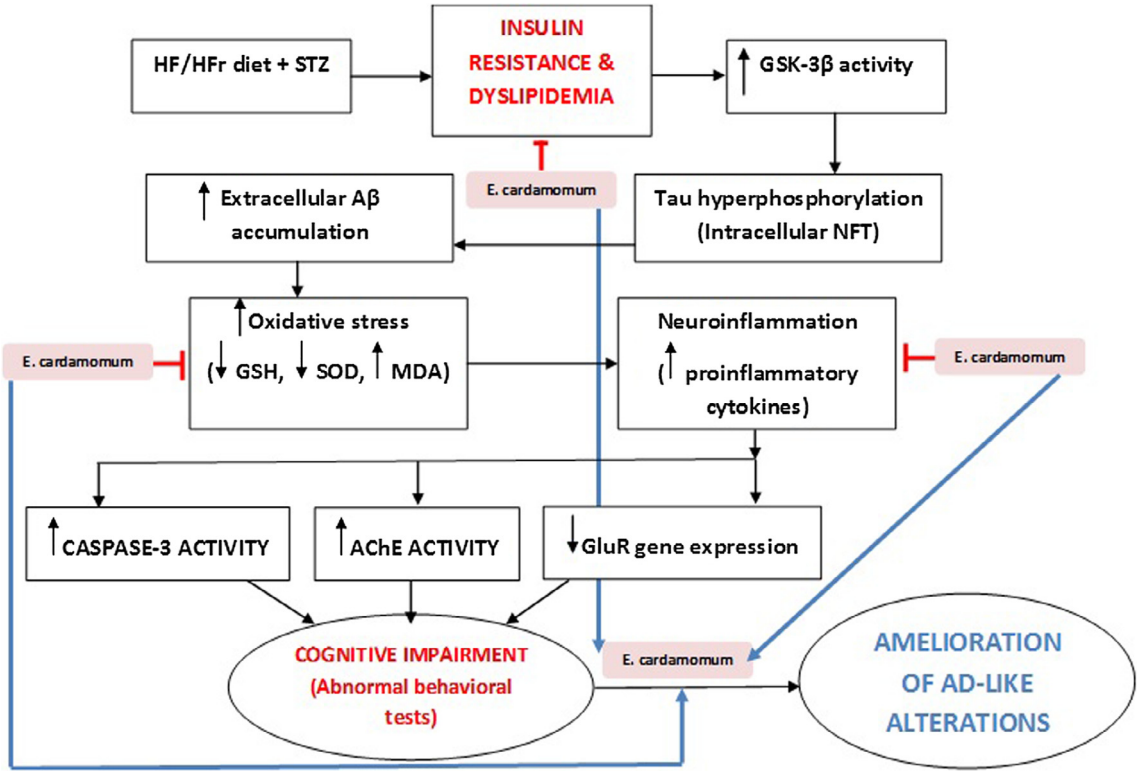

Fig. 6. A proposed pathway and an overview of the obtained results of the present study. EC extract reduced Aβ and p-tau accumulation, acetylcholinesterase activity and brain caspase-3 activity, an indicator of neurodegeneration and increased the attenuated gene expression shown in diabetic rats. In addition it improved impaired cognitive function. These beneficial effects may be attributed to reduction of GSK3β activity or insulin resitance, oxidant stress, and pro-inflammatory cytokines by EC extract.

interact during neurotransmission alterations in the glutamatergic, signaling has been associated with cholinergic disruptions found in the AD. Therefore, our results suggest that EC may has additional neuroprotective activity by modulating glutamate-mediated neuronal excitability.

Further studies are needed before these therapeutic potentials are interpreted. In this study, there are a number of limitations. Only male Wister rats were used in this study. Using female in addition to male may improve the study. A small number of animals used in this study may be another limitation. Pharmacokinetics study will be needed before the clinical use of EC extract. In addition, the research dose of the animal model need to be adjusted for clinical dose of human treatment

5. Conclusion

The findings of the present investigation suggest that EC extract reduced Aβ and p-tau accumulation, acetylcholinesterase activity and brain caspase-3 activity, an indicator of neurodegeneration and increased the attenuated gene expression of the NR2A-NMDARs, NR2B-NMDARs and GlutR1, the subunits of glutamate receptors have a unique role in working memory. It also, improved impaired hippocampal functions, such as learning and memory in diabetic rats. These beneficial effects may be attributed to the insulin-sensitizing, antioxidant, and anti-inflammatory activity of EC extract.

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Conflict of interest

The authors declare no conflicts of interest.

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