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Naglaa H. Eldewany

naglaahossam03@gmail.com

Alexandria University

Seham Z. Nassar

Alexandria University

Abeer Salama

National Research Centre

Sabah G. El-Banna

Alexandria University

Aly B. Okab

Alexandria University

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Neuroprotective Efficacy of Saffron Aqueous Extract on Streptozotocin-Induced Rat Model of Alzheimer's Disease

Naglaa H. Eldewany[a]*, Seham Z. Nassar[b], Abeer Salama[c], Sabah G. El-Banna[a], Aly B.Okab[a]

[a] Department of Environmental Studies, Institute of Graduate Studies and Research, Alexandria University, Alexandria, Egypt. P. O:832-Postal Code: 21526; naglaahossam03@gmail.com, profokab@yahoo.com, sabah_gaber@yahoo.com.

[b] Department of Medical Physiology, Faculty of Medicine, Alexandria University, Alexandria, Egypt. drsehamn2001@yahoo.com

[c] Department of Pharmacology, National Research Centre, Dokki, Cairo, Egypt. P.O., 12622. Berrotec@yahoo.com

Corresponding author

Name: Naglaa H. Eldewany

Email: naglaahossam03@gmail.com

Phone Number: +0201228260193

Abstract

Saffron is regarded as a mood enhancer. Moreover, ethnopharmacological research demonstrates that saffron exhibits efficacy against neurodegenerative illnesses, specifically dementia and Parkinson's disease, due to its bioactive ingredients, The purpose of this work is to assess the potential protective effect of saffron extract in alleviating induced brain damage symptoms in male rats such as myelin basic protein (MBP) and serum Phosphorylated tau-217 (p-Tau 217).

The research conducts that administration of saffron aqueous extract with the three different doses (20,40 and 80 mg/kg) noteworthy decrease the levels of serum phosphorylated tau-217 and in all treated groups. Myelin basic protein significantly decreased in all treated groups. Additionally, there was a significant decrease in the levels of inflammatory markers in the hippocampus tissue homogenate of all saffron treated groups when compared to the induced group. Moreover, all saffron treated groups showed a significant decrease in levels of level of gamma glutamyl transferase, acetylcholinesterase (AChE), beta-site amyloid precursor protein cleaving enzyme-1 (BACE1), and amyloid β-Peptide (1-42) (Aβ 1-42) compared to the induced group. Supplementation with saffron crude extract (SCE) significantly attenuates the formation of Aβ plaque and p-Tau 217 in the hippocampus at different doses. In addition, SCE also counters Alzheimer -induced neuroinflammation.

Keywords: Alzheimer's, Saffron, MBP, p-tau217, amyloid β-Peptide, Behavioural test

1. Introduction

Alzheimer's disease (AD) is a prevalent neurodegenerative disorder. Currently, the precise pathophysiology of Alzheimer's disease remains uncertain. However, research has indicated that the primary features of AD include the deposition of β-amyloid (Aβ), the formation of neurofibrillary tangles, and neuronal death. Hence, the dampening of neuronal apoptosis triggered by Aβ protein is a viable approach for the prevention and management of Alzheimer's disease [1, 2].

The epidemiology of Alzheimer's disease is interconnected with that of all-cause dementia. While Alzheimer's disease is the predominant etiology of dementia, many neurodegenerative or cerebrovascular disorders can also precipitate dementia, especially in elderly individuals. In a study involving 184 individuals who satisfied the neuropathological criteria for Alzheimer's Disease (AD), 31% exhibited solely AD pathology, 22% presented with AD pathology alongside α-synuclein pathology, 29.5% had AD pathology in conjunction with TDP-43 pathology, and 17.5% displayed both α-synuclein and TDP-43 pathology alongside AD pathology. In each of these pathologically defined categories, 29% to 52% of patients exhibited at least one infarct (microinfarct, lacunar infarct, or big infarct) [3].

Streptozotocin (STZ), or 2-deoxy-2-(3-(methyl-3-nitrosoureido)-D-glucopyranose), is a naturally occurring antibiotic synthesized by Streptomyces achromogenes derived from glucosamine nitrosourea. The primary model for sporadic Alzheimer's disease in rats is based on the effects of STZ, which aligns with the sporadic variety in humans. Additionally, STZ induces neuronal injury and hyperphosphorylation of tau, leading to the production of reactive oxygen species (ROS) and reactive nitrogen species [4].

The accumulation of Aβ is a defining pathogenic hallmark in both well-studied autosomal dominant Alzheimer's disease and sporadic late-onset Alzheimer's disease patients. Aβ is produced through the processing of APP, a transmembrane glycoprotein, by sequential cleavage by γ-secretase and β-secretase, a multiprotein complex that includes PS1 or PS2 as catalytic subunits [5].

Myelin basic protein (MBP), the second most abundant protein in the central nervous system, is responsible for adhesion of cytosolic surfaces of multilayered compact myelin. It interacts with polyanionic proteins and negatively charged lipids, potentially acting as a membrane actin-binding protein. MBP may also participate in signalling; oligodendrocytes and myelin use it for different purposes [6].

An accurate blood test for Alzheimer's disease would significantly impact the process of selecting participants for clinical trials. It would enable the pre-screening of non-demented individuals in a way that is minimally invasive and cost-effective. This would reduce the need for additional testing using more invasive and expensive methods such as CSF analyses or PET scans [7].

Phosphorylated tau (p-Tau) is now the most promising candidate for new blood tests for Alzheimer's disease (AD), showing superior precision in diagnosis and specificity for the disease compared to other suggested blood biomarkers [8]. Out of the suggested blood p-Tau biomarkers [9], phosphorylated tau at Threonine 217 (p-Tau217) has consistently demonstrated excellent performance in distinguishing Alzheimer's disease (AD) from other neurodegenerative disorders and in identifying AD pathology in patients with mild cognitive impairment. It is worth mentioning that p-Tau217 shows more significant differences in comparison to p-Tau181 and p-Tau231, often achieving high levels of distinction. Moreover, p-Tau217 has a distinct pattern of change over time in individuals with amyloid, with notable increases that are strongly linked to the progression of cortical atrophy [10].

The scientific community is currently confronted with the formidable task of identifying dependable natural substances that provide practical potential for the treatment of neurological illnesses, such as Alzheimer's disease [11].

Saffron, commonly known as ‘Kesar’ belongs to the dried stigma of Crocus sativus L., family Iridaceae, used as one of the dietary supplements worldwide. It has footprints in many traditional scriptures for its medicinal purposes. In India, saffron mainly grows in Kashmir and is known as Kashmir or Indian Saffron. [12].

Considering their acceptable efficacy and a more favourable safety profile, herbal remedies have attracted more attention as novel promising entities for improving or at least decreasing cognitive deterioration in patients with AD. Effectiveness of herbal compounds in counteracting different central nervous system-affecting disorders and cognitive deficits has been determined in numerous experimental and clinical research. Saffron is the dried stigma of a plant named Crocus sativus L. and has been known as the world's most expensive spice and a widely used medicinal plant. From a chemical standpoint, the main constituents of saffron are carotenoids

(crocin), esters (crocetin), and aldehydes (picrocrocin and safranal). In the recent decades, a growing body of evidence has revealed encouraging beneficial effects for saffron in treating different neuropsychiatric disorders such as depression, seizure, anxiety, and memory disorders. Similarly, promising advantages of saffron in improving different cognitive functions have been demonstrated in multiple preclinical studies. Saffron administration in animals can reverse memory deficits in different behavioural tasks, exerts protective effects against neuronal injury, and positively affects learning behaviour, recognition, spatial memory, and long-term potentiation [13].

A handful of studies have been undertaken to assess the impact of standardised SCE on the dysregulated cholinergic system and associated biochemical and histological alterations in the brain. The present study examined the mitigating effects of standardised SCE supplementation on cognitive impairment and biochemical and immunohistochemical alterations, such as the formation of Aβ plaques and p-Tau 217, generated by Alzheimer's disease induction by (STZ) by 3mg/kg. The study emphasises the significance of SCE phytoconstituents in modulating the dysregulated cholinergic system for use as herbal nutraceuticals.

2. Material and Methods

2.1. Sample preparation for high-performance liquid chromatography (HPLC)

To determine the way of extraction of saffron to obtain the higher concentration of saffron components, ethanolic and water extracts were prepared in the same way:10 mL of ethanol and 10 mL of water were added to 1 g of intact stigmata of saffron and macerated for about 3 days in the dark at ambient temperature with occasional stirring [14]. The crude ethanolic and water extract was analysed by HPLC.

2.1.1. HPLC conditions

Analysis by HPLC was conducted using an Agilent 1260 series. The separation was conducted using a Zorbax Eclipse Plus C8 column (4.6 mm x 250 mm i.d.,5 µm). The mobile phase consisted of water (A) and 0.05% trifluoroacetic acid in acetonitrile (B) at a flow rate of 0.9 mL/min. The mobile phase was programmed consecutively in a linear gradient as follows: 0 min (82% A); 0–1 min (82% A); 1–11 min (75% A); 11–18 min (60% A); 18–22 min (82% A); 22–24 min (82% A). The multi-wavelength detector was monitored at 280 nm. The injection volume was 5 µL for each of the sample solutions. The column temperature was maintained at 40°C.

2.1.2. Preparation of saffron crude aqueous extract

Saffron (*Crocus sativus L.*) was collected from Estahban, 170 km east of Shiraz, Iran, on 20 January 2024. The plant was identified by **Soliman M. Toto,** Associate Professor of Plant Ecology, Department of Botany and Microbiology, Faculty of Science, Alexandria University. A voucher specimen was deposited at the Herbarium of Alexandria University, no. ALEX 4125. The stigma was immersed in distilled water for a duration of 3 days at a temperature of 4°C, without exposure to light, and with constant agitation. The extract underwent filtration using gauze to get a clear extract.

2.2. Total antioxidant capacity

Several assays were developed for the assessment of the total antioxidant capacity of compounds and herbal extracts. The chemical bases of these assays differ significantly, and the results obtained are associated with specific chemical properties of the screened compounds rather than their biological antioxidant capacity [15].

2.2.1. Azino-bis-3-ethylbenzothiazoline-6-suLphonic acid (ABTS) assay

The assay was carried out according to the method of adopting the modifications of Re et al. (1999) [16]: Briefly, the study involved dissolving 192 mg of ABTS in distilled water, adding it to 140 mM potassium persulphate, and then adding methanol to obtain the final ABTS dilution. The reagent was mixed with 10 µL of the sample/compound in a 96-well plate, and the colour intensity was measured at 734 nm. At the end of the incubation time, the decrease in ABTS's colour intensity was measured at 734 nm. Data are represented as means ± SD according to the following equation:

$$\text{Percentage inhibition} = \left(\frac{\text{Average absorbance of blank} - \text{average absorbance of the test}}{\text{Average absorbance of blank test}}\right) * 100$$

2.2.2. Oxygen radical absorbance capacity assay (ORAC)

The assay was carried out according to the method of Ou et al., (2002) [17].

2.2.3. Acetylcholinesterase inhibition assay

The assay was carried out according to the method of Vinutha et al., (2007) [18] with minor modifications. Briefly, 10 µL of the indicator solution (0.4 mM in buffer (1): 100 mM Tris buffer pH 7.5) was transferred to a 96-well plate, followed by 20 µL of enzyme solution (acetylcholine esterase 0.02 U/mL final concentration in buffer (2): 50 mM Tris buffer pH 7.5 containing 0.1% bovine serum albumin). Next, 20 µL of the sample/standard solution was added, followed by 140 µL of buffer (1), and this step was repeated with another 20 µL of the sample/standard solution and an additional 140 µL of buffer (1). The mixture was allowed to stand for 15 min at room temperature. Afterwards, 10 µL of the substrate (0.4 mM acetylcholine iodide buffer) was added immediately to all wells. The plate was incubated in a dark chamber for 20 minutes at room temperature. At the end of the incubation period, the colour was measured at 412 nm. Data are represented as means ± SD.

2.3. Experimental Animals

Forty healthy male albino rats (*Rattus norvegicus*), each averaging 210±10 g and two months old, were obtained from the animal house at the Faculty of Medicine, Alexandria University, and acclimated for two weeks before the experiment. They were assigned to 8 groups and housed in Universal galvanized wire cages at room temperature (22-25°C) and in a photoperiod of 12/12 h/day. Animals were provided with a balanced commercial diet containing 18% crude protein, 14% crude fiber, 2% fat, and 2600 Kcal DE/kg feed.

2.4. Ethics statement

The experiments were approved by the Ethical Committee of the National Research Centre, Egypt, and the Institutional Animal Care and Use Committee, Faculty of Medicine, Alexandria University (ALEXU-IACUC No. AU14-231130-2-12), Alexandria, Egypt. All experiments with animals complied with the ARRIVE (Animal Research:Reporting of In Vivo Experiments) guidelines (https://arriveguidelines.org).

2.5. Experimental Design

Animals were randomly divided into 8 equal groups and assigned as follows:

The sham group received a citrate buffer via intracerebroventricular (ICV) injection, while the induced group was injected once with 3 mg/kg body weight of streptozotocin (STZ) using citrate buffer as a vehicle. After 30 days of intracerebral ventricular (ICV) STZ injection, animals' neurodegenerative alterations induced by ICV-STZ were evaluated [19], Saffron crude extract was administered for one month daily orally through gavage in three different doses: 20 mg/kg (SD20), 40 mg/kg (SD40), and 80 mg/kg (SD80) [20]. The remaining three groups were induced with STZ and after that administered with different doses of saffron extract (ISD20, ISD40, and ISD80, respectively).All experiments and treat with animals where according to the Animal Care Guidelines and accept responsibility for the conduct of the experimental procedures detailed in this proposal in accordance with the guidelines contained in the Guide for the Care and Use of Laboratory Animals 8th Edition 2011

2.6. Induction of experimental dementia of AD in male rats

The animals were initially administered intraperitoneal (i.p.) injections of ketamine (100 mg/kg) and xylazine (10 mg/kg) to induce anesthesia. Streptozotocin was administered unilaterally into the lateral ventricle using a Hamilton syringe, with a dosage of 3 mg/kg, 4 µl per injection site, using stereotaxic surgery [21]. According to BÜTtner-Ennever, (1997) [22] the ICV injection was performed using the following coordinates: 0.8 mm posterior to the bregma, 1.5 mm lateral to the sagittal suture, and 3.6 mm ventral from the surface of the brain. The meninges were meticulously preserved without any injury throughout the surgery. The injection of a 3 mg/kg STZ solution was performed using a Hamilton syringe with a cannula diameter of 0.3 mm. Shortly prior to administration, STZ was dissolved in a citrate buffer (0.05M, pH:4.5) and subsequently injected unilaterally into the ventricle of the brain. After the injection, the cannula was left in place for an additional 5 min to facilitate passive diffusion from the tip of the cannula and reduce the distribution of the substance throughout the injection tract. Subsequently, the cannula was gradually extracted from the scalp and secured using sutures.

2.7. Behavioral tests

2.7.1. Morris water maze

To assess the rats' spatial learning and memory, the Morris Water Maze (MWM) was conducted over the final seven days of the experiment. The maze consists of a grey circular metal tank (180 cm in diameter) that is filled with water at a temperature of approximately 22 ± 3°C. The water depth is 30 cm. The water was rendered opaque by adding non-toxic paint. The pool was partitioned into four quadrants, each delineated by distinct beginning locations on its wall: north, south, east, and west (N–S–E–W). Inside the tank, there is a circular black platform with a diameter of 10 cm. This platform is located in the SW quadrant 20 cm away from the tank's edge and is submerged 1 cm below the water's surface. Extra maze cues were placed around the tank in the room. Each of the experimental rats underwent daily water maze training consisting of four trials per day from different start locations for a duration of six days. Memory recall testing was conducted 24 h following the sixth day, during which the platform was taken away and each rat was given 60 s of unrestricted swimming. The percentage of time is determined by dividing the time spent in the target quadrant by the total time and then multiplying the result by 100. Similarly, the percentage of distance is calculated by dividing the distance swum in the target quadrant by the total distance swum and then multiplying the result by 100 [23, 24].

2.7.2. Novel object recognition (NOR) task

The object recognition task, a behavioural test, was conducted in the last two days of the treatment period. The test assumes that animals explore the novel object as their natural propensity to the novelty and that novel stimuli can change animals' behaviour, provoke stress responses, and elicit approach behaviour. The experimental setup consisted of open-field boxes with dimensions of 20×20×17, which were sparsely lighted. Initially, habituation was conducted by placing each rat individually in one of the boxes for a duration of 15 min. The subsequent day, the familiarisation phase was done by placing a pair of plastic sample objects, positioned roughly 12 inches apart, inside one of the boxes. The rats were then given a 10-min period to explore the objects. Following a 3-h interval, the rats had a retention test in which they were placed back in the open field. However, one of the objects from the training session was replaced with a new object of similar size and complexity. The rats were allowed to explore for 5 min; the total time spent exploring each of the two objects (when the animal's snout was directly towards the object at a distance ≤ 2 cm) was recorded. The recognition index (RI) was employed to quantify memory recognition in the study. RI refers to the percentage of time spent investigating either the familiar object or the novel item out of the total time spent exploring both objects. This calculation is derived from the formula RI = (time spent exploring familiar object or novel object / total time spent exploring both objects) × 100 [25].

2.8. Biochemical analysis

2.8.1. Determination of phosphorylated tau-217 (p-Tau 217) blood-based biomarker

At the end of treatment period in the last week behavioral tests were performed then rats were anesthetized using anhal consist of isoflurane 100% (Anesthetic liquid for inhalation), after that rats were weighed before scarification. Minimal pain and distress are anticipated as the animals will be well-fed, cared for, and anesthetized before the sacrifice.Then they were desiccated, blood was collected from the heart, in Serum Separator tubes to obtain blood serum. Blood was collected in a serum separator tube and then centrifuged at 1000 xg for 15 min. The obtained serum was used to estimate the activities of phosphorylated tau-217 (p-Tau 217), which were determined using ELISA kits procured from Shanghai Ideal Medical Technology Co., Ltd.

2.8.2. Determination of gamma-glutamyl transferase (GGT) and butyryl cholinesterase (BChE) activity

Gamma-glutamyl transferase (GGT; EC 2.3.2.2) and butyryl cholinesterase (BChE; EC: 3.1.1.8) were measured according to the methods of [26], respectively. The kits were obtained from **Bio Diagnostic,** Egypt.

2.9. Preparation of brain hippocampus homogenate

The rats were sacrificed by decapitation. The brains were scooped out, rinsed with normal saline, and the hippocampi were identified and dissected out for demolition in a tissue homogenization using a tissue homogenizer (HG-15D) using 0.01 M phosphate buffer (pH 7.4). The tissue-buffer ratio was 1:9 buffer by weight/volume. Centrifugation of homogenates was conducted at 10000 xg. Supernatants were collected in separate aliquots and stored at -80°C for biochemical analysis.

2.9.1. Estimation of myelin basic protein and acetylcholinesterase in hippocampus homogenate

Myelin Basic Protein (MBP) and Acetylcholinesterase (AChE) levels were on admission in hippocampus homogenate using Enzyme Linked Immunosorbent Assay (ELISA) following the production instructions provided by Sunlong Biotec.

2.9.2. Evaluation of beta-site amyloid precursor protein cleaving enzyme-1 and amyloid β-Peptide (1-42)

The level of beta-site amyloid precursor protein cleaving enzyme-1 (BACE1) and amyloid β-peptide (1-42) (Aβ 1-42) was determined using the respective commercial ELISA kit from Sun Red Co. LTD, Shanghai, China. The kit uses a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) to assay the level of rat β-site APP-cleaving enzyme 1 (BACE1) in samples. Add β-site APP-Cleaving Enzyme 1 (BACE1) to the monoclonal antibody enzyme well, which is pre-coated with Rat β-site APP-Cleaving Enzyme 1 (BACE1) monoclonal antibody, and incubate; then, add the (BACE1) antibody labelled with biotin and combined with Streptavidin-HRP to form an immune complex; then, carry out incubation and washing again to remove the unbound enzyme. Then add Chromogen Solutions A and B; the colour of the liquid changes into blue, and under the effect of acid, the colour finally becomes yellow. The chroma of colour and the concentration of the Rat Substance β-site APP-Cleaving Enzyme 1 (BACE1) of the sample were positively correlated. The same procedure was followed to assay the level of amyloid β-peptide (1-42) in homogenate.

2.9.3. Determination of inflammatory and oxidative stress biomarkers

Hippocampus content of tumour necrosis factor-alpha (TNF-α), interleukin 6 (IL-6)**,** cyclooxygenase 2 (COX-2), and cysteine-aspartic acid protease (Caspase-3) as an apoptotic marker were estimated using ELISA kits according to the manufacturing instructions of Sunlong Biotec Co. LTD, Zhejiang, China.

2.9.4. DNA breakages assay with diphenylamine reaction in hippocampus homogenate

The diphenylamine DNA fragmentation percentage assay is a highly effective approach for evaluating apoptosis because it determines the proportion of DNA fragmentation into oligosomal-sized particles. The amount of soluble DNA released by apoptotic nuclei into the cytoplasm is a quantitative indicator of cellular response. Another feature of the diphenylamine assay is that it may detect apoptotic DNA fragmentation in both adhering and floating cells after treatment with chemotherapeutic or other drugs. The DNA fragmentation percentage assay was performed using the procedure of Wu et al. (2006) [27].

$$\text{Fragmented DNA (\%)} = T \times 100 / (T + B)$$

T: Measure absorbance of Supernatant
B: Measure absorbance of Pellet

2.10. Immunohistochemical Examination of Amyloid β (Aβ) peptide plaques and tubulin-associated unit (Tau)

For determination of Amyloid β (Aβ) peptide plaques and tubulin-associated unit (Tau) in hippocampus brain tissue, the tissue analysis staining technique comprises multiple phases. The slides are initially immersed in a peroxidase blocking solution for 10 minutes, followed by rinsing with wash buffer to eliminate surplus liquid. Subsequently, serum blocking is applied to each tissue segment and incubated for 10 minutes. The primary antibody is produced, incubated for 30 to 60 minutes, and subsequently washed three times. A biotinylated goat secondary antibody is applied, incubated for 10 minutes, and subsequently washed three times. An enzyme conjugate is introduced, incubated for 10 minutes, and subsequently washed three times. Incubation with substrate/chromogen is conducted, followed by counterstaining with Hematoxylin. Mounting and covering lips are subsequently placed using Acu-stainTM Mouse + Rabbit HRP kits (Genemed Biotechnologies, inc, USA) with Catalog No. 52-0003 and 54-0003. (Figure 1) [28].

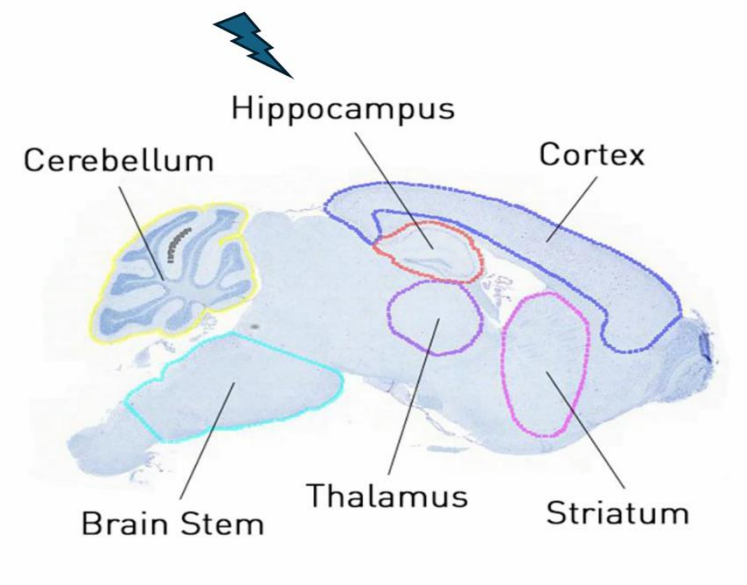

Figure (1): Sagittal mouse brain section with ground truth test image AI segmentation

2.11. Statistical analysis

The data was presented as the mean values together with the standard error of the mean (SEM). An ANOVA was conducted followed by Tukey's post hoc tests to evaluate group differences. The data was analyzed using SPSS version 25. P-values below 0.05 were deemed statistically significant [29].

3.Results

3.1. Phytochemical composition of saffron extracts

The chromatographic conditions used permitted the identification of key components in each sample, resulting in a unique baseline separation. **Table 1 and Figure 2** demonstrate the results of an aqueous extract and an ethanolic extract of saffron. According to our findings, different extracts appeared to have similar chemical compositions but varying concentrations of each component.

Table (1): High-performance liquid chromatography of the phytochemical composition of aqueous and ethanolic saffron extracts

Extract Components	Ethanolic Extract (µg/g)	Aqueous Extract (µg/g)
Gallic acid	29.88	879.58
Chlorogenic acid	115.02	124.48
Catechin	8.24	35.63
Methyl gallate	16.85	266.09
Coffeic acid	26.31	207.46
Syringic acid	452.94	0.00
Pyro catechol	0.00	3854.72
Rutin	1202.03	6012.25
Ellagic acid	4.31	53.01
Coumaric acid	5.46	5.36
Vanillin	38.79	107.04
Ferulic acid	30.87	47.44
Naringenin	30.06	61.23
Rosmarinic acid	28.19	217.21
Daidzein	3.63	8.28
Quercetin	751.44	1783.85
Cinnamic acid	5.04	2.24
Kaempferol	32.89	509.82
Hesperetin	30.67	146.91

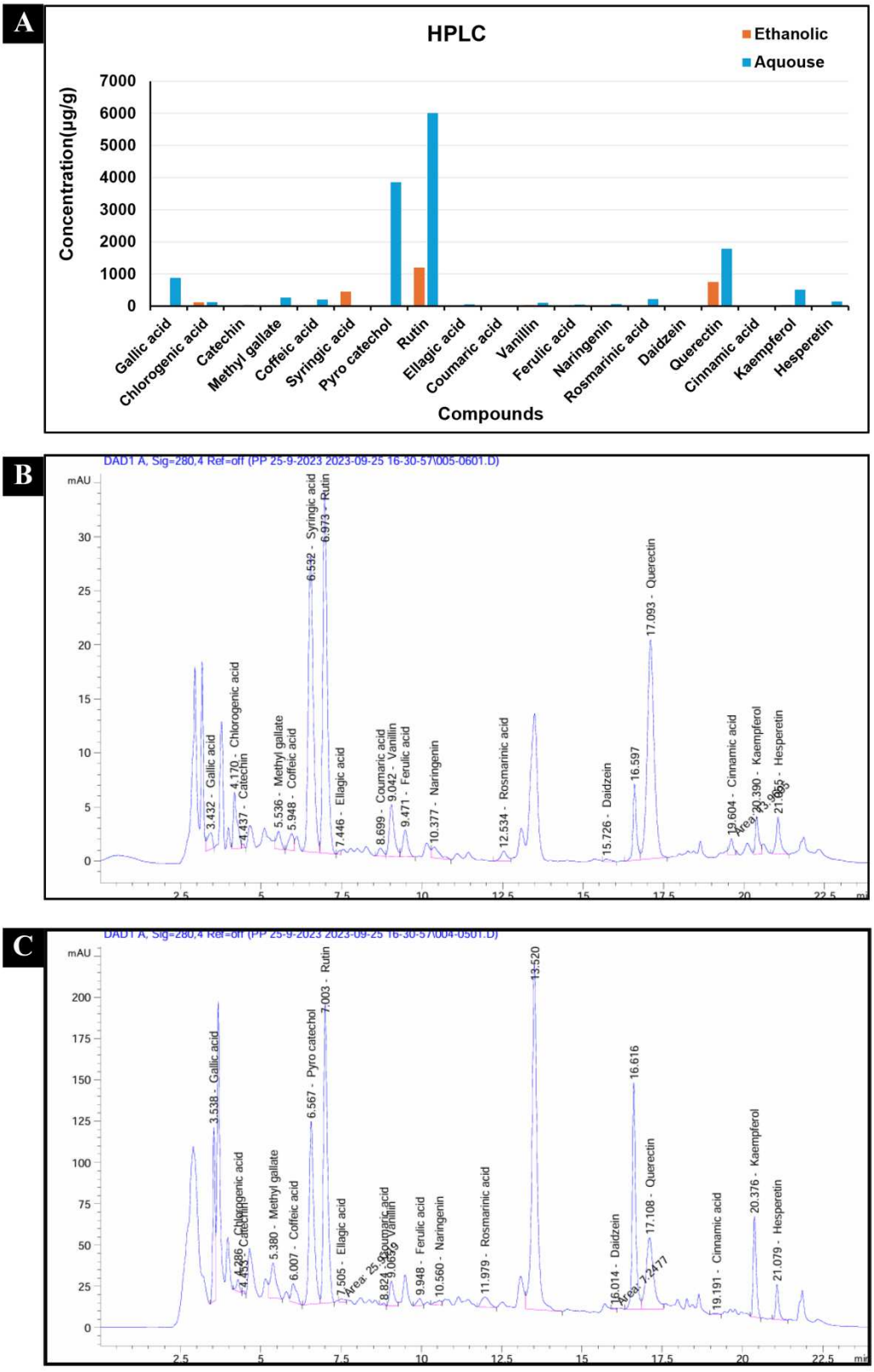

Figure (2): (A, B and C) Chromatograms of water and ethanolic saffron extract obtained using high-performance liquid chromatography (A): Comparison between saffron water and ethanolic extract**, (B):** HPLC chromatogram analysis of saffron ethanolic extract and **(C)**: HPLC chromatogram analysis of saffron water extract.

3.2. The ABTS radical scavenging assay

The ABTS radical scavenging assay is among the most widely employed antioxidant evaluations for plant specimens. Antioxidants utilized in food are chemical substances that can donate hydrogen radicals, hence reducing rancidity and lipid peroxidation in food items. They prolong the shelf life of lipid-rich food without compromising sensory or nutritional integrity [30]. **Figure (3)** indicate that the IC50 of saffron extract is 208.5 µg/mL. The percentage inhibition of saffron extract at several concentrations (31.25, 62.5, 125, 250, 500 µg/mL) in aqueous extract is (7.47%, 16.76%, 29.42%, 54.19%, 81.06%).

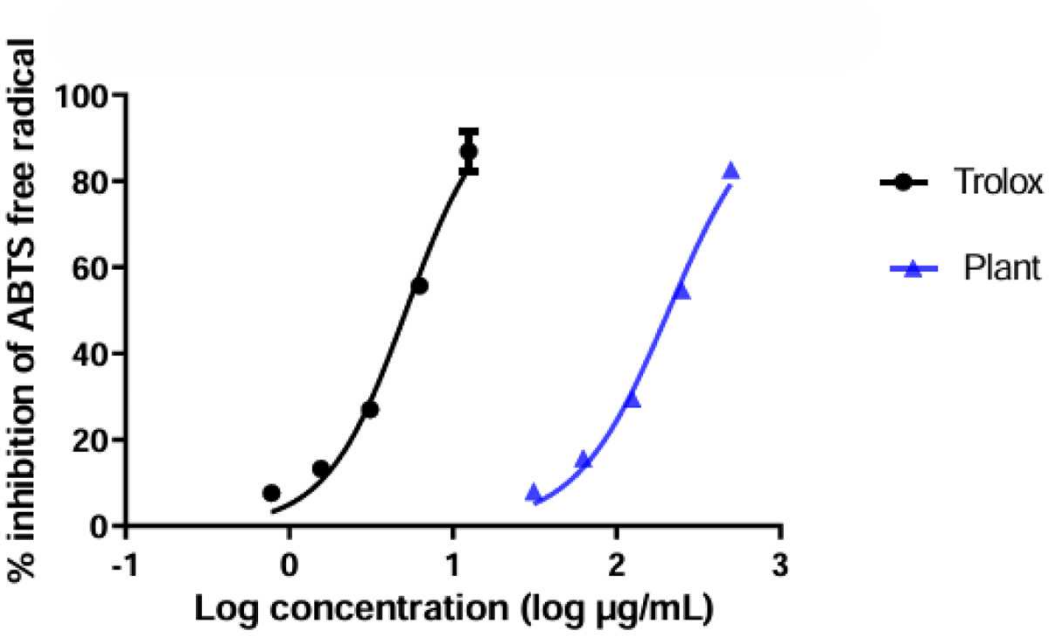

Figure (3): ABTS inhibitory concentration (**IC50**)

3.3. Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay evaluates an antioxidant's effectiveness by analysing the area under the curve of fluorescence intensity over time, allowing for the assessment of peroxyl radical reactions with a specific compound and the determination of the total antioxidant capacity of a food extract [31]. The experiment quantifies the temporal degradation of fluorescence of fluorescein in the presence and absence of antioxidant substances. Antioxidant activity using ORAC assay is 1µg Trolox equivalent (TE) /186.18 mg sample as observed in **Table (2) and Figures (4 and 5)**.

Table (2): Saffron extract effect on oxygen radical absorbance capacity assay

Sample I.D.	Av RFU	Substitution in equation 1 (µ*g*/*mL*)	Antioxidant activity using ORAC assay is (µg TE /mg sample)	Standard Deviation
Saffron extract	**1341887**	**93.09**	**186.18**	18.10

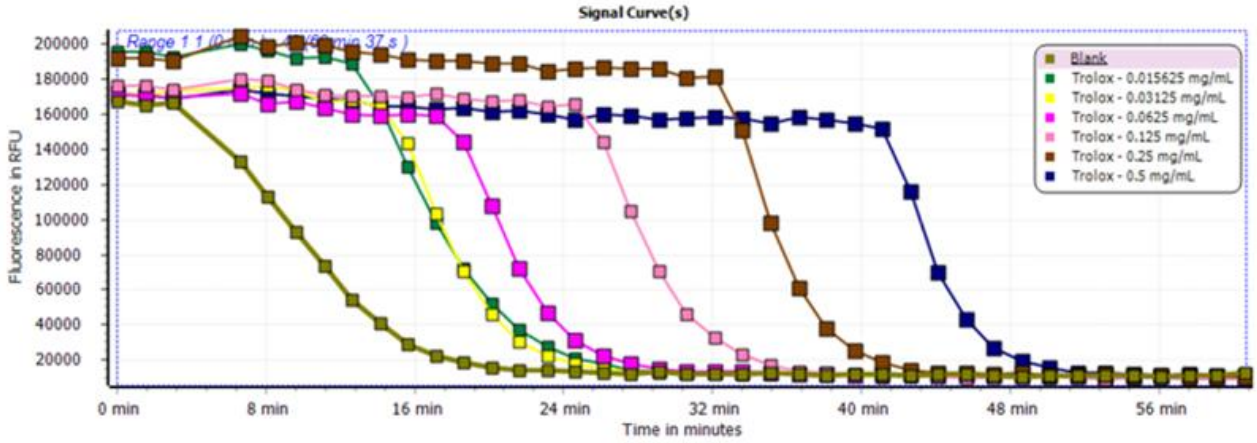

Figure (4): The antioxidant impact of Trolox on fluorescein degradation in the ORAC experiment. Adjusted linear regression curve for Trolox. Signal curves depicting fluorescein deterioration at different Trolox doses, beside a blank control.

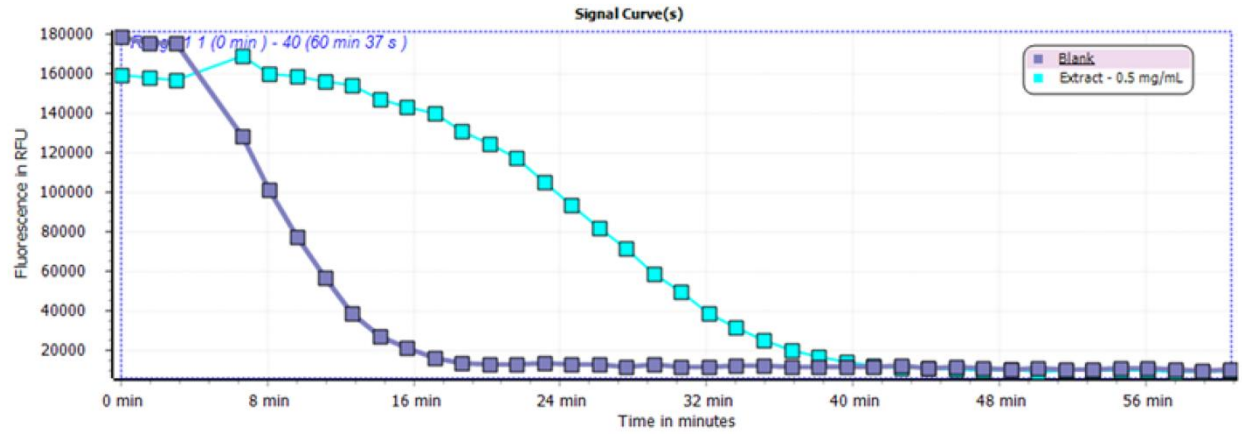

Figure (5): Signal curve of Samples and blank indicating the decay of fluorescein upon applying the samples.

3.4. Acetylcholinesterase inhibitor assay

Inhibition of Acetylcholinesterase (AChE), the fundamental enzyme responsible for the degradation of Acetylcholine, is seen as a potential approach for the treatment of neurological illnesses including Alzheimer's disease, senile dementia, ataxia, and myasthenia gravis. A significant supply of AChE inhibitors is undoubtedly offered by the prevalence of plants in nature [32]. The percentage inhibition of saffron extract in a dose dependant manner as the concentration increase the inhibition increase in 50 and 500 µg/mL the percentage inhibition equal 1.41 % and 4.84 % as observed in **Table (3)**.

Table (3): Acetylcholinesterase percentage inhibition of saffron extract

Sample ID	Mean at 50 µg/mL	SD	Mean at 500 µg/mL	SD
Extract	1.41	0.21	4.84	0.67
Donepezil (µg/mL)	**0.0005 µg/mL**		**0.5 µg/mL**	
	4.61	0.31	96.20	0.24

3.5. Memory tests

Administration of saffron crude extract enhances cognitive abilities related to learning and memory.

3.5.1. Morris water maze

Figure 6 (a, b) illustrates the results of the spatial memory retention test, the healthy control rats spent the longest on average time and swam in the target quadrant, which suggests that memory consolidation functioned effectively in this group. However, the STZ group exhibited considerably reduced time spent and swimming distance in the target quadrant compared to the control group. However, treatment with various dosages of saffron crude extract resulted in a considerable increase in the percentage of time spent in the intended quadrant. This increase was found to be modest when compared to healthy control rats, but significantly greater than untreated rats with STZ-induced AD.

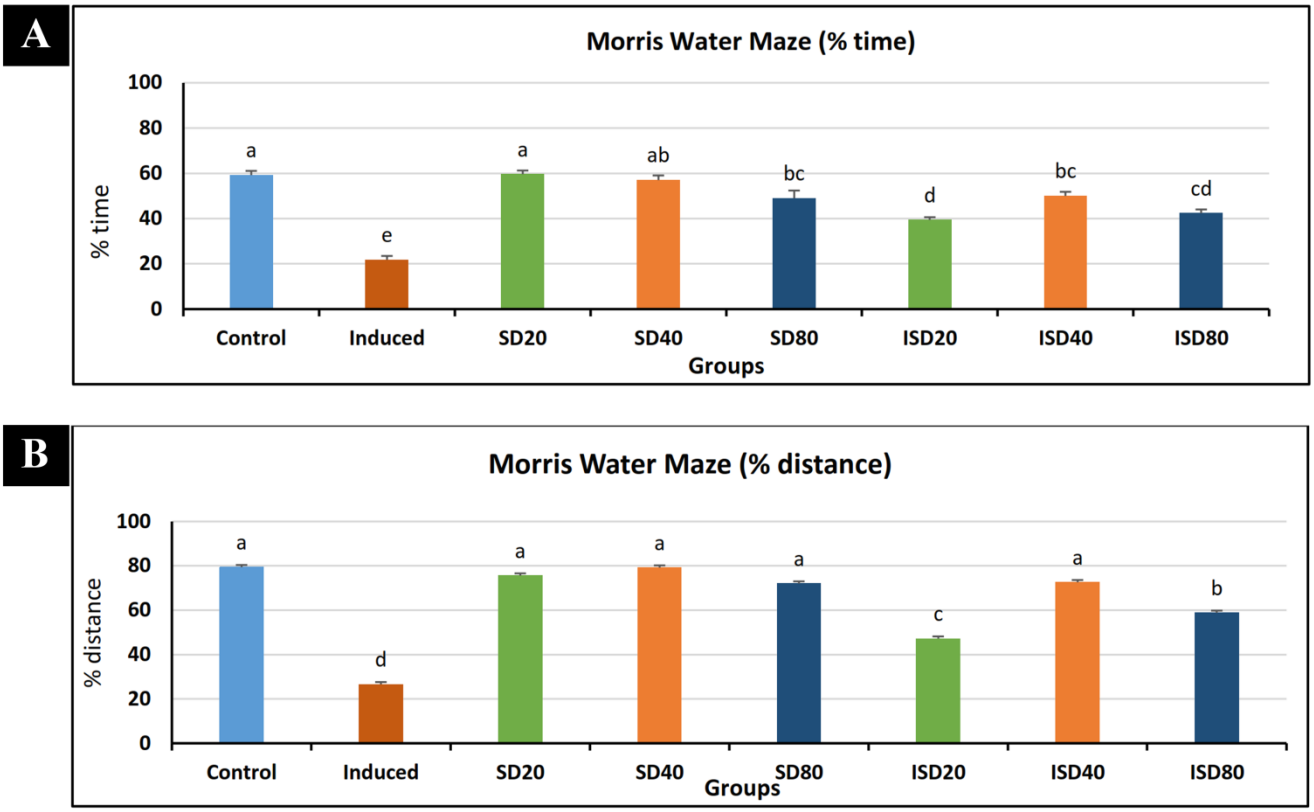

Figure 6: Effect of treatment on learning and memory, **(A)** The mean percent of time spent in the target quadrant in memory retention test, **(B)** The mean percentage of distance swum the target quadrant.

3.5.2. Influences of saffron extract on the memory of recognizing objects

Over the last week, recognition memory was assessed using the object recognition task. Typically, rats exhibit a greater inclination to investigate a new object compared to a familiar one. As shown in **Figure 7 (a, b)** The object recognition memory of STZ untreated rats was notably impaired, as they spent less time investigating the novel objects compared to the known ones. In contrast, rats treated with SCE did not show any difference in interval of time they spent with the novel and familiar objects. Nevertheless, administration of saffron crude extract (20,40&80 mg/kg) to AD rats resulted in a substantial increase in the duration of exploration of the novel object by (68.8%, 90.59%&60.4%) respectively, which was statistically significant compared to the induced group.

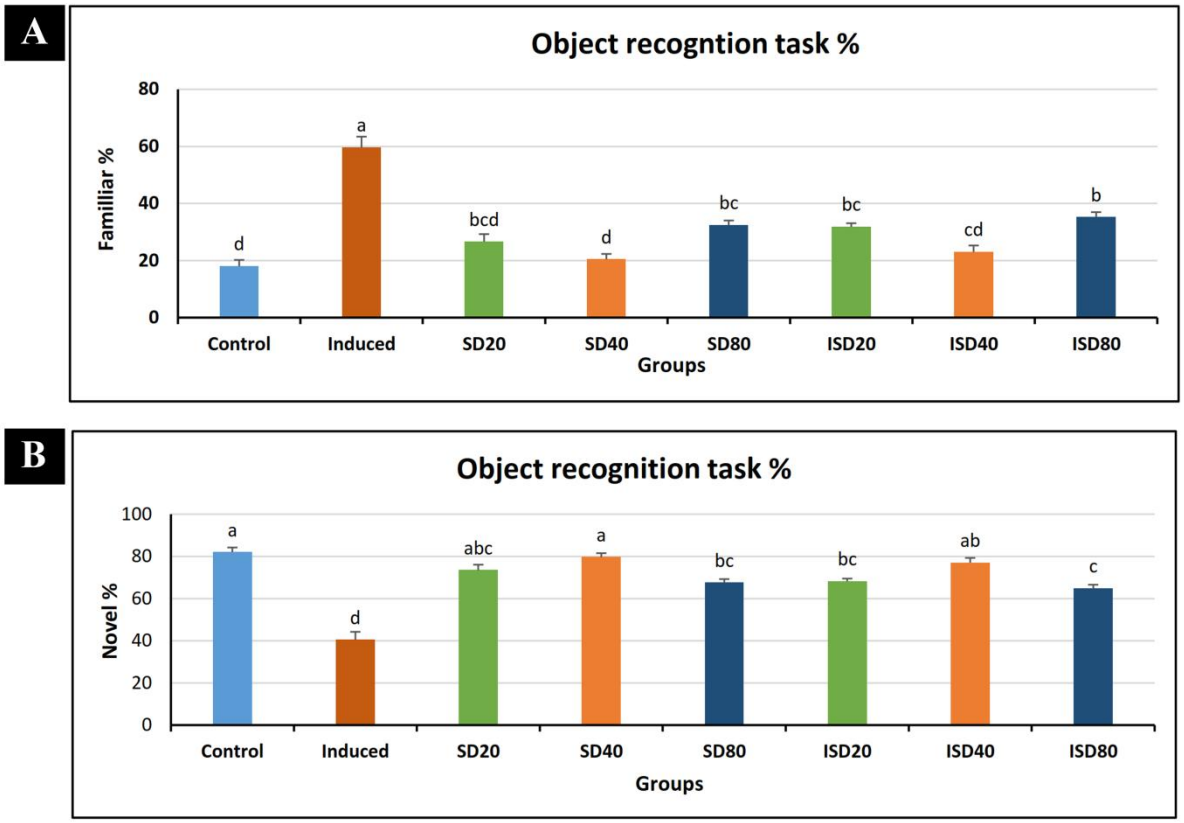

Figure 7: Effect of treatment on object recognition task, **(A)** the percent of time spent in the exploring the familiar object, **(B)** the percent of time spent in the exploring the novel object.

3.6. Saffron extract impacts on serum level of phosphorylated tau 217

Levels of p-tau 217 show a considerable rise in the induced group compared to the sham group. Indeed, p-tau 217 was substantially diminished in all treated groups (20,40&80 mg/kg) compared to the induced groups by (19.9%,55.55%&65.43%) respectively as shown in **Figure 8** in addition in saffron 80 mg/kg returned p-tau 217 to their level to its normal range.

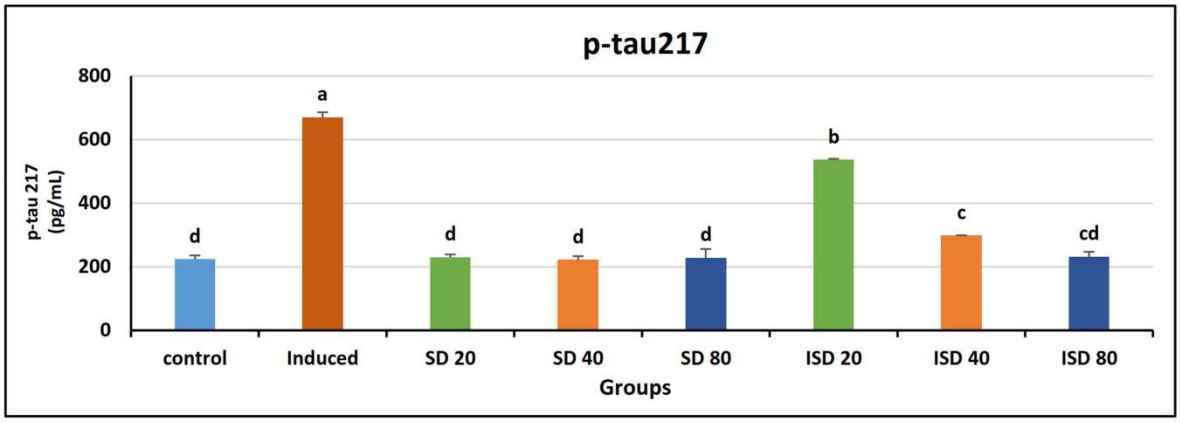

Figure 8: Phosphorylated tau 217 level in hippocampus tissue homogenate.

3.7. Gamma-glutamyl transferase (GGT) and butyryl cholinesterase (BChE) activity in blood serum

The results in **Figure 9 (a and b)** indicated that level of gamma glutamyl transferase (GGT) markedly elevated in the induced group compared to the control group. The injection of saffron crude extract significantly reduced GGT in all treated groups by (69.82%,70.11%&55.22%).

The findings demonstrate that serum butyrylcholinesterase (BChE) significantly diminished in the induced group compared to the control group. The injection of saffron aqueous at varying doses (**20**, **40**, and **80** mg/kg) considerably elevated BchE by (205.14%,259.88%&290.51%), which subsequently normalized in comparison to the stimulated group. Returning them to the normal range compared to the induced group.

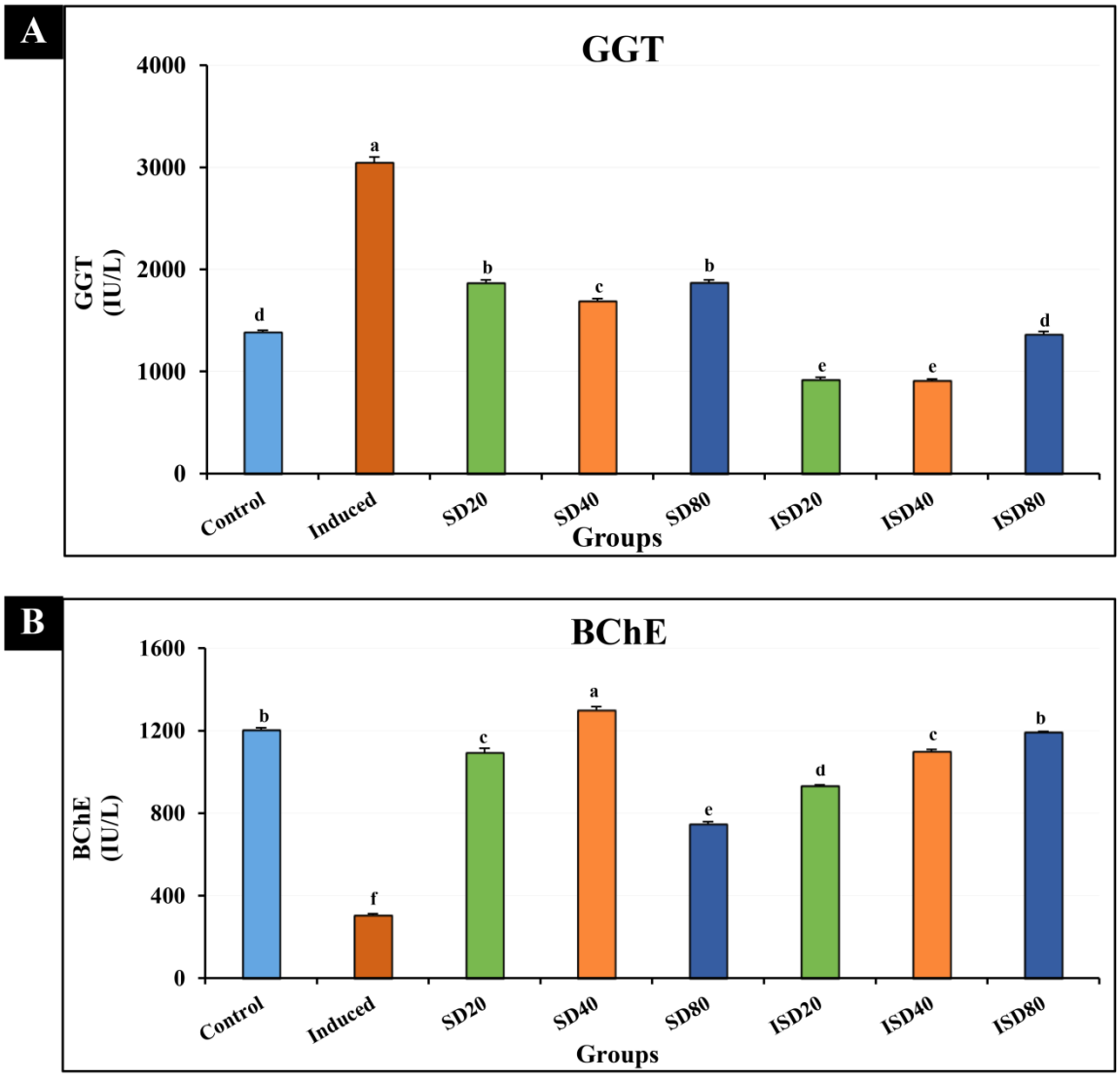

Figure 9: (A and B) Saffron crude aqueous extract administration on (A): Gamma glutamyl transferase (GGT). **(B):** Butyrylchlinesterase (BChE).

3.8. Efficacy of saffron extract on myelin basic protein and acetylcholinesterase in the hippocampus

The results in **Figure 10 (a and b)** revealed that levels of myelin Basic Protein (MBP), and acetylcholinesterase (AChE) were considerably higher in the induced group compared to the sham group. Concurrently, the co-administration of saffron extract (20,40&80mg/kg) notably reduced GFAP by (77.32%,88.37%&92.57%), MBP by (81.48%,85.60%&93.08%) and AChE by (46.15%,59.23%&74.41%) in all treated groups compared to the induced group. In addition, saffron80 returned MBP and AChE to the normal range.

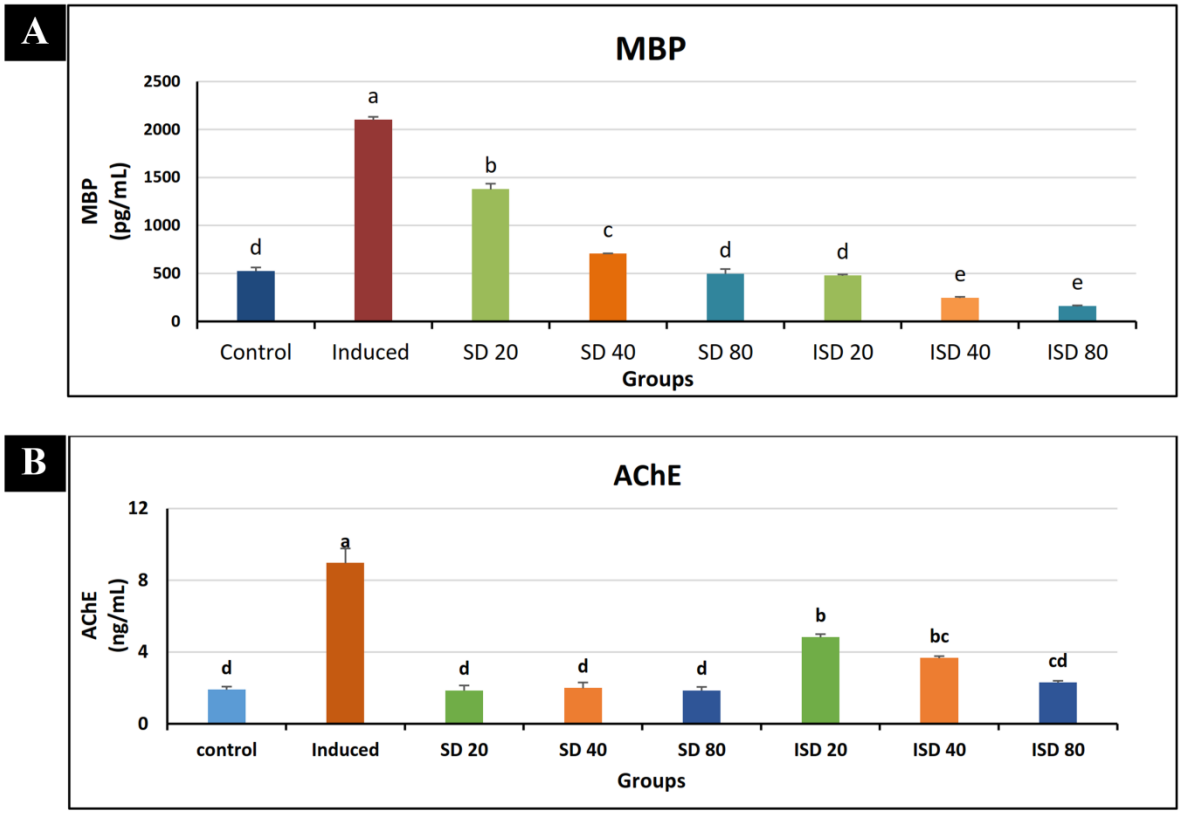

Figure 10: (A nd B): Myelin Basic Protein (MBP) and Acetylcholinesterase (AChE) levels in hippocampus tissue homogenate.

3.9. Attenuating effects of saffron crude extract supplementation on Alzheimer induced amyloid β-Peptide (1-42) (Aβ) plaque accumulation and beta-site amyloid precursor protein cleaving enzyme 1

Figure 11 (a and b) displays the levels of beta-site amyloid precursor protein cleaving enzyme-1 (BACE1) and amyloid β-Peptide (1-42) (Aβ 1-42) in the hippocampal tissue homogenate. The findings demonstrated a substantial rise in BACE1 and Aβ 1-42 levels in the STZ-induced group compared to the control group. Concurrently administering different dosages of SCE (20,40&80 mg/kg) resulted in a substantial decrease (BACE1) by (64.67%,47.59%&36.63%) and (Aβ 1-42) by (62.94%,70.68%68.925) in all treatment groups compared to induced group.

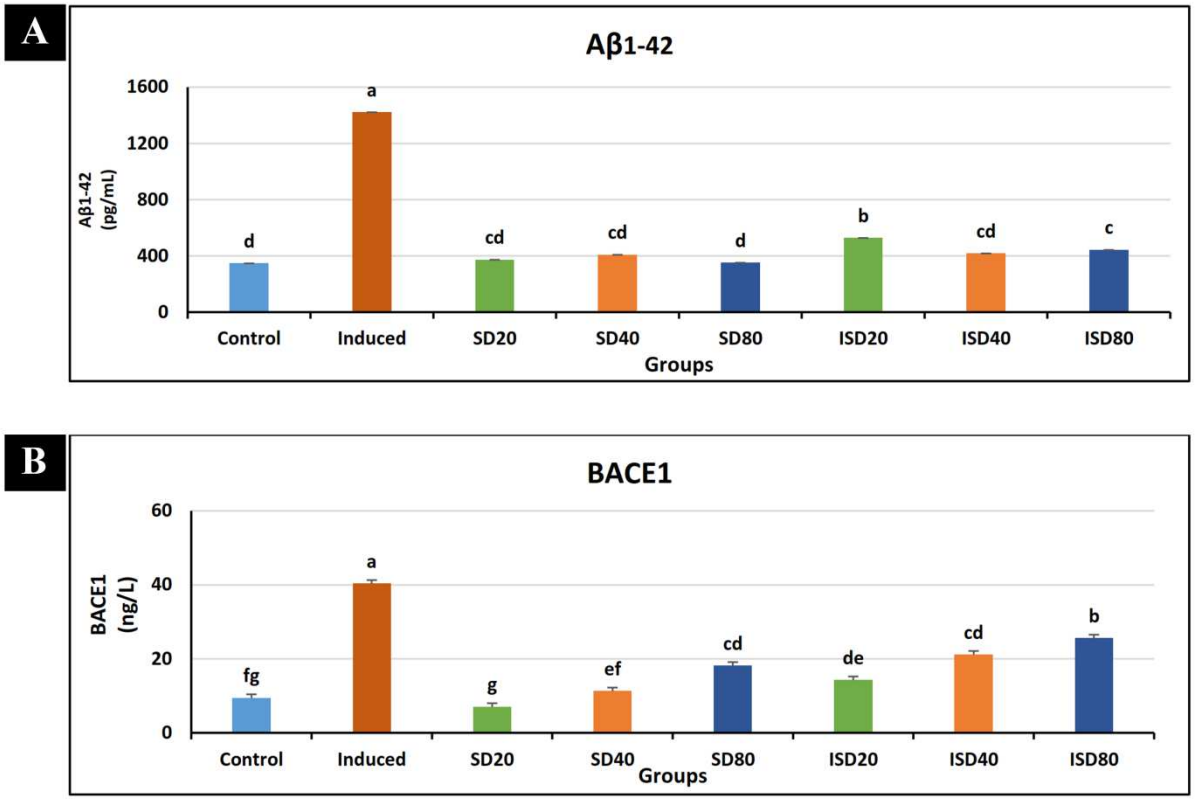

Figure 11: **(A and B)** Amyloid β-Peptide (1-42) (Aβ) plaque accumulation and beta-site amyloid precursor protein cleaving enzyme 1(BACE1) levels in hippocampus tissue homogenate

3.10. Effects of saffron extract administration on pro-inflammatory and apoptotic biomarkers

The findings in **Table (4)** showed an alarming increase in tumor necrosis factor-alpha (TNF-α), interleukin 6 (IL-6), cyclooxygenase- 2 (COX-2), and cysteine-aspartic acid protease (Caspase-3) in the stimulated group compared to the control group. Concurrently, supplementing saffron crude extract (20,40&80mg/kg) notably reduced levels of TNF-α by (16.11%,32.04&55.63%), IL-6 by (64.37%,64.37&89.84%), COX-2 by (82.58%,84.61&94.92%), and Caspase-3 by (14.42%,22.07&32.44%) in all treated groups compared to the induced group. Moreover saffron 80 returned TNF-α, IL-6 and COX-2 to their normal range.

Table (4): Pro-fibrotic and oxidative stress biomarkers

Parameter	*Control*	*Induced*	*SD20*	*SD40*	*SD80*	*ISD20*	*ISD40*	*ISD80*
TNF-α	$103^{c}\pm$ 15.43	$339^{a}\pm$ 8.82	$124^{c}\pm$ 11.48	$102.6^{c}\pm$ 12	$116.2^{c}\pm$ 16.62	$284.4^{b}\pm$ 1.63	$230.4^{b}\pm$ 11.99	$150.4^{c}\pm$ 10.72
IL-6	$592^{c}\pm$ 28.53	$5984^{a}\pm$ 149.18	$489.6^{c}\pm$ 21.73	$577^{c}\pm$ 14.8	$628^{c}\pm$ 14.63	$2132^{b}\pm$ 39.29	$2132^{b}\pm$ 39.30	$607.60^{c}\pm$ 30.43
COX-2	$31.11^{c}\pm$ 2.05	$664^{a}\pm$ 7.78	$32.56^{c}\pm$ 6.80	$33.11^{c}\pm$ 3.03	$32.56^{c}\pm$ 5.57	$115.7^{b}\pm$ 1.65	$102.2^{b}\pm$ 2.25	$33.78^{c}\pm$ 3.81
Caspase-3	$6.618^{e}\pm$ 0.081	$10.77^{a}\pm$ 0.151	$6.68^{e}\pm$ 0.36	$6.67^{e}\pm$ 0.045	$6.63^{e}\pm$ 0.118	$9.23^{b}\pm$ 0.029	$8.39^{c}\pm$ 0.069	$7.27^{d}\pm$ 0.185

Tumor necrosis factor-alpha (TNF-α), interleukin 6 (IL-6), cyclooxygenase- 2 (COX-2), and cysteine-aspartic acid protease (Caspase-3).

The results expressed as (Mean ± SE, n=5)

[abcd] Mean values within columns not sharing common superscript letters were significantly different, $p < 0.05$.

control: sham group (citrate buffer), induced: STZ (3 mg/kg), SD20: saffron extract (20mg/kg), SD40: saffron extract (40mg/kg), SD80: saffron extract (80mg/kg), ISD20: induced + saffron extract (20 mg/kg), ISD40: induced + saffron extract (40 mg/kg) and ISD80: induced + saffron (80 mg/kg).

3.11. Attenuating effect of saffron aqueous extract administration on DNA breakages in brain homogenate

The results summarized in **Figure 12** indicate that the proportion of DNA breakage in the brain significantly increased in the induced group compared to the control group. The administration of saffron crude aqueous extract at varying doses (20, 40, and 80 mg/Kg) significantly reduced the percentage of DNA breakage by (70.05%,73.63%&85.24%) compared to the stimulated group.

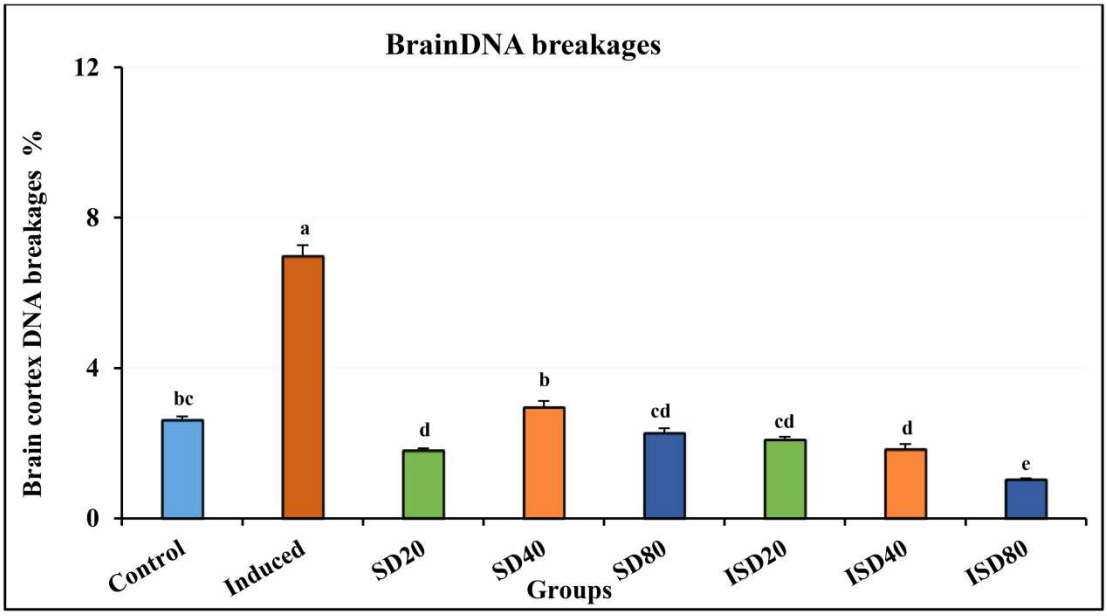

Figure 12: Attenuating effect of saffron aqueous extract administration on DNA breakages in brain homogenate

3.12. Immunohistochemical Findings

a- Amyloid β (Aβ) peptide plaques

Photomicrograph of a **control** rat brain subjected to treatment with ant. β-amyloid protein exhibits negative immunostaining in the majority of neuropile cells inside the hippocampal area, The administration of saffron extract was evaluated for its impact on amyloid plaque deposition in STZ-treated groups. Subsequently, the photomicrograph of the **induced** rat brain exhibits robust immunostaining in the majority of neuropil cells inside the hippocampal area. Endothelial blood vessels exhibited coarse, red-colored granules in the cytoplasm and cell membrane, accompanied with a negative blue hue in the cell nucleus at CA3 demonstrates significant immunostaining density within the hippocampal region in both stratum oriens (So) and stratum radiatum (Sr), manifesting as a mass of red granular structures (R) encircling the dilated myelinated sheaths (S) and blood vessels (BV). Numerous degenerative small and hyperchromatic pyramidal cells are evident as dark red immunostaining in the cytoplasm, with thick, voluminous axons predominantly located in the upper regions of the nuclei (*), alongside a negative blue hue in the neuronal cell nuclei (N). Most degenerative small and hyperchromatic pyramidal cells are evident as dark red immunostaining in the cytoplasm, with thick, voluminous axons predominantly located in the upper region of the nuclei (*), while the neuronal cell nuclei (N) exhibit a negative blue coloration. In the box, an Alzheimer patch of the aggregated pyramidal cells exhibits a dense coloration of beta-amyloid in the cytoplasm above the nuclei of necrotic (n), pyknotic (p), and hyperchromatic (h) cells

Photomicrographs of the **ISD20** rat brain revealed a moderate reduction in immunostaining across the majority of neuropil cells in the hippocampal regions So and Sr, presenting as a uniform yellowish hue. Alterations in the neuronal tissue resulting from the administration of extract in the **ISD40** group rat brain photomicrograph demonstrate a significant reduction in immunostaining in the majority of neuropile cells inside the hippocampal regions So and Sr. Finally, a rat in group **ISD80**, exhibiting robust immunostaining in the majority of neuropile cells inside the So and Sr regions of the hippocampus. The robust immunostaining in the CA3 area neuron cells manifested as clusters of red granules in degenerative pyramidal cells, accompanied by a negative blue hue in the nuclei as shown in **Figure 13 (a, b, c, d and e), respectively**.

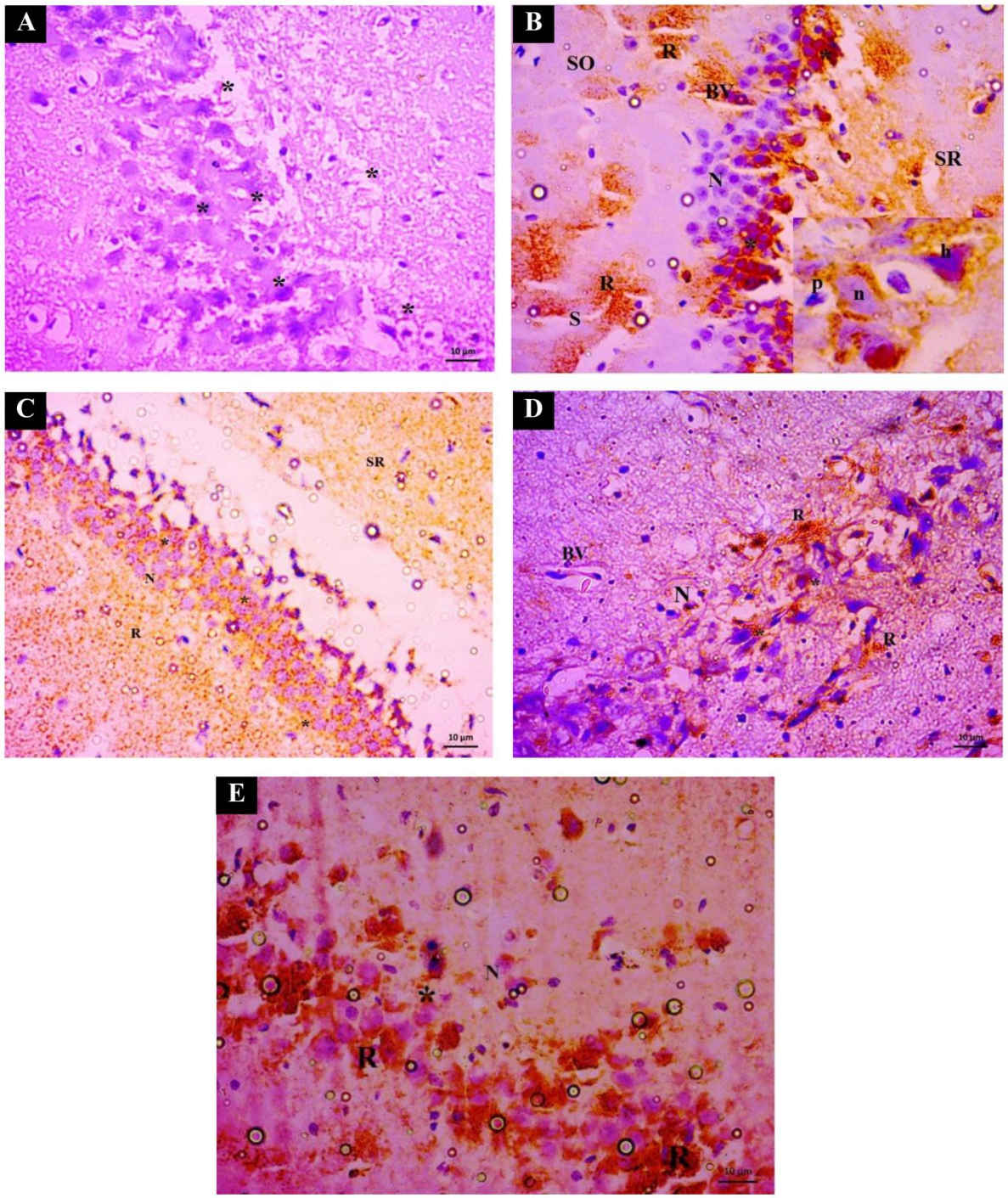

Figure 13: **(A, B, C, D and E)** Photomicrograph rat brain (A): control group, (B): Induced group, (C): ISD20: induced + saffron extract (20 mg/kg), (D): ISD40: induced + saffron extract (40 mg/kg) and (E): ISD80: induced + saffron (80 mg/kg) treated by ant β -amyloid protein and stained by ACU, (β-Am & ACU stain Bar=50µm)

b- Tubulin associated unit (Tau)

To investigate the effect of saffron extract on Alzheimer's disease, immunostaining of Tau protein in the **control** group photomicrograph (Tau & ACU stain Bar=50µm) of the rat brain hippocampus demonstrates negative immunostaining in the majority of neuropile cells within the hippocampal region. Alterations in neuronal tissue resuLting from the administration of streptozotocin to the induced group a photomicrograph revealed pronounced immunostaining in the majority of neuropile cells within the hippocampal region, manifesting as coarse red granuLes in the cytoplasm and diffuse filamentous deposition of staining in the stratum region, accompanied by a negative blue coloration of the cell nuclei. Furthermore, at CA3, revealing pronounced immunostaining density in the hippocampal region, manifested as red granuLar aggregates in the cytoplasm of clustered astrocytes adjacent to degenerative, hyperchromatic pyramidal cells, along with diffuse immunostaining in the cytoplasm of cells and small filaments of neural cells within the stratum region. A negative blue hue of the neuronal cell nuclei (N)

Regarding the potential therapeutic efficacy of saffron extract on tau protein in the **ISD20** ,**ISD40**, and **ISD80** rat brain, moderate immunostaining was observed in the majority of neuropile cells within the hippocampal region, manifesting as coarse red granuLes in the cytoplasm and diffuse filamentous deposition in the stratum region, along with a limited presence of astrocytes in the DG region. Weak immunostaining of fine filament deposition in the stratum region of select astrocytes in the dentate gyrus **Figure 14 (a,b,c,d and e) respectively**.

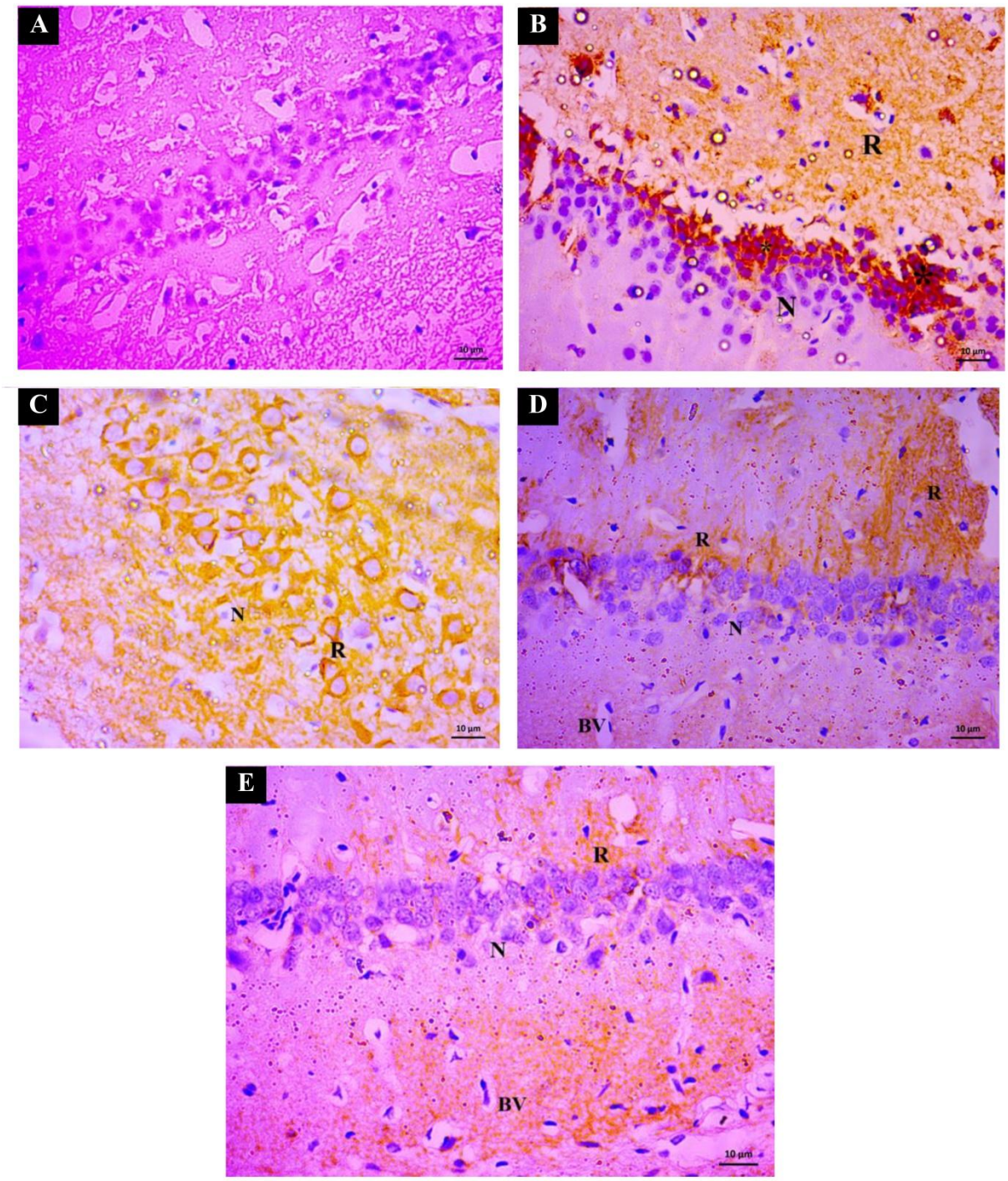

Figure 14: **(A, B, C, D and E)** Photomicrograph rat brain (A): control group, (B): Induced group, (C): ISD20: induced + saffron extract (20 mg/kg), (D): ISD40: induced + saffron extract (40 mg/kg) and (E): ISD80: induced + saffron (80 mg/kg) treated by tau protein and stained by ACU, (Tau & ACU stain Bar=50µm)

4. Discussion

To put things into perspective, Alzheimer's disease (AD) is the most common neurodegenerative cause of dementia. Neurodegeneration is associated with toxic amyloid-beta (Aβ 1-42) oligomers and protein aggregates, intra-neuronal neurofibrillary tangles consisting of hyperphosphorylated microtubule-associated Tau protein, regionally specific reduction of cerebral glucose metabolism, synaptic dysfunction, and mitochondrial dysfunction [33]. Prior research has demonstrated that administering low dosages of STZ directly into the brain through the ICV route disrupts brain homeostasis, insulin signalling and impair in cerebral glucose metabolism. This is characterized by neuropathological and metabolic alterations that resemble those seen in the pathophysiology of AD, including synapse dysfunction, tau hyperphosphorylation, and oxidative stress [34].

In our study, the induction of AD was evident by behavioural and biochemical studies. To assess the effect of SCE on cognitive impairment, the current study has shown that supplementation with aqueous extract of saffron effectively counteracted Alzheimer-induced memory impairment at all supplemented dose levels. Significant differences were observed in spatial and recognition memory in MWM and novel object recognition assessments between the induced group and SCE-supplemented groups.

In the ORAC assessment, the reduction of indicator fluorescence displays a lag phase, wherein the kinetics are defined by the rivalry between the interaction of peroxyl radicals with the indicator and the antioxidant [35]. The lag phase is analogous to that observed for oxygen intake during the suppression of autoxidation by chain-breaking antioxidants and contrasts with competitive tests, such as crocin bleaching. The ORAC assay evaluates an antioxidant's effectiveness by analysing the area under the curve of fluorescence intensity over time, allowing for the assessment of peroxyl radical reactions with a specific compound and the determination of the total antioxidant capacity of a food extract. The ORAC assay presents potential benefits over other methods by employing peroxyl radicals as reactants with similar redox potential and reaction mechanisms (i.e., hydrogen atom versus electron transfer) to physiological oxidants, and by utilizing a physiological pH, enabling antioxidants to react under conditions that reflect the body's overall charge and protonation state. The results derived from individual compounds demonstrate a correlation between the fluorescence bleaching curve in the ORAC and the oxidation potential of the antioxidant. These findings are anticipated to be widely useful for evaluating the total oxidation potential of more intricate combinations of antioxidants, such as those present in food or plant extracts [31].

The Acetylcholinesterase inhibitory activity of saffron extract and its compounds derived from the stigmas of Crocus sativus was assessed in a study to evaluate its potential application in combating Alzheimer's disease. The experiment revealed Acetylcholinesterase inhibitory action in the saffron extract. The IC50 values of safranal, crocetin, and dimethyl crocetin were determined to be within the low micromolar range. A distinct investigation utilized Iris germanica var. Florentina for the extraction of phenolic and flavonoid compounds. Isolated fractions demonstrated suppression of both AChE and BChE, suggesting their possible application in Alzheimer's disease treatment [36].

Our results show that untreated ICV-STZ-injected rats were impaired in the memory recall testing day as they had a significantly lower preference for the target quadrant that had previously contained the platform. Memory impairments in the MWM are among the major behavioural tests of rodent models of AD. This task is a specific for model of AD because it taps hippocampal functions as it requires storing precise representation of spatial relationships to locate a small, hidden goal. Significant differences in escape latency were observed in acquisition and reference memory and novel object recognition assessments between the induced group and SCE-supplemented groups. The percentage of time spent in the target quadrant and the travelled distance to reach the hidden platform in each group were used to assess acquisition of the water maze task. The results showed that in comparison with the control group, the ICV-STZ-injected rats revealed significant decline in spatial learning, with longer latency and distance in reaching the hidden platform. Moreover, during the memory retention test, rats failed to remember the precise location of the platform, spending significantly less time in the target quadrant than the normal groups. Also, STZ rats failed to discriminate novel and familiar objects in object recognition task. On the other hand, supplementation of SCE greatly enhanced the spatial and recognition memory in both MWM and NOR tests. Saffron extract was also reported to have a positive impact on spatial learning memory in morphine-induced memory impairment [37].

The anti-oxidative stress properties of saffron and crocin indicate a potential strategy for preventing stress-induced damage to learning and memory. Saffron possesses have been attributed to many carotenoids that have potent antioxidant properties and have the potential to safeguard central nervous system neurons from a detrimental effect of oxidative stress [38].

In the hippocampal tissue homogenate, the level AChE were higher in the group treated for Alzheimer's disease compared to the control group. However, after administering different dosages of SCE, AChE dramatically decreased in all treated groups.

Acetylcholine, a neurotransmitter, enhances the propagation of nerve impulses and the conveyance of signals related to memory and cognitive abilities. Increased AChE activity can lead to acetylcholine deficiency, resulting in cholinergic dysfunction and cognitive impairments [39-41]. The cholinergic system is crucial for attention, learning, and memory. It promotes nerve conduction and signal transduction related to memory and learning ability [42]. However, the deterioration of cholinergic neuronal function leads to the accumulation of AChE [43], tau phosphorylation, Aβ plaques, neurotoxicity, neuroinflammation, and neuronal death [44] which ultimately results in condition like Alzheimer or dementia.

The result of the current study is consistent with previous finding where active constituent crocin also increases acetylcholine levels which in turn decrease AChE, prevent hippocampal neuronal apoptosis, and improve memory in the rat brain affected by Alzheimer [45]. Further, the molecular docking of extract constituent at the active site of AChE also indicates that SCE constituents effectively inhibit the enzyme and could increase the level of acetylcholine in the brain to improve memory [46].

In our study, we investigate the enzymes in blood serum associated with acetylcholinesterase function and the impact of saffron extract on these enzymes. We assess the levels of gamma-glutamyl transferase (GGT), which were significantly elevated in the induced group, while butyrylcholinesterase (BChE) was significantly reduced in the induced group compared to the control group. Simultaneously, the co-administration of saffron aqueous extract countered this effect in all treatment groups.

Gamma glutamyl transfers (GGT) has demonstrated a neuroprotective impact on neuronal cells. The primary limitation of the vascular endothelial barrier is its failure to obstruct the ingress of lipophilic xenobiotics, whereas Intracellular glutathione production, facilitated by GGT, can detoxify these compounds. Consequently, GGT is pivotal in protecting cells from oxidative damage. Furthermore, elevated GGT levels within the normal range may signify enhanced hepatic capacity to manage oxidative stress. A reduction in oxidative stress levels in the body correlates the deficiency or lack of GGT results in a compromised glutamate cycle, adversely affecting the absorption, transport, and use of amino acids, leading to disorders in glutathione resynthesis and increasing neurological symptoms [47]. Therefore, a better GGT reserve is essential for generating enough glutathione to maintain the redox balance in the body.

Primarily, to examine the impact of butyrylcholinesterase (BChE) on Alzheimer's disease, it is essential to note that BChE hydrolyses acetylcholine (ACh). As BChE is an α-glycoprotein located in the central and peripheral nervous systems, exhibiting a half-life of 12 days. It is a nonspecific or pseudocholinesterase, also known as serum cholinesterase, which hydrolyses both choline and aliphatic esters [48].

The intracerebral infusion of STZ is known to cause memory impairment that closely resembles the cognitive decline observed in sporadic Alzheimer's disease. Streptozotocin injected rats are used as a non-transgenic rodent model to mimic Alzheimer's disease [49]. In this current investigation, thirty days following a single ICV injection of STZ, the level of phosphorylated tau-217 significantly increased in STZ treated rats as blood serum biomarkers that accurately indicate Alzheimer’s disease. Meanwhile, co-administration of SCE significantly decreased p tau-217 in all treated groups. Serum measures of phosphorylated tau have high diagnostic accuracy in differentiating AD from other neuro degenerative disorders in clinical studies, which are validated by postmortem neuropathological studies [50]. Tau proteins constitute a group of brain-specific structural proteins present in microtubules. Their hyperphosphorylation causes abnormal folding and fragmentation, giving rise to toxic insoluble aggregates inside cells, which are known as NFTs [1].

Above all a significant increase was observed in Aβ1-42, MBP and BACE1 in induced group when compared with control group.

The extracellular fibrillar amyloid β (Aβ) peptide plaques represent classical pathological hallmarks of Alzheimer's disease. Aβ plaques form due to changes in amyloid precursor protein [21] metabolism. The cleavage of APP by α-secretase leads to the production of non-amyloidogenic Aβ fragments, while cleavage by β-secretase results in the formation of neurotoxic amyloidogenic Aβ fragments. Aβ aggregation triggers the NFTs, neuronal dysfunction, and dementia [51]. Furthermore, high level of AChE not only down regulates acetylcholine function but also combines with Aβ fibrils and forms more neurotoxic AChE-Aβ complex which further exaggerate neurodegeneration than those of Aβ fibrils alone [52].

The β-cleaving secretase, BACE1, define a family of transmembrane aspartic proteases [53]. BACE1 exhibits all the properties of the β-secretase and is the key rate-limiting enzyme that initiates the formation of Aβ [54]. BACE1 is principally present in neurons in the central nervous system [55]. Higher β-secretase activity, as well as induction of BACE1 protein level, is seen in AD brains. Another interesting observation is that induced BACE1 protein appears to colocalize with markers of apoptosis. This is consistent with the role of apoptosis in AD. In neurodegenerative diseases including AD, signals that initiate neuronal apoptosis, including neurotrophic factor support withdraw, disturbance of calcium homeostasis , increased oxidative stress and metabolic stress, are described [56]. These pro-apoptotic factors may contribute to the over-expression of BACE protein in the AD brain, and interfere with APP processing, thus seeding formation of senile plaques. This BACE activation can then cause further apoptotic signalling, inducing caspase activation and DNA fragmentation, both of which are observed in AD brains [57].

The current study indicate that induction of AD with STZ significantly increase MBP which is one of the most abundant proteins in the myelin sheath and the only structural myelin protein known to be essential for the formation of compact myelin sheaths as well as it is a multifunctional protein that interacts with lipids and a diverse array of proteins [58].Concurrently, the co-administration of SCE resulted in a significant drop in MBP across all dosages. However, in the case of saffron 80, MBP restored to its usual range. Specifically, MBP regulates voltage-gated Ca2+ channels (VGCCs) at the plasma membrane reducing the Ca2+ influx in the oligodendrocyte. Numerous studies have indicated that the absence of this protein leads to myelin vesiculation and subsequent breakdown. However, there is no study describing the opposite effect. It is therefore plausible that an excess accumulation of this protein might influence myelin compaction, subsequently affecting conduction velocity and the delivery of nutrients to the axon [59].

Upregulation of MBP inhibits VGCCs, thereby reducing Ca2+ influx into oligodendrocytes. Ca2+ influx through membrane channels is a critical step in signalling pathways involved in the regulation of growth, maturation and functional plasticity. In addition, elevated Ca2+ levels stimulate MBP synthesis in oligodendrocytes [60] and are critical for myelin sheath extension. The accumulation of MBP in the membrane would inhibit or reduce the number of VGCCs in the membrane, as previously reported [61], thereby affecting to the calcium entry through these channels. Thus, our results suggest that Aβ oligomers disrupt Ca2+ regulation, making oligodendrocytes more susceptible to environmental stimuli that increase intracellular Ca2+ levels and affect myelination.

Increased levels of MBP protein in AD cortex have been reported in at least one previous study [62]. Thus, our results confirm that the levels of MBP and degraded myelin basic protein complex (dMBP) are increased in AD cortex compared to controls. The mechanism by which that MBP levels were higher in AD compared to control remains unclear. It is possible that MBP/myelin injury is associated with remyelination which increases total MBP in AD brain. In addition, we cannot rule out the possibility that the increase in "MBP" observed in AD brain is due to induction of Golli MBP proteins that are normally expressed in neurons [63].

Investigations show a close link between BChE and Aβ, the major pathogenic feature of Alzheimer's disease. According to the Aβ hypothesis, β- and γ-secretases successively digest amyloid precursor protein, producing damaging full-length Aβ1-40/42 peptides. In Alzheimer's disease patients, an imbalance between Aβ production and clearance in the nervous system leads to irreversible accumulation of Aβ plaques and pathological processes. Microglia play a crucial role in clearing extraneuronal Aβ plaques [64]. Based on the aforementioned evidence, inhibiting BChE instead of AChE may represent a more promising and effective therapeutic approach for patients with advanced Alzheimer's disease, without inducing significant side effects.

In the present study potential of saffron crude extract (SCE) to reduce neurodegeneration dysfunction induced cholinergic disturbance and associated changes caused by STZ were investigated. Further, the impact of SCE on AChE, and proinflammatory cytokine i.e., IL-6, COX-2, Caspase-3 and TNF-α were explored.

The brain's cellular membrane is rich in polyunsaturated fatty acids (PUFA), making it more vulnerable to neuroinflammation [65], which in turn lead amyloid and tau pathologies [66]. Streptozotocin administration releases of pro-inflammatory cytokines and accelerates neurodegeneration [67]. In this study, STZ significantly elevated IL-6, COX-2, Caspase 3 and TNF-α which was countered by SCE supplementation which significantly decreases in all dose levels. This is also supported by previous literature in which saffron extract and crocin has shown a neuroprotective role against methamphetamine neurotoxicity in rats by arresting proinflammatory cytokines production [68].

Oxidative stress is increasingly thought to be involved in various neurodegenerative diseases. Elevated oxidative damage was identified as a common characteristic in neurons and peripheral cells from both sporadic and familial Alzheimer's disease patients. Data suggests that oxidative stress manifests early in Alzheimer's disease, somewhat prior to the emergence of pathological markers such as neurofibrillary tangles and senile plaques [69]. The deposition of insoluble Aβ forms is considered a cause of oxidative stress in Alzheimer's disease, with Aβ hypothesized as the originator of this process. The accumulation of hyperphosphorylated tau, synaptic loss, and axonal degeneration are pathogenic characteristics of Alzheimer's disease. Tau not only stabilizes microtubules and promotes neurite development but also regulates the movement of cellular components via molecular motors along microtubules. Misregulation of tau protein leads to the progressive retraction of cellular organelles, including mitochondria and peroxisomes, resulting in synaptic starvation and heightened oxidative stress due to the lack of catalase in peroxisomes [70]. It has been noted that Aβ deposition occurs subsequent to elevated levels of 8-Hydroxy-2-deoxyguanosine (8-OHdG), and that 8-OHdG levels return to baseline following the formation of Aβ plaques. Oxidative damage refers to the formation of reactive oxygen species (ROS), which can alter the structure of proteins, lipids, and nucleic acids through interaction. The comet assay, a novel methodology for evaluating DNA damage, is an effective test for genotoxicity and serves as a crucial instrument for exploring fundamental elements of DNA damage and subsequent cellular responses [71].

Saffron and its constituents can safeguard DNA and RNA from destructive chemical reactions [72]. The constituents of saffron demonstrate potential antioxidant properties, allowing free radicals to withdraw a hydrogen atom from the antioxidant molecule rather than from polyunsaturated fatty acids (PUFA), thus disrupting the sequence of free radical reactions, resulting in an antioxidant radical that is a relatively nonreactive entity [73] both ethanolic and aqueous extracts of saffron have been shown to possess antioxidant activity through various in vitro methods, specifically employing three experimental approaches: the deoxyribose assay, erythrocyte membrane peroxidation, and rat liver microsomal lipid peroxidation induced by Fe2+/ascorbate. Crocin, a primary carotenoid in saffron, has exhibited supplementary roles concerning the antioxidant properties of saffron. Safranal, a monoterpene aldehyde and the primary constituent of saffron's essential oil, also exhibits antioxidant properties.

Multiple investigations have demonstrated that the active compound of Crocus sativus reduces Aβ fibril production and neuronal apoptosis [74], alters the Aβ aggregation pathway, and forms stable fibrils by changing the distribution of Aβ1-40 [75]. Crocin lowers Aβ, and p-tau levels via altering MAPK signalling pathways [76] and enhancing the expression of Aβ-degrading enzymes. The acetylcholinesterase inhibitory action of CSE components in the active site of the galantamine binding receptor resembles that of conventional medications rivastigmine and galantamine. These results come in align with our results.

Conclusion

To summarize, this study shows that the standardized aqueous SCE has the ability to inhibit the activity of acetylcholinesterase and operate as an antioxidant. Additionally, it has been found to reduce the negative effects on learning and memory caused by Alzheimer. Administration of SCE effectively reduces the occurrence of Aβ plaque and the level of phosphorylated tau-217 and MBP in the hippocampus at various dosage levels. Furthermore, SCE also mitigates the inflammation caused by Alzheimer disease. The findings suggest that a botanical supplement derived from Saffron may have potential in the treatment of senile dementia.

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Author contributions:

Aly B. Okab: propose the research point, design the experiment and supervising the practical parts, supervising the physiological studies; Sabah G. Elbanna: propose the research point, design the experiment and supervising the practical parts, supervising the biochemical studies, and supervising analysis of results; Abeer Salama: Contributing in methodology, Resources and statistical analysis ;Seham Z. Nassar: Shared in the conceptualization of the study, stereotaxic technique for ICV injection and the behavioural studies conduction and interpretation; Naglaa H. Eldewany: Propose the research point , conducted the experiments and the practical part, interpretation of data, Statistical analysis and wrote the manuscript; Aly B. Okab, Sabah G. Elbanna, Abeer Salama, Seham Z. Nassar and Naglaa H. Eldewany: All authors finally reviewed, editing and agreed with the content of the manuscript.

Data availability statement:

Data will be made available on request.

Approval of animal experiments:

The experiments were approved by the Ethical Committee of the National Research Centre, Egypt, and the Institutional Animal Care and Use Committee, Faculty of Medicine, Alexandria University (ALEXU-IACUC No. AU14-231130-2-12), Alexandria, Egypt.

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