**Protective effect of a standardized** ***Allium jesdianum* extractin an Alzheimer's disease induced rat model**

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**Abstract**s

**Background:** Alzheimer's disease (AD) is a complex disorder with multiple underlying mechanisms.  Existing treatment options mostly address symptom management and are associated with numerous side effects. Therefore, exploring alternative therapeutic agents derived from medicinal plants, which contain various bioactive compounds with diverse pharmacological effects, holds promise for AD treatment. This study aims to assess the protective effects of the hydroalcoholic extract of *Allium jesdianum* on cognitive dysfunction, mitochondrial and cellular parameters, as well as genetic parameters in an intracerebroventricular Streptozotocin (icv-STZ) induced rat model of AD. **Methods:**  Male Wistar rats were injected with a single dose of STZ (3 mg/kg, icv) to establish a sporadic AD model. *A. jesdianum* extract (100, 200, and 400 mg/kg/day) and donepezil (5 mg/kg/day) were orally administered for 14 days following model induction. Cognitive function was evaluated using the radial arm water maze test. Mitochondrial toxicity parameters, including succinate dehydrogenase (SDH) activity, mitochondrial ROS formation, mitochondrial membrane potential (MMP) decline, mitochondrial swelling, and cytochrome c efflux in various brain regions (whole brain, frontal cortex, hippocampus, and cerebellum) were assessed. Gene expression analysis of miR-330, miR-132, Bax, and Bcl-2 in isolated rat brain neurons was performed using RT-qPCR.analysis were evaluated. **Results**: *A. jesdianum* extract significantly attenuated cognitive dysfunction and mitigated mitochondrial toxicity induced by icv-STZ administration. Following STZ injection, there was upregulation of Bax gene expression and downregulation of miR-330, miR-132, and Bcl-2 gene expression. However, treatment with *A. jesdianum* extract resulted in the reversal of the expression of these microRNAs and genes, indicating its potential for improving AD and reducing neuronal apoptosis. **Conclusion:** This study demonstrates the neuroprotective capabilities of *A. jesdianum* against STZ-induced oxidative stress and cognitive impairment in rats, highlighting its therapeutic potential in the management of AD. Further investigations are warranted to elucidate the underlying molecular mechanisms and potential clinical applications of A. jesdianum in AD treatment.

**Keywords**: Alzheimer’s disease (AD), *Allium jesdianum*, Radial Arm Water Maze,Mitochondria, microRNA (miRNA), Bax, Bcl-2

**1 | Introduction**

Alzheimer's disease (AD) is a multifactorial and progressive neurodegenerative disorder, accounting for the majority of dementia cases worldwide. The exact etiology of AD remains incompletely understood, and curative treatments have yet to be developed (1). AD is characterized by a range of pathological factors, including the formation of amyloid beta plaques, neurofibrillary tangles (NFTs), dysfunction of the cholinergic system, oxidative stress, neuroinflammation, synaptic plasticity impairment, and eventual neuronal loss (2). These hallmark features may be triggered by mitochondrial dysfunction and oxidative stress. Clinical studies have established a correlation between neuronal loss and mitochondrial dysfunction, leading to memory impairment in affected individuals (3). Notably, mitochondrial dysfunction has been implicated in synaptic transmission impairments observed in various neurodegenerative conditions, including Alzheimer's, Huntington's, and Parkinson's diseases (4). MicroRNAs (miRNAs) are small non-coding RNAs that post-transcriptionally regulate gene expression and are vital for neuronal function and survival. The brain expresses approximately 70% of identified miRNAs, which directly contribute to the regulation of synaptic function and specific signaling pathways involved in memory formation. Furthermore, miRNAs show promise as sensitive biomarkers for AD detection, management, and prevention (5, 6). Recent evidence suggests that dysregulation of specific miRNAs occurs in Alzheimer's disease, impacting the expression of key genes associated with the disease, such as APP, BACE1, and MAPT (7). Additionally, miRNAs have been implicated in the development of amyloid beta (Aβ) peptide accumulation and tau phosphorylation, which are central to AD pathogenesis. Oxidative stress, a significant contributor to AD development, influences the expression of numerous miRNAs, and conversely, miRNAs can modulate the oxidative stress response (8). Considering that AD is a multifactorial disease, single-target drugs that affect only one enzyme or one receptor are not suitable for its treatment. Existing treatment options for AD primarily focus on alleviating cognitive symptoms and do not halt or delay disease progression. Moreover, these medications often come with side effects. Therefore, there is a growing need for natural and safe therapeutic alternatives (9, 10). In recent years, various compounds with therapeutic potential have been extracted from plants, animals, and microorganisms, demonstrating beneficial effects in targeting multiple pathogenic mechanisms associated with AD (11, 12). Thus, Medicinal plants offer an alternative to standard AD drugs. *Allium jesdianum* Boiss & Bushe is a species of bulbous plant in the genus Allium and belongs to the Alliaceae family and is grown in the Zagros Mountains of Iran.

*A. jesdianum* has been extensively studied for its therapeutic and pharmacological activities.For instance, the hydroalcoholic extract of *A. jesdianum* has shown anti-anxiety and anti-depressant effects in rat models, as evidenced by elevated plus maze, open field, and forced swim (13). Additionally, *A. jesdianum* has demonstrated hepatoprotective effects against acetaminophen-induced oxidative stress markers in rat liver tissue (14). The antioxidant properties of the hydroalcoholic extract of *A. jesdianum* have been reported in a mouse model of nephrotoxicity induced by carbon tetrachloride (15). Moreover, *A. jesdianum* has exhibited analgesic, antipyretic, anti-bacterial, anti-fungal, and anti-cancer properties, and its usage has been beneficial in the treatment of genitourinary disorders and the removal of renal stones (16, 17).

Despite the nutritional and pharmacological value of A. jesdianum and its antioxidant properties, its potential anti-Alzheimer's effects in experimental AD models have not been investigated. Thus, the aim of this study is to assess the effects of A. jesdianum hydroalcoholic extract on learning and memory impairment, as well as mitochondrial, cellular, and genetic parameters in an intracerebroventricular streptozotocin (icv-STZ) induced rat model of AD. By elucidating the potential therapeutic properties of A. jesdianum in AD, this research may contribute to the development of novel treatment strategies for this multifactorial disease.

**2 | Materials and methods**

**2.1 | Preparation of the extract**

The *A. jesdianum* was found and collected near in the Zagros Mountains of Shahrekord, Iran, in April 2021(32° 30' 30" N, 56° 27' 33" E). The Iran's Jondishapur University herbarium unit identified the plant and samples were stored there with the code number of A-0138. Extract of the whole plant of *A. jesdianum* was prepared by maceration method using ethanol: water (80:20 v/v) solution as a solvent and a solid-to-solvent ratio of 1:10 (w/v) for four days. A rotary vacuum was used to concentrate the extract until a crude solid was produced (18).

**2.2 | Standardization of extract**

Total phenolic components in the *A. jesdianum* hydroalcoholic extract were calculated using Folin Ciocalteu's colorimetric assay (19). The calibration curve was established using gallic acid, and the total phenolic content (TPC) was reported as milligram gallic acid equivalent per milliliter extract. The total flavonoid of*A. jesdianum* was evaluated by the aluminum chloride colorimetric method using a UV spectrophotometer (20). Through the quercetin calibration curve, the quantity of total flavonoids was expressed as milligram of quercetin equivalent per milliliter of the samples.

**2.3 | Animals**

All of the Wistar rats (4-6 month old) used in this study were male and weighed between 230 - 270 g and were obtained from the Shahid Beheshti University of Medical Sciences. The rats were kept in an air-conditioned room with a controlled temperature of 25± 2 °C, a 12:12-hour light/dark cycle, 50 to 60 percent humidity, and free access to food and water. All experiments were conducted based on animal research international guidelines and were authorized by the Ethics Committee of Shahid Beheshti University of Medical Sciences (ethics code: IR.SBMU.PHARMACY.REC.1400.143).

**2.4 | Study design**

Randomly, the animals were sepreted into six groups (*n*=8). Group I was Sham (received normal saline icv + oral gavage of vehicle solution for two weeks); Group II received STZ (STZ 3 mg/kg icv once + oral gavage of vehicle solution for two weeks); Group III received STZ (STZ 3 mg/kg icv once) and treated with *A. jesdianum* (AJ) (100 mg/ kg/day, p.o. for two weeks); Group IV received STZ (STZ 3 mg/kg icv once) and treated with AJ (200 mg/kg/day, p.o. for two weeks); Group V received STZ (STZ 3 mg/kg icv once) and treated with AJ (400 mg/kg/day, p.o for two weeks). Group VI received STZ (STZ 3 mg/kg icv once) and treated with donepezil (5 mg/kg/day, p.o. for two weeks). The doses of AJ were selected based on previous studies (14, 21).

**2.5 | Experimental induction of the AD model**

In order to anesthetize the rats before the surgical operation, they were given a combination of ketamine (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). Following this, the animal's head was positioned in a stereotaxic apparatus, and the scalp was shaved. The skull was exposed after a midline sagittal incision was made in the scalp. The stereotaxic coordinates for the lateral ventricles region were 0.8 mm posterior to bregma, 1.5 mm lateral to the sagittal suture and 3.6 mm beneath the cortical surface set according to the rat brain atlas (22). Using a Hamilton syringe needle a burr hole created in the skull and 5 μl of STZ was slowly infused into each lateral ventricle. STZ solution was prepared in normal saline instantly before injection (23).We used appropriate anti-inflammatory and antibiotic agents after stereotaxic surgery (24). Animals received daily oral gavage of *A. jesdianum* extract and donepezil 24 hours after the surgery for 14 days.

**2.6 | Behavioral assessment**

The behavioral test period began 15 days following the STZ injection. Radial arm water maze (RAWM) test was done on days 15-17. In addition to other studies, our preliminary experiments established learning and memory impairments two weekspost-STZ administration (25, 26).

**2.7 | Radial Arm Water Maze Test**

The Radial Arm Water Maze (RAWM) test evaluated the animals' spatial learning and memory. The RAWM apparatus consists of a water tank and six (59×13 cm) arms. Visual cues were positioned at a certain distance from each arm. The water temperature was kept at 25 ˚C and the water depth was 50 cm. There were three consecutive days of testing for each animal. On the first two days, the animals were trained 15 times to learn the hidden platform location guided by the visual cues placed at the end of each arm. During each trial, the platform was placed in one of the six arms, 2 cm below the water surface (target arm). The animals were placed in one of the arms (starting arm) and allowed 60 s to reach the platform. The latency to find the platform and the number of entries to the non-target arm were recorded. On the last day, as the probe or test day, each rat was located in the starting arm and the hidden platform was removed from the target arm. The latency to enter in the target arm, the number of entries to the target arm, the time spent in the target arm, total distance traveled and velocity were recorded. The Ethovision (Noldus, the Netherlands) tracking software was utilized for analyzing all data captured by digital cameras (27, 28).

**2.8 | Biochemical measurements**

Rats were sacrificed 24 hours after the probe test, and their brains were quickly taken out, cleaned with saline that was icy cold and three cerebral areas include the hippocampus, frontal cortex and cerebellum were dissected. Then, the mitochondria were isolated, and mitochondrial toxicity parameters were evaluated. Animal brain neurons were isolated for evaluation of miR-330, miR-132, Bax and Bcl-2 genes expression via real-time PCR.

**2.9 | Isolation of Mitochondria**

The brain tissue was immersed in an ice-cold isolation buffer containing 225 mM mannitol, 75 mM sucrose, 5 mM HEPES, 1 mg/ml fatty acid-free BSA, and 3 mM EGTA. The solution had a pH of 7.4 and was isotonized with KOH**.** Using a hand-held homogenizer, the tissues were minced and homogenized, and then, by performing differential centrifugation technique, mitochondria of the collected tissues were extracted. Before anything else, the wrecked cell debris and cores were centrifuged (2000 g, 10 min, 4 °C) and deposited in sediment from the specimens. A second centrifugation (at 12000 g for 10 minutes at 4 °C) was applied to the supernatant. The top layer was removed, and the mitochondrial pellet was washed before being put in the extraction medium and centrifuged again (12000 g, 10 min, 4 °C). Then, the mitochondrial pellet was homogenized and kept cold by an ice shower until the tests. Coomassie blue protein-binding method, introduced as Bradford test, was used to estimate protein concentrations. The mitochondrial samples were normalized based on protein concentration (0.5 mg mitochondrial protein per ml). All isolation procedures were carried out on ice to maintain mitochondrial quality during processing (29).

**2.10 | Succinate dehydrogenase activity**

To evaluate the activity of the Succinate dehydrogenase (SDH) in the isolated mitochondria,

the reduction of MTT to formazan based on Zhaoet al*.* 2010; methodwas performed (30).

**2.11 | Mitochondrial ROS Level Measurement**

Based on the Gao et al. 2009; method, the amount of ROS in neuron mitochondria was measured using DCFH-DA fluorescence probe and Shimadzu RF-5000U fluorescence spectrophotometer (31).

**2.12 | Mitochondrial Membrane Potential Measurement**

To calculate the mitochondrial membrane potentials (MMP), the cationic fluorescent dye, Rhodamine 123 (Rh123), and fluorescence spectrophotometer were used (32).

**2.13 | Determination of mitochondrial swelling**

To determine mitochondrial swelling, light scattering changes at 540 nm were evaluated spectrophotometrically (30). The absorbance was measured at 540 nm using an ELISA reader equipment after the isolated brain mitochondria were suspended in swelling buffer. The presence of swollen mitochondria was indicated by a decline in the absorbance.

**2.14 | Release of cytochrome C**

The Quantikine Rat/Mouse Cytochrome c Immunoassay kit from R&D Systems, Inc., Minneapolis, MN, USA, was used to measure cytochrome c release. This kit employs the quantitative sandwich enzyme immunoassay technique (33).

**2.15 | Rat neurons isolation**

Brewer et al.'s method was used to isolate neurons from rats (34). The cortex, hippocampus, and other parts were dissected briefly. After that, 0.5-mm slices were cut, and papain was used to digest them for 30 minutes at 30 °C. The cells were triturated for release in the subsequent step. Density gradients were utilized to purify the neurons. The cells were concentrated and resuspended in the desired medium in the next step. After that, the neurons were plated on a poly-Lys-coated glass substrate and placed in the Neurobasal/B27 with growth factors added. At 37 degrees Celsius, they were then incubated with 9 percent oxygen and 5 percent CO2.

**2.16 | Quantitative real-time PCR**

The expressions of miR-132, miR-330, Bcl-2 and Bax genes were determined using RT-qPCR. The RNeasy Mini Kit (QIAGEN, Hilden, Germany) and miRNA Isolation Kit (Yekta Tajhiz Co., Iran) were used to isolate total RNA from rat brain neurons according to the directions given by the manufacturer. miRNAs were transcribed into complementary DNA (cDNA) by using the stem-loop RT primer hybridization, based on the M-MLV Reverse Transcriptase Kit (Yekta Tajhiz Co., Iran). The stem-loop RT structures were designed according to the previous studies (35, 36). A Mic qPCR Cycler (Biomolecular Systems, Australia) and a SYBR Green kit (Yekta Tajhiz Co., Iran) were utilized for the real-time quantitative RT-PCR analysis and amplification of cDNA. The thermal cycle conditions for miRNAs were 15 s at 95°C and 30 s at 60°C for 40 cycles and for Bax and Bcl-2 were as follows: 10 s at 95 °C, 20 s at 60 °C, and 15 s at 72 °C for 40 cycles. To normalize the cDNA variation, β-actin was used as a housekeeping gene forBcl-2 and Bax genes and the relative amount of miRNAs were normalized by U6 small nuclear RNA. The Pffafl method was utilized for relative quantification. Analysis using the Pffafle method indicates that a ratio more than 2 indicates Overexpression, whereas a ratio less than 0.5 indicates Low Expression (37). Table 1 shows the primer sequences.

|  |  |  |
| --- | --- | --- |
| **Sequence of primer 5′ 3** | **Gene** |  |
| F: CCCGAGAGGTCTTTTTCCGAG  R: CCAGCCCATGATGGTTCTGAT | Bax |  |
| F: GGTGGGGTCATGTGTGTGTGG  R: CGGTTCAGGTACTCAGTCATCC | Bcl-2 |  |
| F: AACGGTGAAGGTGACAGCAGTCG  R: GGCAAGGGACTTCCTGTAACAACG | ACTB |  |
| F:AAGCGCTCTCTGGGCCTGTG  R:CAGTGCGTGTCGTGTCGTGGAG | miR-330 |  |
| F:CGCTAACAGTCTACAGCC  R:GCAGGGTCCGAGGTATTC | miR-132 |  |
| F:GCTTCGGCAGCACATATACTAAAAT  R:CGCTTCACGAATTTGCGTGTCAT | U6 |  |
| GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCGACCAT | RT-miR132 |  |
| GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACGCCTAA | RT-miR-330 |  |

**2.17 | Statistical Analysis**

Graph Pad Prism was utilized for each and every statistical analysis (Graph Pad Prism software, version 8). Two-way ANOVA followed by Bonferroni's post hoc test was used for parameters of the training days of RAWM and one-way ANOVA followed by Turkey's post hoc test for parameters of the probe test. The one-way ANOVA was used to compare various mitochondrial parameters across the experimental groups, followed by Turkey's post hoc test. For RT-qPCR, the one-way ANOVA test and Turkey's post hoc test were performed. Data are reported as mean ± SEM. Statistical significance was set at P < 0.05.

Table1. PCR primer sequences for reverse transcription and qRT-PCR

**3 | Results**

**3.1 | Standardization and Characterization of *A. jesdianum* hydroalcoholic extract**

We found that the total phenolic compounds and total flavonoid of the *A. jesdianum* extract were 157.6 ± 13.4 mg of gallic acid equivalent per ml and 114.7 ± 10 mg of quercetin equivalent per ml, respectively.

In a study conducted by Alidadi et al. 2021; 12 Phyto-components of hydro-alcoholic *A. jesdianum* extract were identified by gas chromatography and mass spectroscopy (GCMS).

The major bioactive compounds present in the *A. jesdianum* extract were Dimethyl- sulfide, Allyll alcohol, Allicin, Cyclopentasiloxane-decamethyl, β-Pinene, α-Pinene, 2-pentylfuran, 2-phenyl-5-methylindole, Methyl ethyl cyclopentene , γ-Tocopherol , Pentasiloxane and dodecamethyl, Bicyclo[4.3.0]nonane, 3-methylene (38).

**3.2 | RAWM results**

In order to assess the *A.jesdianum*'s protective efficacy against STZ-induced memory loss in rats, the radial arm water maze assay (RAWM) were performed. Figure 1 displays the RAWM results of all the experimental groups in the first two days of the training experiment. In the majority of trials, animals in the STZ group required considerably longer time to locate the position of the platform, and the frequency of reference memory errors in the STZ group was significantly greater than the sham group throughout the training test days.

As shown in Fig. 1A, the animals that received the dose of 200 mg/kg *A. jesdianum* extract in trial 12 and the animals treated with donepezil in trials 6, 12 and 24 found the platform significantly faster than the STZ group. Figure 2B shows that the *A.jesdianum* at a dose of 200 mg/kg in trial 3, the dose of 400 mg/kg in trials 12 and 30 and donepezil in trials 27 and 30 were able to significantly reduce the number of entries into the non-target arm comparing to the STZ group.





**Figure 1:** Effect of *A. jesdianum* and donepezil on latency to locate platform (A) and reference memory errors number (B) on training days in the radial arm water maze test. Two‑way ANOVA estimated differences. Mean ± SEM (n = 8) are the forms of data that presented; \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 compared to the STZ group. #p<0.05, ##p<0.01, ###p<0.001, #### p<0.0001 compared to the sham group.

To access memory recall, the platform was removed on probe day. The STZ group had a considerably significant latency to locate the target arm than the sham group (P< 0.0001). One‑way analysis of variance showed a lower latency to locate the target arm in animals treated with *A. jesdianum* (400 mg/kg) and donepezil compared to the STZ group (P< 0.05 and P < 0.01 respectively; Fig. 2A).

  

**Figure 2:** Effect of *A.jesdianum* and donepezil on latency to locate target arm (A), entry frequencies to target arm (B), duration in target arm(C), Velocity (cm/s) (D) and Total distance traveled (cm) (E) in the probe day in the radial arm water maze test. One‑way ANOVA estimated differences. Mean ± SEM (n = 8) are the forms of data that presented; \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 compared to the STZ group. ###p<0.001, p<0.001, #### p<0.0001 compared to the sham group.

Also, the STZ group notably had fewer entries to the target arm on probe day than the sham group, according to the results (P < 0.001). In comparison with the STZ group, medium and high dosages of *A. jesdianum* (200 and 400 mg/kg) and donepezil increased target arm entries significantly (P< 0.05, P < 0.01 and P < 0.01 respectively; Fig. 2B). Finally, the duration the target arm significantly decreased in the STZ group when compared to the sham group (P< 0.0001). Animals administered 400 mg/kg of *A. jesdianum* and donepezil spent more time in the target arm comparing to those in the STZ group (P<0.01 and P < 0.001 respectively) (Figure 2C). Velocity and total distance traveled was recorded on the third day of the RAWM test. The difference between the experimental groups was not significant. (Figure 2D, E). It means that the treatments did not affect the locomotor activity of the subjects in the RAWM test.

**3.3 | Mitochondrial function**

**3.3.1 | Succinate dehydrogenase activity**

The mitochondrial viability related to succinate dehydrogenase activity in the whole brain, frontal cortex, hippocampus and cerebellum was found to be significantly reduced in STZ- induced animals in comparison to sham animals (P<0.0001). *A. jesdianum* at a low dose (100 mg/kg) showed no improvement in reduced the viability. *A. Jesdianum* was able to dramatically enhance the lowered MTT levels in the whole brain, frontal cortex, and cerebellar area at a dosage of 200 mg/kg (whole brain and cerebellum: P< 0.05 and frontal cortex: P < 0.01). Supplementation at a high dosage (400 mg/kg) is more successful (P<0.001) than STZ group in restoring the reduced MTT levels in the whole brain and frontal cortex, hippocampus, and cerebellum (P< 0.01). SDH activity was significantly higher in the donepezil group compared to the STZ group in the whole brain, frontal cortex and hippocampus (P< 0.001 for the whole brain and hippocampus and P < 0.0001 for the frontal cortex), but not in the cerebellum (Fig. 3).





**Figure 3:** The mitochondrial SDH activity, isolated from the whole brain (A), hippocampus (B), frontal cortex (C) and cerebellum (D) of STZ-induced AD rats treated with *A.jesdianum* and donepezil. One‑way ANOVA estimated differences. Mean ± SEM (n = 8) are the forms of data that presented; \*P<0.05, \*\*P<0.01, \*\*\*P<0.001, \*\*\*\*P<0.0001 in comparison with the STZ group. #### P<0.0001 in comparison with the sham group.

**3.3.2 | Mitochondrial ROS formation**

The results of ROS generation test represented that the mitochondrial ROS levels, isolated from the whole brain, frontal cortex (P < 0.001), hippocampus and cerebellum (P < 0.0001) significantly increased by STZ injection in comparison to the sham group.

Additionally, the ROS level in the whole brain, frontal cortex, hippocampus and cerebellum regions was significantly reduced by all doses of *A. jesdianum* than the STZ group. *A jesdianum*, at dose of 100 mg/kg, significantly reduced the ROS level in the whole brain, frontal cortex, hippocampus and cerebellum regions compared to the STZ group (P< 0.05).

The ROS formation notably decreased by 200 and 400 mg/kg doses of *A. jesdianum* treatment in the mitochondria isolated from the whole brain (P< 0.01), cerebellum (P< 0.05 and P < 0.01 respectively), hippocampus (P< 0.01and P < 0.001 respectively) and frontal cortex (P < 0.001). Treatment with donepezil tended to bring the ROS level towards normal values in the whole brain, frontal cortex (P< 0.001) and hippocampus (P < 0.01) regions compared to STZ-treated animals, while it was found non-effec­tive in the cerebellum (Fig. 4).





**Figure 4:** The mitochondrial ROS levels, isolated from the whole brain (A) hippocampus (B) frontal cortex (C) and cerebellum (D) of STZ-induced AD rats treated with *A. jesdianum* and donepezil*.* One‑way ANOVA estimated differences. Mean ± SEM (n = 8) are the forms of data that presented; \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001 compared to the STZ group. ### P<0.001, #### P<0.0001 compared to the sham group.

**3.3.3 | Mitochondrial membrane potential**

We found the STZ groups reported significantly less MMP than the sham group (whole brain and frontal cortex, P < 0.0001; hippocampus and cerebellum, P < 0.001). A signifi­cant inhibition of MMP collapse, induced by STZ, is apparentin the mitochondria isolated from the whole brain, frontal cortex (P < 0.01); hippocampus and cerebellum (P < 0.05) in 200 mg/kg *A. jesdianum* treatment comparing the STZ-treated group. Moreover, high dose of *A. jesdianum* (400 mg/kg) was able to increase MMP in all the three brain regions and the whole brain (whole brain and hippocampus, P < 0.01; frontal cortex and cerebellum, P < 0.05). Analogous with pre­vious findings, donepezil is showing promising results to restore mitochondrial membrane damage in the whole brain, frontal cortex and hippocampus (P<0.001), while in the cerebellum region, it showed a non-significant effect (Fig. 5).





**Figure 5:** Mitochondrial membrane potential (MMP) collapse rate, isolated from the whole brain (A), hippocampus (B), frontal cortex (C) and cerebellum (D) of STZ-induced AD rats treated with *A. jesdianum* and donepezil. One‑way ANOVA estimated differences. Mean ± SEM (n = 8) are the forms of data that presented; \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001 compared to the STZ group. . ###P<0.001, ####P<0.0001 compared to the sham group.

**3.3.4 | Mitochondrial swelling**

In order to evaluate the swelling of mitochondria, absorbance changes at 540 nm were selected, which is a sign of permeability in the membrane of mitochondria. STZ injection to the rats significantly increased mitochondrial swelling versus the sham group (Whole brain, P < 0.0001; all the three regions of the brain, P < 0.001).What stands out in the Figure 6 is that the mitochondrial swelling in the whole brain, frontal cortex and cerebellum locales in the group receiving200 mg/kg *A. jesdianum* was significantly reduced compared to the sham group but failed to have any effect in hippocampus region (whole brain, P < 0.01, frontal cortex and cerebellum, P < 0.05), as the post hoc examination uncovered 400 mg/kg *A. jesdianum* in STZ-treated animals, significantly decreased the swelling in the whole brain and different parts of the brain compared to the STZ group (whole brain, frontal cortex and cerebellum, (P < 0.01); hippocampus, P < 0.05). These results indicate that donepezil ameliorated the mitochondrial swelling in the whole brain (P < 0.001), frontal cortex and hippocampus (P < 0.01). However, donepezil showed an insignificant effect in cerebellum comparing to the STZ group.





**Figure 6:**  The mitochondrial swelling level, isolated from the whole brain (A) hippocampus (B) frontal cortex (C) and cerebellum (D) of STZ-induced AD rats treated with *A. jesdianum* and donepezil. One‑way ANOVA estimated differences. Mean ± SEM (n = 8) are the forms of data that presented; \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001 compared to the STZ group. ###P<0.001, ####P<0.0001 compared to the sham group.

**3.4 | Cytochrome c release**

As shown in Figure 7, the icv injection of STZ significantly induced the release of cytochrome c in the mitochondria isolated from the whole brain, frontal cortex, cerebellum (P < 0.0001) and hippocampus (P < 0.001) compared to the sham group. Whereas, cytochrome c release was significantly inhibited during 200 mg/kg *A. jesdianum* treatment in whole brain, frontal cortex (P < 0.01) and cerebellum (P < 0.05). High dose (400 mg/kg) of *A. jesdianum*, as an MPT inhibitor,reduced STZ-induced cytochrome c release comparing to the STZ group in whole brain, hippocampus, frontal cortex and cerebellum (P < 0.01). We found that donepezil significantly inhibited cytochrome c release comparing to the STZ group in the whole brain, frontal cortex and hippocampus (P < 0.001); however, no significant difference in the level of cytochrome c in the cerebellum between the two groupswas evident.





**Figure 7:** Mitochondrial cytochrome c release rates, isolated from the whole brain (A), hippocampus (B), frontal cortex (C) and cerebellum (D) of STZ-induced AD rats treated with *A. jesdianum* and donepezil. One‑way ANOVA estimated differences. Mean ± SEM (n = 8) are the forms of data that presented; \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001 compared to the STZ group. ###P<0.001, ####P<0.0001 compared to the sham group.

**3.5 | Determination of miRNAs, Bax and Bcl-2 level**

Isolated neurons from the rat brain showed significantly lower expression of miR-132 and miR-330 after STZ treatment compared to the sham group (P < 0.01, P < 0.05, respectively). Meanwhile, these miRNAs expression was significantly increased after treatment with higher doses of *A. jesdianum* and donepezil (Figure 8).



**Figure 8:** MiR-132 (A) and miR-330 (B) relative expression in the neurons isolated from the brain that treated with *A. Jesdianum* and donepezilusing qRT-PCR. One‑way ANOVA estimated differences. Mean ± SD (n = 3) are the forms of data that presented; \*\*\*P<0.001 and \*\*\*\*P<0.0001 compared to the STZ group. #P<0.05, ##P<0.01 compared to the sham group.

Comparisons with the Sham group demonstrated a significant reduction of the anti-apoptotic Bcl2 gene in the STZ group (p<0.001) (Figure 9A).Additionally, the *A. jesdianum* supplementations (200 mg/kg and 400 mg/kg) and donepezil significantly increased the expression of this gene comparing to the STZ group (P<0.001, P<0.001 and P<0.0001, respectively). According to Figure 9B, the real-time PCR data demonstrated a significant increase expression of Bax gene in the STZ group as compared with the sham group (p<0.0001). It was also revealed that 200 mg/kg and 400 mg/kg doses of *A. jesdianum* (P<0.0001) and donepezil (P<0.001) significantly reduced the overexpression of the Bax compared to the STZ group.



**Figure 9:** Relative Bcl-2 (A) and Bax (B) genes expression in the neurons isolated from the brain that treated with *A. Jesdianum* and donepezilusing qRT-PCR. One‑way ANOVA estimated differences. Mean ± SD (n = 3) are the forms of data that presented; \*\*\*P<0.001 and \*\*\*\*P<0.0001 compared to the STZ group. ###P<0.001, ####P<0.0001 compared to the sham group.

**4 | Discussion**

Spices and medicinal herbs with antioxidants and anticholinesterase have gained a lot of attention as potential treatments for neurodegenerative disorders such as AD(41). The antioxidant properties and therapeutic effects of *Allium. jesdianum* against various diseases have already been widely studied (18, 19) and According to previous studies, the protective effects of some members of the Allium genus have been proven in AD (41, 42). However, no data exist on the effectiveness of *A. jesdianum* in AD. Considering the complexity of the disease, we assessed the effects of *A. jesdianum* hydroalcoholic extract on a few obsessive parts of AD, including learning and spatial memory, mitochondrial parameters and oxidative stress in the whole brain and three brain regions (hippocampus, frontal cortex, cerebellum) and expression changes of miR-330, miR-132, Bax and Bcl-2 genes in brain neurons isolated from the rat model of AD.

The sub-diabetogenic dose of STZ injected i.c.v. at a dose of 3 mg/kg provides a comparable model for the sporadic type of AD, which affects more than 90% of AD patients globally (43). It is reported that i.c.v.-STZ induces insulin resistance in the brain, brain glucose metabolism reduction, oxidative stress, cholinergic dysfunction, gliosis, learning and memory problems and accumulation of tau and Aβ proteins (44).

In accordance with earlier research, this study also found that STZ caused oxidative damage and cognitive impairment. To evaluate STZ-induced impairments in learning and memory, the RAWM assay, a reliable behavioral model in rats, was utilized in this study (45). Similar to earlier results, STZ administration during the first two days of the training trial dramatically enhanced both the latency to locate platform and the frequency of reference memory errors (46, 47). Confirmation of STZ-induced cognitive decline was identified when the STZ groups, compared to the sham group, showed a marked increase in the latency to locate the platform, a significant decrease of the duration in target arms and the number of entries to the target arm in the probe test. While the oral administration of *A. jesdianum* extract had positive effects on enhancement learning ability and memory consolidation in the animals. In other words, *A. jesdianum* dramatically restored the STZ-induced impairment in learning and memory.

Many studies have shown that plants with antioxidants and mitochondria-targeting effects, including *Allium sativum, Allium cepa* and *Allium hirtifolium*, have potential impact on neurodegenerative diseases, including AD (4, 41, 42). As previously indicated, dysfunction of mitochondria and oxidative stress are known as central AD mechanisms (2). It has been demonstrated that i.c.v.-STZ induces mitochondrial dysfunction and oxidative stress through an increase in MDA and a decrease in glutathione levels (3, 48).

Farbood et al. showed that STZ exposure increased the production of reactive oxygen species (ROS) in the isolated mitochondria of the rat brain (49).

Similarly, our results indicated that STZ significantly increases the amount of ROS in the whole brain, hippocampus, frontal cortex and cerebellum and creates oxidative stress, indicating the role of ROS as one of the pathological factors of memory impairment. *A. jesdianum* extract at all three doses (100, 200 and 400 mg/kg) significantly decreased ROS production comparing to the STZ group and indicates the antioxidant properties of the extract. Sohrabinezhad et al. showed that the *A. jesdianum* extract improves oxidant/antioxidant balance in hepatic tissue in APAP-induced hepatic failure (16).

Researchers have found that complexes II, III, and IV in the mitochondria of the brains of AD models are directly inhibited by free radicals like ONOO- and NO (4). Consistent with previous research, STZ administration significantly decreased mitochondrial complex II enzymatic activities and mitochondrial viability. Medium and high doses of *A. jesdianum* in the whole brain, frontal cortex and cerebellum and High dose of *A. jesdianum* (400 mg/kg) in the hippocampus significantly increased the survival rate compared to the STZ group.

I.c.v.-STZ injection induces numerous bioenergetic defects in the rats' hippocampus and cerebral cortex. These defects include a decrease in oxygen uptake, complex respiratory activities, respiratory control ratio, ATP synthesis, and MMP (50).

Increase in ROS production leads to the MMP collapse, the mitochondrial membrane integrity disruption, and ultimately mitochondrial swelling (34). In this regard, we found MMP decline and mitochondrial swelling in the whole brain, hippocampus, frontal cortex and cerebellum after treatment with STZ. Our results indicated a significant reduction of STZ-induced mitochondrial swelling and MMP collapse after treatment with *A. jesdianum.*

Cytochrome c is a heme-containing protein in mitochondria's inner membrane space that is encoded by nuclear DNA. The cytochrome C released level is one of the most key determinants of mitochondrial dysfunction and cell apoptosis (51). A previous report demonstrated that i.c.v.-STZ led to a substantial rise in the levels of the amyloid beta peptide 1-42 (Aβ1-42) and the release of cytochrome c in cerebellar, prefrontal, and hippocampal neurons (52). Altogether, our results demonstrated that STZ was accountable for the considerable removal of cytochrome c from mitochondria. A treatment with *A. jesdianum* extract was capable of preventing the STZ-induced cytochrome c release from isolated mitochondria in the whole brain, hippocampus, frontal cortex, and cerebellum. This lends credence to the hypothesis that STZ induces apoptosis via mitochondrial permeability transition pore (MPTP) opening and oxidative stress. Our results indicated that, for hippocampus, only the highest dose of *A. jesdianum* (400 mg/kg) was effective fordegradation of STZ-induced cytochrome c release; however, the lower dose of *A. jesdianum* were also effective for the whole brain, frontal cortex and cerebellum. Therefore, we can conclude that the rate of degradation by STZ is higher in the hippocampus.

In the current investigation, donepezil, an AChE inhibitor, was utilized as a positive control group. According to Saxena et al., both tacrine and donepezil, in addition to inhibiting AChE, may also reduce oxidative stress and produce a considerable improvement in cognitive impairment caused by i.c.v.-STZ (53). In mitochondria isolated from the brains of APP/PS1 transgenic rats deficient in the AChE enzyme, donepezil improves mitochondrial swelling and the decreases the ATP produced by Aβ1-42. It shows that in addition to inhibiting the AChE enzyme, donepezil may be involved in another non-cholinergic mechanism as a neuroprotective to alleviate mitochondria-related disorders (54).

We found that donepezil significantly attenuated STZ-induced increases in ROS generation, mitochondrial swelling, MMP collapse, and cytochrome c release in the whole brain, hippocampus, and frontal cortex, it had no such effect in the cerebellum. Donepezil is expected to be most effective on cholinergic neurons that are located in the basal nuclei of the forebrain (including the nuclei of the middle septum and Broca's area in the frontal lobe that extends to the hippocampus and the basalis nucleus of Meinert) and brainstem. Also, small amount of the cholinergic neurons are located in the cerebral cortex and olfactory bulb (55). Our findings support previous research that the cerebellum contains relatively low density cholinergic markers (56), so it could be the reason for the weak effect of donepezil on the cerebellum in our study.

Various studies have reported that, in AD, miRNAs influence levels of amyloid beta (Aβ), phosphorylated tau, synaptic damage and also biological processes including affect cellular senescence, neuroinflammation and dysfunction of mitochondria (8). For instance, overexpression of miR-330 in AD decreases Aβ aggregation, oxidative stress, and mitochondrial dysfunction via targeting VAV1 gene through the MAPK signaling pathway (57). Our results indicated that the expression level of miR-330 in STZ-induced AD rats is decreased significantly, and higher doses of *A. jesdianum* increases the expression of this miRNA which has beneficial properties for improving AD.

The STZ and *A. jesdianum* effects on miR-132 expression were evaluated in the current research. It has been shown in an AD mouse model that miR-132-3p regulates histone deacetylase 3 (HDAC3), resulting in neuroprotection. Moreover, in the hippocampus, upregulation of miR-132 reduces spatial memory impairment in the  Morris Water Maze **(**MWM) test, Aβ1-42 deposition and apoptosis (58). Our results indicated a significant decrease of miR-132 in the AD group, and the higher doses of *A. jesdianum* significantly increased the expression of this miRNA.

Previous studies have shown that upregulation of Bcl-2 results in reduction of the cleavage of APP, tau protein and the amount of extracellular Aβ deposits (59). Lannert et al. reported Bcl-2 protein expression was decreased in rats that received STZ intraventricularly (60). Our results demonstrated that STZ significantly decreased Bcl-2 gene expression and *A. jesdianum* extract was able to increase Bcl-2 expression, increasing neuronal survival, and preventing programmed cell death.

Moreover, our study demonstrated that the expression of the Bax, as a proapoptotic gene, increased after STZ treatment, and *A. jesdianum* significantly reduced the expression of this gene and prevented the apoptosis of neurons due to STZ injection. In line with our results, Baek et al demonstrated that, in the hippocampus, STZ results in reduction of Bcl-2 expression and increment of Bax and caspase-3 expression, which leads to increase of Bax to Bcl-2 ratio (61).

In conclusion, this study revealed that *A. jesdianum* may prevent STZ-indiced cognitive dysfunction in the animal models of Alzheimer's disease through free radical scavenging activity, improvement of mitochondrial function and overexpression of miRNAs as effective agents for treatment of AD. These findings offer new opportunities for drug development against AD. In the future studies it is recommended that to analyze and assess the effect of the extract on levels of beta-amyloids in the brain of animal models of AD.

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