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Antioxidant properties of *Trifolium resupinatum* and its therapeutic potential for Alzheimer's disease

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Abstract

Introduction: *Alzheimer's disease (AD) is the most common cause of dementia and is characterized by a progressive deterioration in cognitive function, which typically begins with impairment in memory. Persian clover (Trifolium resupinatum) is an annual plant found in central Asia. Due to its contents (high flavonoid and isoflavones), extensive researches have been done on its therapeutic properties, such as multiple sclerosis (MS) treatment. In this study, we investigate the neuroprotective effects of this plant on Streptozotocin (STZ)-induced AD in rats.*

Material and methods: *This research aimed to evaluate the neuroprotective effect of Trifolium resupinatum on the spatial learning and memory, superoxide dismutase (SOD), expressions of β amyloid 1-42 ($A\beta_{1-42}$), and β amyloid 1-40 ($A\beta_{1-40}$) in the hippocampus of STZ-induced Alzheimer rats.*

Results: *Our data showed that Trifolium resupinatum extract administration for two weeks before and one week after AD induction significantly improves maze escape latency (p = 0.027, 0.001 and 0.02 in 100, 200, and 300 mg of the extract, respectively) and maze retention time (p = 0.003, 0.04 and 0.001 in 100, 200, and 300 mg of the extract, respectively). Also, the administration of this extract significantly increases the SOD levels from 1.72+0.20 to 2.31+0.45 (p = 0.009), 2.48+0.32 (p = 0.001) and 2.33+0.32 (p = 0.007) and decreases the expressions of $A\beta_{1-42}$) (p = 0.001 in all concentrations of the extract) and $A\beta_{1-40}$) (p = 0.001 in all concentrations of the extract) in the rat's hippocampus.*

Conclusions: *This study suggests that the alcoholic extract of Trifolium resupinatum has anti-Alzheimer and neuroprotective effects on rats.*

Key words: *Alzheimer's disease, Trifolium resupinatum, spatial memory, amyloid peptide, SOD.*

Introduction

Alzheimer's disease (AD) is an age-related progressive neurodegenerative ailment responsible for 60% of dementia cases. Six million patients are diagnosed with AD each year, most in less developed or developing countries [27]. Despite many advances in managing symptoms and delaying the onset of symptoms, no effective cure has been discovered [11]. Recently, numerous studies have shown cellular and experimental evidence supporting oxidative stress's impact on AD pathogenesis [7,21,33]. Molecular studies on AD patients' neurons demonstrate the abnormally high amounts of oxidatively modified compounds (such as proteins, lipids, and DNA); which cause molecular damage and lead to the formation of senile plaques and neurofibrillary tangles. Oxidative damage due to reactive oxygen species (ROS) such as amyloid β-protein (Aβ) has been implicated in the pathogenesis of AD and other neurodegenerative diseases. Usually, ROS are scavenged by enzymatic and non-enzymatic antioxidants. Presumably, the imbalance in ROS-antioxidant status could lead to cell damage [8].

The golden standard test for AD diagnosis is the presence of intracellular neurofibrillary tangles and extracellular senile plaques in the biopsy. The amyloid hypothesis suggests that the formation of Aβ, a peptide that varies in size from 36-43 amino acids, in the

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senile plaques of AD patients' brain is crucial in the pathogenesis of AD [21]. Many current therapeutic strategies are based on this hypothesis, such as γ, β, or α secretase inhibitors [17].

Although there is no approved treatment for AD, some drugs such as Tacrine, Donepezil, Rivastigmine, Galanthamine and Memantine have been used in treating AD. In the absence of definitive treatments for this neurodegenerative disease, herbal and experimental therapies are widely used. Among these, the most promising treatments are those with an anti-inflammatory effects which reduce inflammation of the hippocampal neurons and thus slow the progression of the disease. This effect has been seen in the administration of Radix Ginseng, Radix Scutellariae and Scutellaria Baicalensis Georgi, which has been shown to counteract AD in several studies [18,43,44]. Phytochemical studies performed on all the mentioned plants show very high levels of flavonoids in these plants. Studies show that flavonoids could have a therapeutic effect in AD patients due to their antioxidant effects [1]. Accordingly, numerous other plants with rich flavonoid components have been studied for anti-Alzheimer effects, such as *satureja cuneifolia*, *Centella asiatica*, and *Trifolium pratense L.* [24,36,40].

According to phytochemical studies, *Trifolium resupinatum* (Persian clover) has rich flavonoid compounds. Persian clover is an annual plant that nowadays grows all over the world [28]. Phytochemical investigations on the alcoholic extract of this plant have confirmed the existence of at least seven types of flavonoids [16]. The flavonoids of this plant are also likely to have antioxidant effects because of their radical scavenging activity [16]. Studies have claimed that this plant has hepatoprotective activity and reduces oxidative stress. Congener plants of *Trifolium resupinatum*, including red clover (*Trifolium pratense L.*) and their flavonoids, have shown good antioxidant and neuroprotective effects (especially on cholinergic neurons) [25].

Studies suggest different methods for AD inducing in animal models, but most of them demonstrate that an intraventricular injection of Streptozotocin (STZ) decreases cerebral glucose uptake and produces multiple other effects that modulate the molecular, pathological, and behavioural features of AD [12]. STZ is harvested by low-affinity glucose transporter 2 (GLUT 2) located in the neurons' membrane and induces cellular necrosis by DNA alkylation [31].

Despite various phytochemical studies on *Keywords*, this plant's preventative and restorative effects are almost unknown. For the first time, this study aimed to investigate the therapeutic effects of *Trifolium resupinatum* extract on AD induced by STZ in an animal model; and evaluate the spatial memory, antioxidant, and protein factors in them.

Material and methods

Identifying the plant and preparing the extract

Persian clover (*Trifolium resupinatum*) was identified and collected by the Medicinal Plants Institute of Karaj Academic Center for Education, Culture, and Research. The plant was cut and dried in the shade (22°C), and the plant's aerial parts were separated. Then, it was powdered by a mill and used for the following stages. The percolation method and ethanol solvent (50%) were used for extraction [32]. The crushed plant material was dampened with 30% of the mentioned solvent before entering the percolator, and the resulting mass was left by itself for 24 hours. The moistened plant material was then inserted into the percolator through special sieves in a uniform fashion. Thus, the cellular walls were prepared to accept the extracting solvent after the initial soaking. The surface of the wet plants was covered with cotton, and by placing a few glass cylinders on it, the movement of plant particles was prevented. Then, the rest of the solvent was gradually added to the plant mass so that the solvent evenly penetrated throughout the plant mass. The percolation process was terminated when the material removed from the device was free of any isoflavonoids. The extracted liquid result was dried using a freeze dryer.

Ultra-performance liquid chromatography

The phenolic fraction of *Trifolium resupinatum* extract was isolated with Stochmal's method [35]. Briefly, ground aerial parts of the plant material were extracted with 80% (v/v) ethanol at room temperature for 24 h. After filtration, the extract was concentrated at 35°C under reduced pressure. The crude extract was dissolved in distilled water and separated on an RP18 preparative column (60 × 100 mm, 40-60 µm, Merck). First, the column was washed with water to remove carbohydrates, and then the phenolic fraction was eluted with 40% (v/v) ethanol.

Qualitative and quantitative analyses of the compounds in this fraction were done by using ultra-performance liquid chromatography (UPLC) (solvent, 1% (v/v) acetic acid → 40% (v/v) acetonitrile over 10 min; column C18 50.0 × 2.1 mm, UPLC BEH; column temperature 50°C; flow rate 0.35 ml/min).

DPPH free radical scavenging assay

The DPPH method was used to determine each sample's free radical scavenging activity [19]. We mixed 1.4 ml DPPH (2.2-diphenyl-1-picrylhydrazyl) solution (0.0062 g/100 ml MeOH) with 0.2 ml of the

plant extract dissolved in water. The reaction mixture was shaken and incubated in the dark at room temperature for 30 min. Absorbance (A) was measured at 536 nm against the blank (UV/VIS Perkin-Elmer Lambda 35 spectrophotometer). Controls were prepared for the test group except that the antioxidant solution was replaced with the corresponding extraction solvent. Inhibition of the DPPH radical by the sample was calculated according to the following formula:

DPPH scavenging activity (%) = [A0 – A1/A1] × 100,

where A0 is the absorbance of the control and A1 is the absorbance of the sample. Free radical scavenging activity is expressed as the percentage of DPPH decrease.

The half maximal inhibitory concentration (IC_{50}) value, the amount of antioxidant necessary to halve the initial DPPH concentration, was calculated from the results and used to compare the antioxidant quality of the extract with standard solutions. Vitamin C (5-100 µg/ml) and BHA (butylated hydroxyanisole) (5-5000 µg/ml) were used as standards.

Animals

Adult male Wistar rats (180-200 g with eight weeks of age) were purchased from the Pasteur Research and Production Institute of Iran in Karaj, Alborz Province, and were housed in groups of a maximum of five in standard metal cages at 12°C with a light-dark cycle of 12 hours, and all animals had full access to water and food.

All rats were sacrificed after completing the project, following ethical points approved by the Ethics Committee of Arak University of Medical Sciences, and all efforts were made to minimize their pain and suffering.

Dose adjustment

The acute toxicity dose (LD_{50}) was measured by Lorke's method. In the first phase, nine Wistar male rats were randomly divided into three groups (three animals in each group). 10, 100, and 1000 mg/kg of *Trifolium resupinatum* extract were administered to each group. The rats were returned to their cages and were placed under observation for 24 hours. In the second phase, three animals were administered 1600, 2900, and 5000 mg/kg of the extract and were observed for 24 hours. At last, the LD_{50} was calculated by the formula:

$$LD_{50} = \sqrt{(D_0 \times D_{100})}$$

D_0 = highest dose that gave no mortality,
D_{100} = lowest dose that produced mortality [29].

No behaviour change or death was seen in the first phase or at 1600 mg/kg, but the rats administered by 2900 and 5000 mg/kg of the extract died in 24 hours. According to Lorke's formula, the LD_{50} was estimated at 2150 mg/kg. According to LD_{50} and similar experiments on this herb [16], three doses were selected for the experiment as 100, 200, and 300 mg/kg.

Experiment protocol

Forty-two rats were randomly placed in 6 random groups (n = 7). Control group: no treatment or surgery

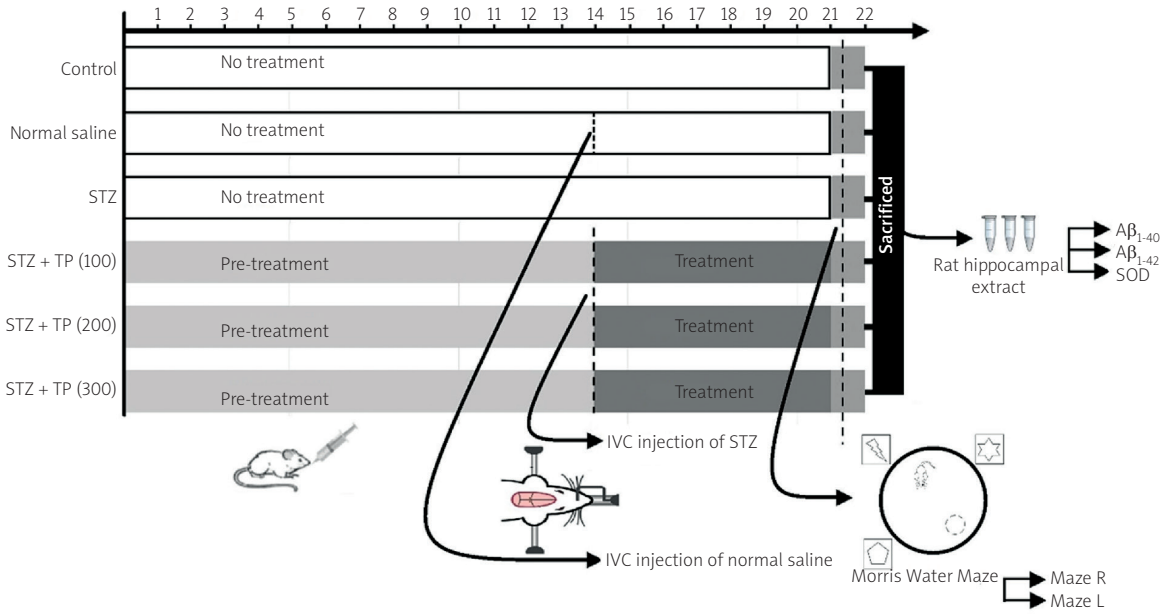

Fig. 1. Experiment protocol.

Fig. 2. Intraventricular injection. Animals' heads were fixed in a stereotaxic brain surgery device; after a long incision in the posterior part of the head, the skull became visible. Skull drilled in frontal-anterior –0.8 mm; internal-lateral ±1.8 mm relative to bregma and dorsal-ventral –3.6 mm relative to the dura. Then the injection cannula was slowly inserted into the ventricles.

was done on the rats of this group. Normal saline: rats in this group received normal saline following intraventricular injection surgery. STZ group: AD was induced in animals by intraventricular injection of STZ. STZ + therapeutic (TP) (100) group: for two weeks before and one week after the induction of AD (by intraventricular injection of Streptozotocin); rats received *Trifolium resupinatum* (100 mg/kg body weight [BW]) extract, in edible form by gavage. STZ + TP (200) group: for two weeks before and one week after the induction of AD (by intraventricular injection of STZ), rats of this group received *Trifolium resupinatum* (200 mg/kg BW) extract in edible form by gavage. STZ + TP (300) group: for two weeks before and one week after the induction of AD (by intraventricular injection of STZ) rats received *Trifolium resupinatum* (300 mg/kg BW) extract, in edible form by gavage (Fig. 1).

Intraventricular injection of Streptozotocin

Animals were anesthetized by intraperitoneal injection of Sodium pentobarbital at a dose of 60 mg/kg BW. Then, their heads were fixed in a stereotaxic brain surgery device after disinfection of the area; the skull became visible with a long incision in the posterior part of the head. After specifying stereotaxic coordinates according to Paxinos atlas and pilot studies for lateral cerebral ventricles (frontal-anterior –0.8 mm; internal-lateral ±1.8 mm relative to bregma and dorsal-ventral –3.6 mm relative to dura) with the help of a drill, the skull was perforated. The injection cannula was slowly inserted into the ventricles. In STZ, STZ + TP (100), STZ + TP (200), and STZ + TP (300) groups; using Hamilton syringes, 5 µl of Streptozotocin (1.5 mg/kg BW) was injected. Also, in the normal saline group, each rat received 5 µl of normal intraventricular saline (Fig. 2).

Morris water maze test

The water maze consisted of a metal pond (170 cm in diameter and 58 m high) that was filled up to 40 cm with 22°C water and opaque with non-toxic materials and divided into four different parts (SW, NE, NW, and SE) by two hypothetical lines passing through the centre of the pond. In the centre of the NW quarter, there was a movable platform with a diameter of 10 cm, placed 1 cm below the cloudy water surface. The position of the platform was the same for each rat during the training period. Several symbols were placed around the pond so that the rats would remember the platform location (none of the signs were directly referring to the platform location). Each animal was gently and randomly placed at the edge of one section into the pond, and then the animal was allowed to swim in the pond, find the platform, and climb up. During the animal training, if they did not find the platform within 60 seconds, the examiner placed the animal on the platform and remained there for 15 seconds, then removed it from the pond. The duration of climbing the platform by each animal was considered as the Escape latency

(Maze L), and the rat's swimming time around the previous platform location was considered as Retention time (Maze R) [41]. All animals were quickly dried with towels after each experiment and then returned to the cage. All experiments were performed at least 45 min after the gavage of *Trifolium resupinatum* extract.

Rat hippocampal extract preparation

The animals were eliminated by the Ethics Committee of the Arak University of Medical Sciences approved procedures, their brains were removed, and their hippocampus was separated and weighed. Phosphate-buffered saline (PBS; pH = 7.4) was poured over the tissue samples, and they were immediately frozen with a liquid nitrogen tank. The samples were then thawed to reach 5°C, then more PBS was added and homogenized by the homogenizer and then centrifuged at 2500 RPM (round per minute) for 20 minutes, and the supernatant was carefully collected.

Measurement of β-amyloid 1-40 and 1-42 levels

The hippocampal extract, according to the instruction of Rat Amyloid Beta Peptide 1-42 ELISA Kit (Laboratory BT, Rat Amyloid Beta Peptide 1-42 ELISA Kit) and Rat Amyloid Beta Peptide 1-40 ELISA Kit (Laboratory BT, Rat Amyloid Beta Peptide 1-40 ELISA Kit) from Bioassay Technology Laboratory, for 60 minutes at 37°C, reacted with the standard solution and ELISA solution. Then the plate was washed five times, and chromogen solutions A and B were added to the solution. Afterwards, the plate was placed in a 37°C incubator for 10 minutes. Finally, the stop solution was added, and immediately, the optical density (OD) level was measured with an ELISA reader (420 nm), and the protein level was calculated based on the related graph.

SOD activity measurement

Superoxide dismutase (SOD) activity level measurement was done according to the instruction of Super Oxide Dismutase activity Assay Kit of ZellBio (Germany, Super Oxide Dismutase Assay kit (96/48 Tests). In summary, 10 µl of the tissue extract was mixed adequately with Diluted R1 (250 µl), R2 (10 µl), dd-Water (10 µl) and Chromogen (20 µl) for measured sample and dd-Water (20 µl) for the blank sample and at 0 min and 2 min was placed in ELISA reader (420 nm). The SOD activity level was measured with the corresponding formula.

Statistical analysis

All the data are reported in the form of mean ±SD. The differences between other groups are measured by ANOVA and later on with the Duncan test, and *p*-value under 0.05 is considered significant.

Results

Phytochemical analysis

By chemical analysis of this extract by ultra-performance liquid chromatography, the presence of four groups of phenolic substances was determined (Table I). These data showed that the ethanolic extract of Trifolium resupinatum was particularly rich in flavonoids (21.30 mg/g d.m.).

The antioxidant activity of the Persian clover extract was assessed by spectrophotometry of the presence of the DPPH radical. DPPH is a stable free radical which dissolves in methanol and shows characteristic absorption at 536 nm. When an antioxidant scavenges free radicals by hydrogen donation, the DPPH assay solution becomes lighter in colour. The sample was analysed compared to vitamin C and BHA in the same conditions.

The quality of the antioxidants in the extracts was determined by the IC_{50} values, denoting the concentration of the sample required to scavenge 50% of the DPPH free radicals (Table I).

Effects of the extract on the study of spatial learning and memory

The performance of rats in the Morris water maze demonstrates that induction of AD decreases Maze R and increases Maze E significantly ($p > 0.05$). Also, the effects of extract's different doses on rat's water maze performance were observed (Fig. 3).

Effects of *Trifolium resupinatum* extract on Aβ concentration

As Table II shows, induction of AD significantly increases the concentration of $A\beta_{1-42}$, and $A\beta_{1-40}$ in the STZ group, compared to the control group ($p < 0.05$). Our data showed that *Trifolium resupinatum* extract

Table I. Phytochemical analysis of *Trifolium resupinatum* extract

Active components	Content
Flavonoids (mg/g d.m.)	21.30
Isoflavones (mg/g d.m.)	17.97
Phenolic acids (mg/g d.m.)	1.55
Clovamides (mg/g d.m.)	9.73
Total phenolic content (mg/g d.m.)	50.55
IC50 (µg/ml)	87.56 ±0.003
Ascorbic acid (µg/ml)	5.676 ±0.001

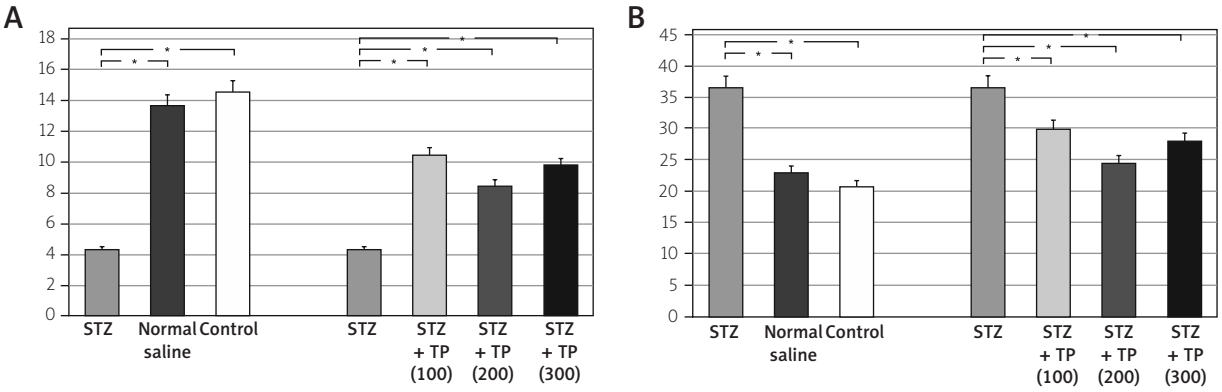

Fig. 3. The performance of rats in the Morris water maze got worse by induction of Alzheimer's disease (AD), and improves after Trifolium resupinatum extract consumption. Maze R indicates the rat's swimming time around the previous platform location which significantly decreased by intraventricular injection of STZ (**A**). Maze L is the duration of climbing the platform by each animal. The higher numbers indicate worse spatial memory (**B**). Bars represent mean ±SD and *p*-value < 0.05 is considered significant (*n* = 7 for each group).

Table II. Effect of intraventricular injection of Streptozotocin

	Control group (*n* = 7)	Normal saline (*n* = 7)	STZ group (*n* = 7)
SOD	2.79 ±0.17	2.71 ±0.19	1.72 ±0.20
p-value		0.98	0.001
1-40 β	51.1 ±4.5	52.1 ±6.1	76.2 ±4.2
p-value		0.99	0.001
1-42 β	51.33 ±3.5	54.66 ±4.17	102 ±5.17
p-value		0.23	0.001
Maze R	14.52 ±2.01	13.68 ±1.03	4.28 ±2.9
p-value		0.345	0.001
Maze E	20.64 ±1.48	22.85 ±2.41	36.52 ±5.02
p-value		0.06	0.001

could significantly decrease $A\beta_{1\text{-}42}$ and $A\beta_{1\text{-}40}$ in all doses ($p < 0.05$) (Fig. 4). However, there was no significant difference between the doses used in the experiment ($p > 0.05$).

Effects of *Trifolium resupinatum* extract on antioxidant markers

Induction of AD with an intraventricular injection of STZ significantly decreases the level of antioxidant markers (SOD activity) in the STZ group (17.2 ±0.02) compared to the control group (2.79 ±0.17) ($p < 0.05$). The effects of receiving the *Trifolium resupinatum* extract on the SOD level are shown in Table III. Receiving this extract reduced the level of antioxidant markers significantly ($p < 0.05$).

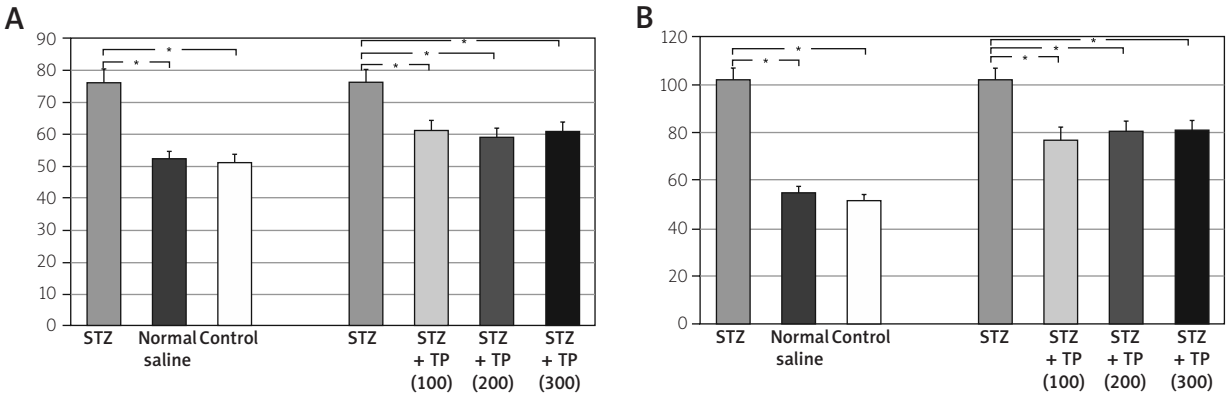

Fig. 4. Effect of intraventricular injection of STZ and treatment with *Trifolium resupinatum* on $A\beta_{1\text{-}40}$ (**A**), and $A\beta_{1\text{-}42}$ (**B**). The peptide level was measured by ELISA Kits in Control, Normal Saline, STZ, STZ + TP (100), STZ + TP (200), and STZ + TP (300) groups. Bars represent mean ±SD and *p*-value < 0.05 is considered significant (*n* = 7 for each group).

Table III. Effect of different doses of Trifolium resupinatum extract

	STZ (*n* = 7)	STZ + TP (100) (*n* = 7)	STZ + TP (200) (*n* = 7)	STZ + TP (300) (*n* = 7)
SOD	1.72 ±0.20	2.31 ±0.45	2.48 ±0.32	2.33 ±0.32
p-value		0.009	0.001	0.007
1-40 β	76.2 ±4.4	61.2 ±4.2	59.0 ±3.6	60.7 ±3.7
p-value		0.001	0.001	0.001
1-42 β	102 ±5.17	76.66 ±3.07	80.5 ±3.08	81 ±2.75
p-value		0.001	0.001	0.001
Maze R	4.28 ±2.9	10.4 ±1.35	8.44 ±4.01	9.77 ±1.86
p-value		0.003	0.04	0.001
Maze E	36.5 ±5.02	29.8 ±5.04	24.38 ±4.48	27.84 ±6.98
p-value		0.027	0.001	0.02

Discussion

Oxidative stress plays a crucial role in neurodegenerative diseases such as AD [6]. The imbalance between production and purging oxidative products, such as ROS, may lead to aggregation of Aβ that could cause intracellular neurofibrillary tangles and extracellular senile plaques (AD pathological hallmarks). Production of ROS may contribute to oxidative damage on both Aβ peptide itself and surrounding molecules (proteins, lipids, ...) [37]. Flavonoids' radical scavenging activity is believed to act through hydrogen atom transfer (HAT), sequential proton loss electron transfer (SPLET), and single-electron transfer followed by proton transfer (SET-PT) mechanisms shown in previous studies [15]. Studies suggest that the presence of flavonoids in different herbs, such as *Centella Asiatica*, and *Desmodium Gangeticum*, could accelerate their anti-Alzheimer effect [23,40]. Phytochemical studies on *Trifolium resupinatum* demonstrate six known flavonoids as: 8-geranyl-4',5,7-trihydroxyflavone; 45, 46, 3'-methoxy-6-prenyl-4',5,7-trihydroxyflavanone; 47,3'-geranyl-4',5,7-trihydroxyflavone; 48,3'- methoxy6,8-diprenyl-3,4',5,7-tetrahydroxyflavone; 49,8-methyl-6-prenyl-3',4',5,7-tetrahydroxyflavanone; and 6,8-diprenyl-3',4',5,7-tetrahydroxyflavanone.

These flavonoids, especially 45, 46, 3'-methoxy-6-prenyl-4',5,7-trihydroxyflavanone, have a tremendous anti-oxidative effect that can slow the aggregation of Aβ. Nevertheless, Kamel *et al*. found a flavonoid that has been found only in this plant, as 3'-geranyl-6-prenyl-2',4',7-trihydroxyflavanone [16]. The scavenging activity of these flavonoids against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals, determined by the method of Brand-Williams *et al*. [4], was significantly higher than others, which can explain the anti-Alzheimer effect of this extract [16].

Neuronal degeneration secondary to AD may lower acetylcholine levels, preventing the brain's normal function and AD symptoms [20]. One of the current therapeutic strategies in AD patients is to maintain ACh by acetylcholinesterase (AChE) inhibitors, such as galantamine, rivastigmine, and donepezil [22]. Studies show that an extract of different herbs such as *Salvia lavandulifolia Vahl*, *Adhatoda Vasica*, and *Peganum Harmala* can inhibit the AChE [39]. Also, herbs like *Achyrocline Tomentosa* (Marcela) (*Asteraceae*) and *Eupatorium Viscidum* (Common boneset) (*Asteraceae*) and *Ruprechtia Apetala* (Manzano del campo) (*Polygonaceae*) and *Trichocline Reptans* (arnica) (*Asteraceae*), and *Zanthoxylum Coco* (cochucho, coco) (*Rutaceae*) have been investigated due to their anticholinesterase properties [20]. In comparison, members of the Trifolium genus, such as *Trifolium angustifolium, have a* remarkable inhibitory effect against acetylcholinesterase enzyme [10]. This result is perhaps due to caffeic acid and chlorogenic acid, higher in *Trifolium resupinatum* than *Trifolium angustifolium*.

Because of the many neurological similarities between Wistar rats and humans, this animal is frequently used in neurological studies [9]. Alzheimer's disease induction is performed by several methods, including intraventricular injection of STZ, D-galactose, and $A\beta_{1\text{-}42}$, but due to the remarkable efficacy of STZ injection, this method has been chosen [30].

Alkaloids are known as toxic components for humans. Considering the alkaloid nature of galantamine, rivastigmine (The Food and Drug Administration [FDA]-approved for AD), and rhynchophylline (third stage of the clinical trial), neuroprotective effects of alkaloids can be seen in appropriate doses [13,45]. These neuroprotective effects indicate the need for further studies on alkaloids. Although there is no approved study on alkaloid properties of *Trifolium resupinatum,* researchers demonstrate high levels of alkaloid in *Trifolium alexandrinum* and *Trifolium pratense L.*

The present study suggests that *Trifolium resupinatum* extract could decrease memory loss and degeneration of hippocampal neurons in an animal model of STZ-induced AD. The data obtained from this study did not show a close relationship between the enhancement of cognitive function and the neuroprotective effect of this extract, suggesting several other mechanisms involved in enhancing cognitive function. For example, studies point to the influence of cerebral blood flow, oxidative stress status, and balanced function of several neurotransmitters, including acetylcholine, serotonin, catecholamine, γ-aminobutyric acid (GABA), and glutamate, in spatial memory [38]. According to the data of the present study and similar studies, the alcoholic extract of this plant may significantly reduce the effects of AD induction by increasing acetylcholine levels, increasing cerebral blood flow, and decreasing oxidative stress.

Due to the linear association between flavonoid and isoflavonoid levels and the anti-Alzheimer effect, higher doses of this extract were expected to be less diminutive than acute toxicity dose (LD_{50}), which might be less diminutive, lead to better anti-Alzheimer outcomes. Still, our study showed that the only amount which could significantly reduce the retention time of the water maze was 200 mg/kg BW. *Trifolium resupinatum* extract contains other compounds, including soyasaponin I, soyasapogenol B, and 3-O-b-D-glucopyranosyl sitosterol. As a result, an increased dose of the extract may conceal the effects of its active ingredient by increasing the other compounds of *Trifolium resupinatum*. This may explain that the results of this extract are not dose-dependent.

The precise mechanism of the *Trifolium resupinatum* anti-Alzheimer effect is out of the scope of this study, but our data suggest that an increased level of superoxide dismutase (SOD) activity had a protective impact on rats' neurons (especially hippocampus). Although some of the anticholinesterase and neuroprotective effects of this extract may be due to its alkaloid properties, further phytochemical researches are necessary for the exact determination of alkaloid levels.

Generally, AD is associated with amyloid plaques formed by Aβ proteins and is found between the neurons and neurofibrillary tangles. Aβ peptides are created by the proteolysis of the β-amyloid precursor protein by the activity of the secretase family enzymes [3,42]; nowadays, inhibiting these enzymes is one of the therapeutic strategies. Most processes related to the neurotoxic effects of β-amyloid are of type 1-42, which may damage synaptic activity [2,5,14,34]. Our findings show that the extract of *Trifolium resupinatum* with its neuroprotective effect significantly reduces the level of β-amyloid peptides in the hippocampus of rats.

Oxidative stress is one of the crucial chains in the pathogenesis of AD, so we evaluate an antioxidant marker (SOD). The toxicity of reactive oxygen species (ROS) is one of the main factors involved in the degeneration of hippocampal neurons [26]. One of the essential antioxidant mechanisms of the body against the attack of reactive oxygen species is the presence and activity of SOD. Phytochemical analysis of this extract showed a deficient IC_{50} level for both. IC_{50} represents a sample concentration that causes 50% inhibition in radical capacity and is obtained by plotting different radical scavenging activity (RSA) values according to different sample concentrations and calculating the regression line equation. Lower IC_{50} values indicate more potent antioxidant properties of the extract. Significant increases in the SOD level in rats and the low level of IC_{50} in this herb may indicate its beneficial practical use in antioxidant therapy for Alzheimer's patients.

However, we should also note that the therapeutic mechanism of *Trifolium resupinatum* extract in treating AD by scavenging oxygen free radicals or improving the activity of related enzymes, or both, remains unclear. The therapeutic effect of this extract was approximately the same in all doses. The mechanism through which *Trifolium resupinatum* extract plays a therapeutic role in treating rats may be dose-independent. Increasing SOD and lowering $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ might be just one mechanism of this extract in treating AD. As we know, the body is not a one-compartment model but rather a multi-compartment model. Therefore, the concentration of observed factors in the blood may or may not reflect their level in the brain. Other factors may influence oxidative stress formation in other tissues, especially muscle. Also, it should be noted that immunohistochemistry has its limitations. Therefore, further experiments should be conducted to uncover more detailed mechanisms. In conclusion, the data of this study demonstrate that *Trifolium resupinatum* extract has a remarkable amount of anti-Alzheimer substances and can have significant effects on the behavioural (maze escape latency and maze retention time) and molecular factors of AD.

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Disclosure

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