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Thymus daenensis extract prevents scopolamine-induced memory impairment through declining oxidative stress in rats

Mahdieh Anoush[1,2], Nasrin Tayebi[2], Soroush Bijani[1,2], Mina Ebrahimi[1,2], Ali Reza Yazdinezhad[3], Mir-Jamal Hosseini[1,2]*

[1] Zanjan Applied Pharmacology Research Center, Zanjan University of Medical Sciences, Zanjan, Iran,
[2] Department of Pharmacology and Toxicology, School of Pharmacy, Zanjan University of Medical Sciences, Zanjan, Iran,
[3] Department of Pharmacognosy, School of Pharmacy, Zanjan University of Medical Sciences, Zanjan, Iran,
** Email: jamal_hossini@yahoo.com; mhosseini@zums.ac.ir*

Memory and cognitive impairment induced by oxidative stress are among the main hallmarks of Alzheimer's disease's (AD) pathology. The present study aimed to investigate the potential neuroprotective effects of *Thymus daenensis (T. daenensis)* extract against scopolamine-induced memory impairment and oxidative stress in rats. *T. daenensis*, widely distributed in Iran and Europe, is known to be a rich source of natural antioxidants and has been traditionally used for various medical purposes. The present study investigated the post-treatment effects of *T. daenensis* on learning and memory functions, antioxidant cellular defense, and oxidative stress using the scopolamine rat model of AD. The experiments were performed by intraperitoneal injection of scopolamine for 10 consecutive days in Wistar male rats (180–220 g). Additionally, the animals received T. *daenensis* extract (50-200 mg/kg) by gavage for 14 consecutive days after induction of memory impairment. The animals were divided into 8 groups, namely: control, 200 mg/kg of *T. daenensis* extract (D200), donepezil (DON), scopolamine (ALZ), ALZ animals treated with different doses of the extract (ALZ+D50 or 100 or 200 mg/kg) and ALZ animals treated with (ALZ+DON). The animals were then subjected to the Morris water maze (MWM) paradigm as a standard criterion for memory function assessment, and after extracting the brain tissues, the related biochemical oxidative stress parameters were determined in the brain. Our results indicated that *T. daenensis* extract significantly improved animals' performance in the MWM while significantly reducing oxidative stress and antioxidant imbalance. Furthermore, the extract did not show hepatotoxic effects on treated animals. In addition, the extract treatment significantly decreased both cellular malondialdehyde (MDA) and protein carbonyl (PCO) content while conversely increasing the total reduced glutathione (GSH) content and also the levels of total and endogenous antioxidants in the ferric reducing antioxidant power (FRAP) assay. It seems that the administration of *T. daenensis* significantly improved both cellular biochemical aspects and memory performance in animal models. Conclusively, it could be beneficial for scopolamine-induced neurotoxicity.

Key words: scopolamine, *Thymus daenensis* extract, oxidative stress, memory impairment, Alzheimer's disease, rat

INTRODUCTION

Alzheimer's disease (AD) is the most common type of dementia with augmenting impairment of cognition and memory that would highly affect the daily lifestyle (Berahmand et al., 2020). Over the past decades, the prevalence of AD has drastically risen to 47 million cases as of 2015, which is predicted to be tripled by 2050 (Mielke et al., 2014). Many of the symptoms share the same indicators as depressive disorders, and no pathological difference has been discovered between different types of AD (Cobos and Rodríguez, 2012). From a pathological point of view, these mechanisms have been determined for AD: beta-amyloid plaques forma-

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tion, intracellular neurofibrillary tangles, and sclerosis of hippocampal tissue, which count as the hallmarks of AD occurrence (Garre-Olmo, 2018). Tau protein and microtubules play a crucial role in maintaining neural structure and axonal transport (Lasagna-Reeves et al., 2015). Augmented kinase and declined phosphatase enzyme levels minimize the aggregation of Tau protein and microtubules (Wang et al., 2015); the result of the aforementioned is cellular death which happens in correlation with beta-amyloid plaques formation (Roberson et al., 2007).

Oxidative stress markers such as reactive oxygen species (ROS) play a critical role in AD. These memory and cognitive impairments induced by scopolamine might have caused alterations in synapses and nerve fibers, neuronal structure, neurotransmitter levels, and oxidative stress, ultimately leading to brain damage (Butterfield and Boyd-Kimball, 2004). Beta-amyloid peptides have suppressive effects on cytochrome C oxidase and Krebs cycle enzymes such as pyruvate dehydrogenase, leading to mitochondrial dysfunction. Besides, the significant reduction in ATP levels results in an accumulation of peroxide and superoxide anions in mitochondria and subsequently a significant rise in free radicals, which facilitates the phosphorylation of Tau protein (Spuch et al., 2012). Beta-amyloid escalation leads to activation of astrocytes and microglia and increased cytokines like IL-1β, IL-8, TNF-α, and MIP-1α (Murgas et al., 2012). IL-1β alone causes high increases of ROS, which stimulates mitogen-activated protein kinase and caspase 3, followed by activation of apoptotic cell death signaling and the neural synapses count dropping (Sheng et al., 2001). In addition, IL-1β has an inhibitory effect on acetylcholine's secretion in the synaptic area, which accelerates AD's cognitive impairments (Fiala et al., 1998).

In many health-threatening disorders, ROS desynchronizes the cellular calcium pathways; as a result, the accumulation of calcium ions activates the glutamate receptor, which increases the chance of cellular apoptosis; this mechanism is significant in the progression of both Parkinson and Alzheimer's disease (Mattson, 2002; Goodwin et al., 2013). The vast majority of medications available to treat AD belong to the acetyl-cholinesterase-inhibitors group, namely donepezil, which improves cognitive and decreases inflammatory cytokines, which may not be directly related to the antioxidant effects of donepezil (Jelic and Darreh-Shori, 2010; Klugman et al., 2012). Additionally, memantine is another widely used medicine in AD complications as an uncompetitive NMDA antagonist, which in advanced stages of AD can be used in combination therapy (Atri et al., 2013; Tan et al., 2014). Previous data confirm that coenzyme Q_{10} and vitamin E have proven effective in ameliorating oxidative stress, resetting cellular energy production, and recovering the endogenous antioxidant levels (Hong et al., 2004; Andalib et al., 2019).

T. daenensis is an aromatic plant from the Lamiaceae family, indigenously growing in Europe, Asia, and North Africa. *T. daenensis's* rich source of thymol and carvacrol are both potent antioxidant compounds (Pirbalouti et al., 2013). Furthermore, limited data suggested that *T. daenensis* extract demonstrated promising results as a free radical scavenger due to its high content of phenolic compounds (Dadashpour et al., 2011; Ghasemi Pirbalouti et al., 2014). Considering the limited data about *T. daenensis* extract in *in vivo* and *in vitro* studies, we planned to study the protective effects of *T. daenensis* extract in AD, specifically by measuring memory performance using the MWM test and evaluating the oxidative stress via biochemical assays in the brain. Finally, to confirm the probable toxicity of the extract, these markers were also measured in the animals' livers.

METHODS

Collection, identification, and extraction of *T. daenensis*

T. daenensis was collected from its natural habitat in Zanjan (Voucher no. ZUMS-1317). First, the aerial parts of the plant samples were powdered by mechanical milling after drying at room temperature. Then, 200 g of the obtained powder was soaked in 400 ml of 70% ethanol at room temperature overnight to distill and extract the active ingredients. Following this, solution evaporation was carried out using the rotary evaporator (IKA-RV05). Lastly, the obtained extract (26.13 g) was stored in sealed dark bottles in the refrigerator for further utilization.

Total phenolic compounds determination

The total phenolic content was determined spectrophotometrically using the Folin–Ciocalteu method with gallic acid solutions (50, 100, 250, 500, 750, and 1000 mg/L), which were used as the standard equivalent, according to our previous study (Kianpour et al., 2021). Briefly, in each 96-well of a multi-well plate, 25 µl of the sample was mixed with 125 µl of Folin–Ciocalteau's reagent (Merck, Germany). After 3 to 5 min, the mixture was neutralized by 100 µl of 7.5% sodium carbonate (w/v) and then left for 90 min at room temperature. The absorbance was measured by a spectrophotometer at 765 nm and compared to a blank sample. The results

were expressed as microgram (µg) of gallic acid equivalents per ml of sample.

Animal treatment

In this study, 58 male Wistar rats (Pasteur Institute, Tehran, Iran) were used, weighing ~150-180 g. After acclimatization, the animals were housed in standard Plexiglas® cages in the animal laboratory with access to food and water ad libitum, according to NIH guidelines. All experiments and procedures were approved by the Animal Ethics Committee of Zanjan University of Medical Sciences (Ethical code: ZUMS.REC.1399.068), adopted by the National Institute of Health (NIH) Guideline.

Experimental design

Animals were randomly distributed into 8 groups (6 rats in each group), namely: control (only received normal saline intravenously), D group (received 200 mg/kg of *T. daenensis* extract by gavage), DON group (received 1 mg/kg donepezil by IV route), ALZ group (received 2 mg/kg scopolamine by intraperitoneal route), ALZ+DON group (received 2 mg/kg IP scopolamine then received 1 mg/kg IV donepezil), ALZ+D groups (first received 2 mg/kg IP scopolamine and then each group treated with 50, 100 and 200 mg/kg of *T. daenensis* extract by gavage for 10 days). All the animals were anesthetized and culled at the end of the treatment courses. Their whole brain and livers were removed immediately and stored at -80°C for further studies.

Water maze test

The Morris water maze (MWM) is a behavioral test to assess spatial learning memory ability. During each run, animals were gently floated in a circular dark pool from an exact point randomly determined by the computer. The escape platform was submerged under the surface of the water. On training days, rats were trained for 4 consecutive days to find the escape platform. A 60-second countdown was used for each animal to swim freely in the pool. In the cases where animals found the platform before 60 s, the software ended the trial. At the end of each turn, animals were dried and transformed into a warm cage. On the 5th day, the platform was removed from the pool. Again, animals were allowed to swim for 60 s. The swimming path patterns and time spent in different quadrants, especially the target quadrant, were counted as criteria for spatial memory assessment. (Kandimalla et al., 2018; Berahmand et al., 2020).

Evaluation of biochemical factors

It has been firmly proven that cellular ROS accumulation results in LPO induction. This spectrophotometric test was done based on the reaction of MDA, as the main product of LPO, with thiobarbituric acid, after which the absorbance was read at 532 nm. In this method, tetramethoxypropane (TEP) was used as the standard, and MDA content was expressed as nmol/mg protein (Ahmadi et al., 2021).

This standard test assesses the antioxidant power, using TPTZ [2,4,6-Tri(2-pyridyl)-s-triazine] as the sensitive reagent. The absorbance of each sample was recorded at 593 nm. The obtained results were assumed as hallmarks of antioxidant activity of the generated ferrous ions as described by a previous study, and data were expressed as mmol/g tissue (Benzie and Szeto, 1999).

GSH is one of the main cellular indigenous antioxidants and one of the first affected parameters in AD. The GSH amount was evaluated by 5,5'-Dithiobis (2-nitrobenzoic acid) which developed a yellow hue at 412 nm and expressed as µg/mg protein (Amiri et al., 2017; Mozafari et al., 2020).

Protein carbonyl levels are the most general and essential biomarker of severe oxidative protein damage in AD. Therefore, the amount of carbonylated protein was measured via its reaction with DNPH (2,4-dinitrophenylhydrazine) reagent at 365 nm as described in the previously accepted method (Dalle-Donne et al., 2003; Ahmari et al., 2020).

Statistical analysis

Results were expressed as mean ± SD, and R studio programming software was used for statistical analyses. Six rats were used in behavioral tests and 3 rats in molecular tests. Comparisons between the groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* tests. $P<0.05$ was considered statistically significant.

RESULTS

The random swimming patterns of some rats in the MWM test are shown in Fig. 1 for all study design groups. As shown in Fig. 2, the final effects of treatments were demonstrated on the test day. Results of Fig. 2 revealed

that injection of scopolamine induced memory impairment and significantly dropped animals' Q_2 time percentage compared to control rats ($F_{7,40}$=21.666; P<0.001). On the other hand, donepezil (DON) and *T. daenensis* (200 mg/kg) treatment demonstrated no significant difference compared to the control group (P>0.05). In addition, ALZ+ (200 mg/kg) groups showed a significant increase in the presence of the Q_2 zone compared to the ALZ group (P<0.01).

Furthermore, the control, DON, D (200 mg/kg), and the ALZ+ D (200 mg/kg) groups did not differ significantly from each other in time spent on the main track of their swimming in the target zone (Q_2); while the pattern of swimming for rats that received scopolamine was observed to be randomized with a significant decrease in the time spent in Q_2 (Fig. 2). On the other hand, post-treatment with DON and D (200 mg/kg) demonstrated a significant increase in the Q_2 time percentage. Therefore, it can be deduced that DON and *T. daenensis* (200 mg/kg) extract could effectively inhibit scopolamine's destructive and harmful effects on the cholinergic system.

As depicted in Fig. 3 A and B, administration of scopolamine has significantly increased the MDA levels, both in brain and liver tissues ($F_{7,16}$=29.118; P<0.001 in brain and $F_{7,16}$=16.721; P<0.001 in the liver). Furthermore, treatment with *T. daenensis* has significantly decreased MDA levels in the brain compared to the ALZ group (P<0.001). In addition, there has not been a noticeable difference between treatment groups in both tissues. Interestingly, treatment of ALZ animals with *T. daenensis* (50 and 100 mg/kg) demonstrated no significant difference from the control group with intact healthy animals' liver samples. Similarly, administration of *T. daenensis* and DON in healthy rats had no significant effect on the MDA tissue amount.

As presented in Fig. 4 A and B, the ALZ group indicated a lower Frap level (enzymatic and non-enzymatic antioxidant level) than the control group rats in both tissues ($F_{7,16}$=29.699; P<0.001 in the brain and $F_{7,16}$=11.006; P<0.001 in the liver). On the other hand, administration of *T. daenensis* (100 and 200 mg/kg) on ALZ rats showed a significant rise in Frap levels compared to the ALZ group (P<0.05). In addition, treatment of ALZ rats with DON (currently the gold standard drug) resulted in a significant increase in the total antioxidant levels both in the brain and liver (P<0.01 and P<0.05, respectively). Lastly, normal healthy rats that received D (200 mg/kg) and DON showed no significant alteration in Frap levels in the brain and liver (P>0.05).

As shown in Fig. 5 A and B, scopolamine injection significantly reduced glutathione levels in the brain and liver ($F_{7,16}$=54.338; P<0.001 in the brain and $F_{7,16}$=5.644; P=0.002 in the liver). On the other hand, DON treatment significantly increased the GSH levels in the brain and liver compared to the control group. Our data showed a significant rise in GSH levels in ALZ+ D (100 mg/kg) treated group in comparison with the ALZ group in both tissues (P<0.01 in the brain and P<0.05 in the liver). Furthermore, D (200 mg/kg) and DON in normal healthy rats did not significantly alter GSH levels. Interesting data are related to the GSH lev-

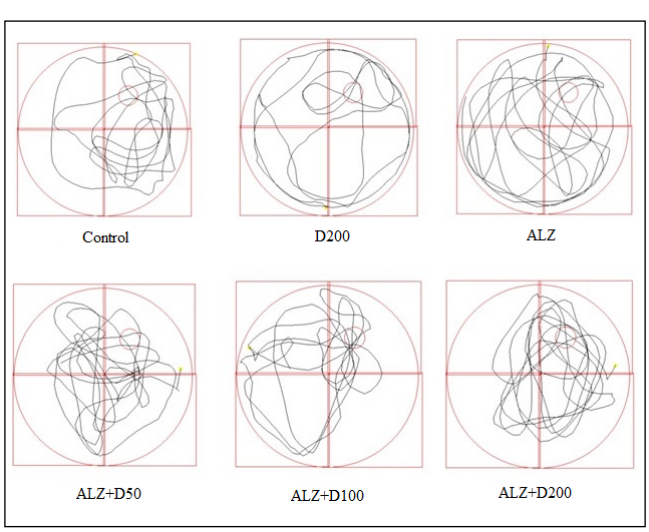

Fig. 1. Randomized selection of swimming patterns in the water maze task.

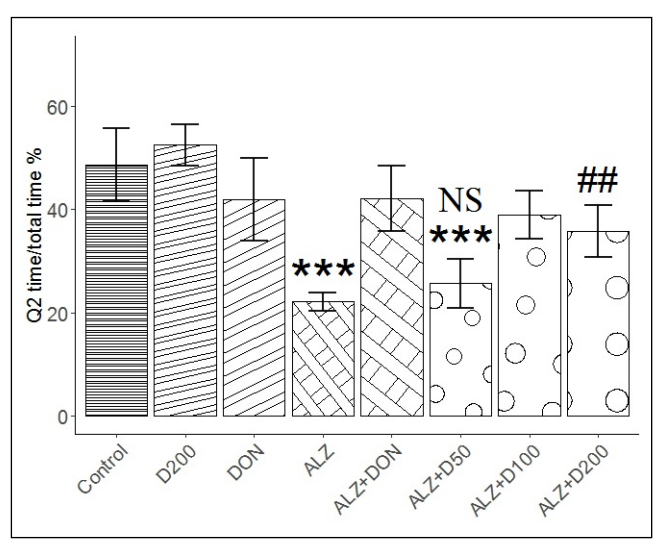

Fig. 2. Effects of *T. daenensis* extract and donepezil on Q_2 time / total time percentage in all groups on test day. Values are expressed as the mean ± SD and were analyzed using one-way ANOVA followed by Tukey's *post hoc* test. (n=7-8). * P<0.05; ** P<0.01 and *** P<0.001 compared with control group. # P<0.05; ## P<0.01 and ### P<0.001 compared with ALZ rats. Scopolamine induced rats (ALZ); Donepezil (DON); *T. daenensis* extract (D); NS: Non-significant.

els (non-enzymatic antioxidant) which is consistent with Frap data, demonstrating the involvement of enzymatic antioxidant as the main antioxidant source in this extract.

As presented in Fig. 6 A and B, administration of scopolamine increased protein carbonyl (PCO) levels in comparison to normal rats ($F_{7,16}$=49.164; P<0.001 in brain and $F_{7,16}$=86.577; P<0.001 in the liver), while DON treatment in these rats had a significant suppressive influence on PCO levels compared to ALZ group (P<0.001). Besides, ALZ+D (100 mg/kg) and ALZ+D (200 mg/kg) groups showed a decreasing pattern in PCO both in the brain and liver compared to the ALZ group (P<0.05 and P<0.001 for brain and liver samples respectively). On the other hand, D (200 mg/kg) and DON treatments in normal rats did not alter the factors mentioned above.

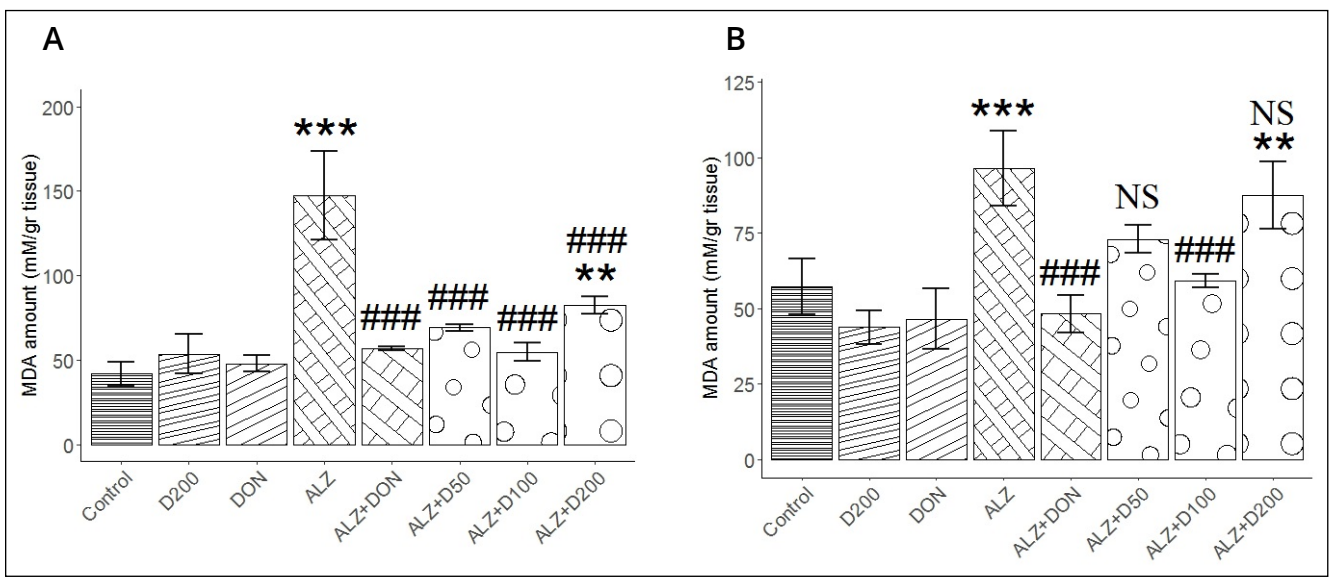

Fig. 3. Effects of *T. daenensis* extract and donepezil on MDA amount of (A) brain and (B) liver samples. Values are expressed as the mean ± SD and were analyzed using one-way ANOVA followed by Tukey's *post hoc* test (n=3). * P<0.05; ** P<0.01 and *** P<0.001 compared with control group. # P<0.05; ## P<0.01 and ### P<0.001 compared with ALZ rats. Scopolamine induced rats (ALZ); Donepezil (DON); *T. daenensis* extract (D); NS: Non-significant.

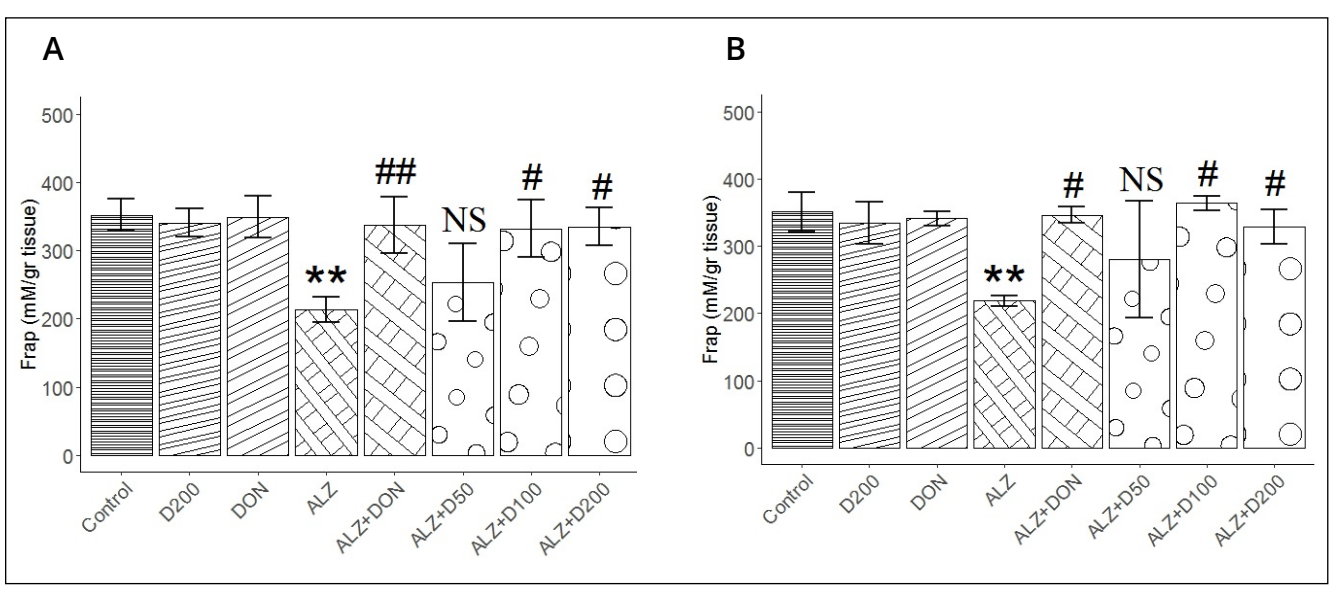

Fig. 4. Effects of *T. daenensis* extract and donepezil on Frap amount of (A) brain and (B) liver samples. Values are expressed as the mean ± SD and were analyzed using one-way ANOVA followed by Tukey's *post hoc* test (n=3). * P<0.05; ** P<0.01 and *** P<0.001 compared with control group. # P<0.05; ## P<0.01 and ### P<0.001 compared with ALZ rats. Scopolamine induced rats (ALZ); Donepezil (DON); *T. daenensis* extract (D); NS: Non-significant.

DISCUSSION

Neurodegeneration in AD patients is the main reason for memory loss and performance impairments (Niedzielska et al., 2016). Amalgamating various research lines indicated that AD symptoms are known to be related to the low synaptic levels of acetylcholine (ACH). In this context, scopolamine demonstrated an increase in amyloid-β deposition and cholinergic dysfunction in animal models (Chen and Yeong, 2020). In our study, administration of scopolamine had a significant influence on lowering endogenous cellular an-

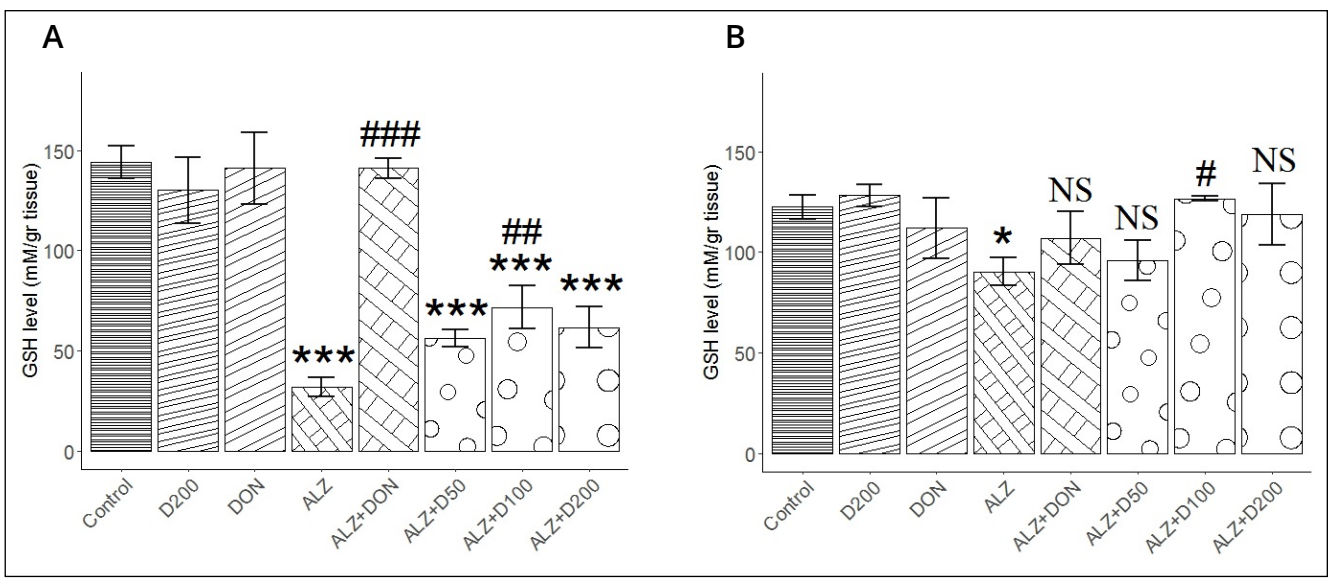

Fig. 5. Effects of *T. daenensis* extract and donepezil on GSH amount of (A) brain and (B) liver samples. Values are expressed as the mean± SD and were analyzed using one-way ANOVA followed by Tukey's *post hoc* test (n=3). * P<0.05; ** P<0.01 and *** P<0.001 compared with control group. # P<0.05; ## P<0.01 and ### P<0.001 compared with ALZ rats. Scopolamine induced rats (ALZ); Donepezil (DON); *T. daenensis* extract (D); NS: Non-significant.

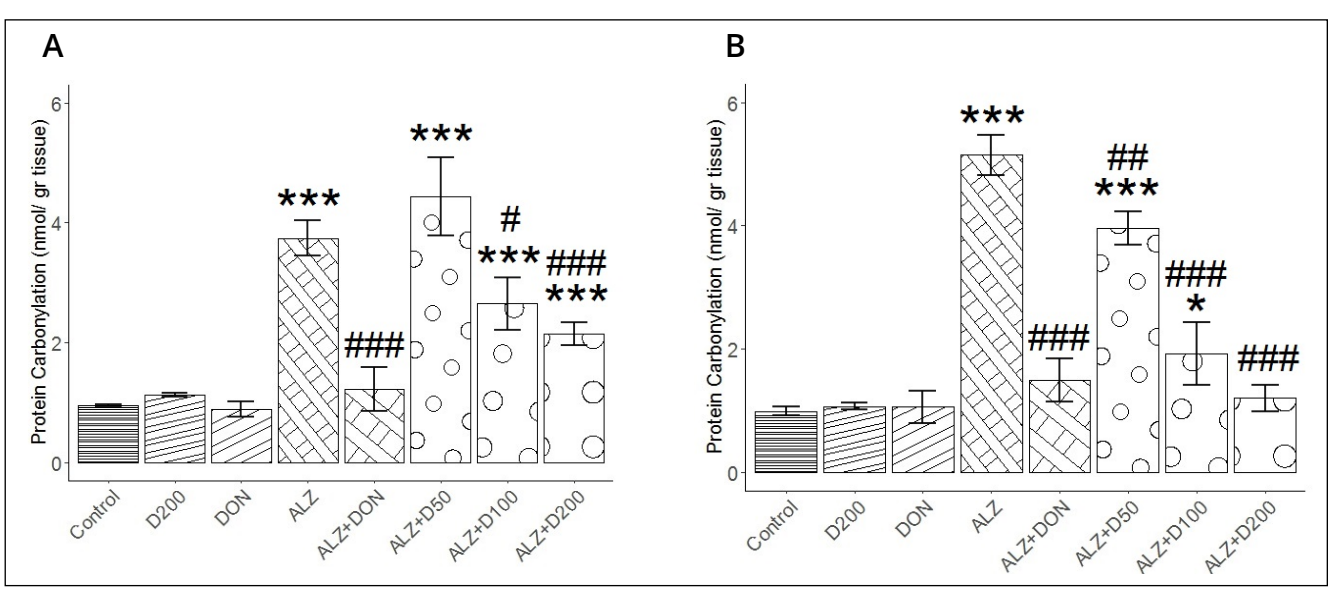

Fig. 6 Effects of administration of *T. daenensis* extract and donepezil on PCO amount of (A) Brain samples and (B) liver samples. Values are expressed as the mean± SD and were analyzed using one-way ANOVA followed by Tukey's *post hoc* test (n =3). * P<0.05; ** P<0.01 and *** P<0.001 compared with control group. # P<0.05; ## P<0.01 and ### P<0.001 compared with ALZ rats. Scopolamine induced rats (ALZ); Donepezil (DON); *T. daenensis* extract (D); NS: Non-significant.

tioxidants and increasing the production of MDA and protein carbonyl as a result of escalation in ROS levels in both brain and liver tissues which share different potentials in encountering oxidative stress in rats with memory impairments (Klinkenberg and Blokland, 2010; Khosravi-Farsani et al., 2016). Our present data confirm the relationship between oxidative stress and neurodegenerative disorders like AD, similar to previous studies (Niedzielska et al., 2016). In addition, lower presence in the platform quadrant in scopolamine-received rats confirmed the previously reported memory impairments in rodents and destruction in the cholinergic system after scopolamine administration. The aforementioned suggests that scopolamine alters gene expression involved in synaptic plasticity, the cholinergic system, and calcium-dependent memory-related genes leading to oxidative stress and apoptotic cell death signaling activation (Bastola et al., 2020; Ponne et al., 2020).

Donepezil (DON), a gold standard medication for mild to moderate AD treatment, was utilized in the present study with positive effects on amelioration of memory impairment, recovery of enzymatic and non-enzymatic antioxidant levels, and declining ROS byproducts such as MDA (Saxena et al., 2008). However, DON was not capable of increasing GSH levels in the liver. This result may be due to the indirect antioxidant effects of DON, which took effect by improving synaptic interaction and neural performance (Klugman et al., 2012). Affirmatively, studies on patients confirmed the effect of Donepezil on recovering Frap and PCO by altering redox homeostasis (Atukeren et al., 2017).

T. daenensis extract is a rich source of terpenoids, phenolic contents, and flavonoids, demonstrating strong antioxidant and antibacterial potency (Bistgani and Sefidkon, 2019). According to the results of this research, the total phenolic content of the extract was 47.3 ± 0.8 µg/ml. In other studies, *T. vulgaris* extract, another member of this family, showed promising effects in restoring memory impairment in passive avoidance and the MWM test per our results (Rabiei et al., 2015). However, in our study, high dose administration of *T. daenensis* extract did not alter the memory performance of samples and the antioxidant capacity and oxidative factors of liver tissue. The overall results of the current study on liver tissues confirmed that treatment with *T. daenensis* showed hepatoprotective effects, which in turn suggested the augmented cellular endogenous antioxidant capacity, scavenging of free radicals, and modulating TNF-α and IL-6 levels (Soosani and Sazegar, 2017). Furthermore, at the highest dose (200 mg/kg), *T. daenensis* extract did not induce any hepatotoxic effect. The reason liver tissues were studied in this study is correlated to the unique position of the liver in the circulatory system and its vital role in the biotransformation of compounds and metabolites.

Oxidative stress is the first influenced marker in neurodegeneration, which could deplete cellular antioxidant levels while augmenting ROS, MDA, and PCO levels, leading to an interruption in the electron transport chain, which is critical for ATP production (Mozafari et al., 2020). Thymus species are rich sources of natural antioxidants and AChE inhibitors, which were previously suggested to be useful in preventing and treatment of AD (Kindl et al., 2015). Our data confirm that *T. daenensis* extract managed a significant rise in memory impairment induced by scopolamine. This effect coincides with an increase in neural FRAP and GSH and a decrease in MDA and PCO levels compared to the ALZ group. Also, treatment of ALZ rats with *T. daenensis* (100 mg/kg) demonstrated more promising results in recovering memory impairment and biochemical parameters. Moreover, it has been suggested that thymol as the main monoterpene of *T. daenensis* extract demonstrated a neuroprotective effect in rodent models (Asadbegi et al., 2017).

Furthermore, geraniol and carvacrol, as the main component in essential oil extract of *T. daenensis* alongside thymol (Hashemi et al., 2010), have been revealed to possess positive effects in controlling oxidative stress and mitochondrial function by carrying out free radicals excavation impact and recovering the levels of antioxidant enzymes, namely Superoxide dismutase and catalase (Prasad 2014; Mehrjerdi et al., 2020). Hydroalcoholic extract of Thymus is also a rich source of rutoside and quercetin; both have powerful antioxidant properties. Rutoside manages to switch cellular energy production from anaerobic glycolysis to oxidative phosphorylation; this boost in energy production may increase Aβ clearance and attenuate neuroinflammation (Pan et al., 2019). Moreover, quercetin demonstrated an anti-neuroinflammatory effect by modulating inflammatory cytokines such as IL-1β, IL-6, IL-12, TNF-α, and COX-2 (Khan et al., 2020).

Compared to scopolamine received animals, *T. daenensis* treatment showed a significant increase in FRAP levels and a decline in PCO and MDA levels. Furthermore, *T. vulgaris,* as a more widely distributed member of the Thymus species, demonstrated similar effects on the regulation of cholinesterase (AChE and BChE) and arginase activities which confirm improvement in memory function. In addition, the modulating effect of this extract on adenosine hydrolyzing and arginase enzymes has been introduced. These enzymes are critical in ATP metabolism and nitric oxide production, which affect memory performance in animal models (Hosseini et al., 2018; Adefegha et al., 2020). In conclusion, based on the MWM test and biochemical as-

says, we found that *T. daenensis* extract has remarkable cognitive-enhancing properties and redox homeostasis effects by inhibition of harmful influences of scopolamine in male Wistar rats.

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