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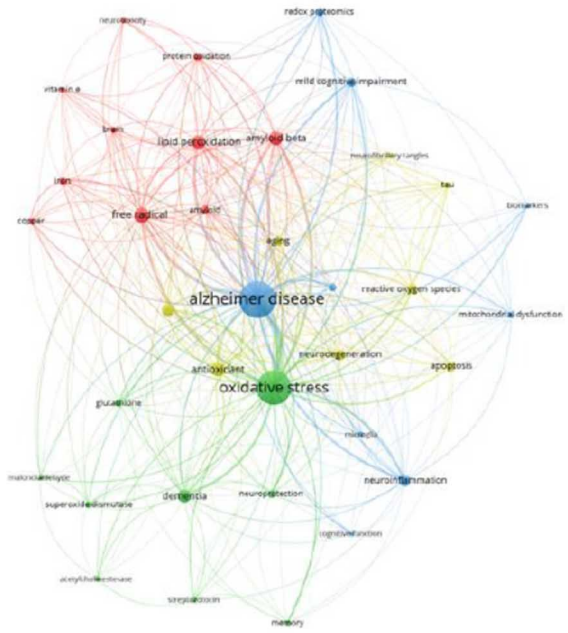

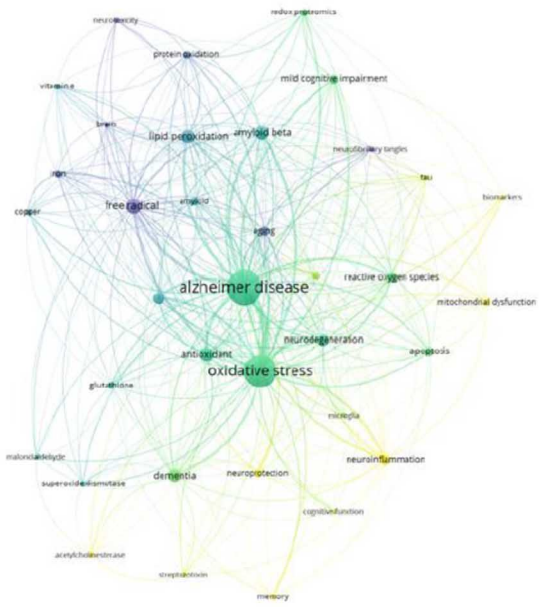

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AIM AND SCOPES
Journal of Cellular Neuroscience and Oxidative Stress is an online journal that publishes original research articles, reviews and short reviews on the molecular basis of biophysical, physiological and pharmacological processes that regulate cellular function, and the control or alteration of these processes by the action of receptors, neurotransmitters, second messengers, cation, anions, drugs or disease.

Areas of particular interest are four topics. They are;

A- Ion Channels (Na^+- K^+ Channels, Cl^- channels, Ca^{2+} channels, ADP-Ribose and metabolism of NAD^+, Patch-Clamp applications)

B- Oxidative Stress (Antioxidant vitamins, antioxidant enzymes, metabolism of nitric oxide, oxidative stress, biophysics, biochemistry and physiology of free oxygen radicals)

C- Interaction Between Oxidative Stress and Ion Channels in Neuroscience
(Effects of the oxidative stress on the activation of the voltage sensitive cation channels, effect of ADP-Ribose and NAD^+ on activation of the cation channels which are sensitive to voltage, effect of the oxidative stress on activation of the TRP channels in neurodegenerative diseases such Parkinson's and Alzheimer's diseases)

D- Gene and Oxidative Stress
(Gene abnormalities. Interaction between gene and free radicals. Gene anomalies and iron. Role of radiation and cancer on gene polymorphism)

READERSHIP

Biophysics	Biochemistry
Biology	Biomedical Engineering
Pharmacology	PhysiologyGenetics
Cardiology	Neurology
Oncology	Psychiatry
Neuroscience	Neuropharmacology

Keywords
Ion channels, cell biochemistry, biophysics, calcium signaling, cellular function, cellular physiology, metabolism, apoptosis, lipid peroxidation, nitric oxide, ageing, antioxidants, neuropathy, traumatic brain injury, pain, spinal cord injury, Alzheimer's Disease, Parkinson's Disease.

RESEARCH ARTICLE

J Cell Neurosci Oxid Stress 2021;13(1): 985-993.

Effects of *Thymus vulgaris* on passive avoidance learning and oxidative stress in pentylenetetrazole-induced model of memory impairment in the male Wistar rats

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List of Abbreviations;

AEDs, *Anti-epileptic drugs;* ***CAT,*** *Catalase;* ***GPx,*** *Glutathione peroxidase;* ***GSH,*** *Glutathione;* ***GTCS,*** *Generalized tonic-clonic seizure;* ***HO-1,*** *Heme oxygenase-1;* ***IL,*** *Initial latency;* ***NO,*** *Nitric oxide;* ***Nrf2,*** *Nuclear factor erythroid 2–related factor 2;* ***OS,*** *Oxidative stress;* ***PA,*** *Passive avoidance test;* ***PTZ,*** *Pentylenetetrazole;* ***RL,*** *Retention latency;* ***SN,*** *Number of shocks;* ***SOD,*** *Superoxide dismutase;* ***TLC,*** *Total light compartment;* ***TVE,*** *Thymus vulgaris extract;* ***VAL,*** *Sodium valproate.*

Abstract

Thyme with the scientific moniker of *Thymus vulgaris* has been used in traditional medicine for treatment of respiratory and gastrointestinal disease. Its extract has also been adopted to treat epilepsy and other central nervous system disorders. To evaluate the effect of *T. vulgaris* extract (TVE), the experimental model of memory impairment caused by seizures was performed in rats. Seizures in the rats (200-250 g) were induced with a challenge dose (60 mg/kg) of pentylenetetrazole (PTZ). Before PTZ administration, the animals were intraperitoneally treated (30 min) by the treatments of TVE (100 mg/kg), sodium valproate (100 mg/kg), and their combination. The effect of memory was assessed using passive avoidance test (PA). At the end of the experiment, the serum samples from all rats were taken for the assays of oxidant (nitric oxide, NO))/antioxidant assay (catalase, CAT and superoxide dismutase, SOD). PTZ-treated groups revealed memory deficit seizures compared with the control group. TVE exhibited protection at the dose of 100 mg/kg in the PTZ-induced seizures. TVE also reduced seizure-induced memory impairment, which resulted in a significant improvement in memory retention in PA compared with the PTZ-

treated group. In addition, TVE treatment protects the seizure-induced memory deficit by lowering NO and restore the antioxidant enzyme CAT and SOD levels. The findings revealed that TVE exhibits significant inhibitory activity and impedes seizure-induced memory impairment by inhibiting oxidative stress damage.

Keywords: *Thymus vulgaris*; passive avoidance; learning and memory; seizure; oxidative stress

Introduction

Seizure is a condition in which nerve cells make sudden and simultaneous nerve discharges and is often accompanied by changes in the network and neural function. The term epilepsy is also defined as the presence of two or more seizures, which is one of the most common diseases in the world (Katyayan and Diaz-Medina 2021). Along with seizures, epilepsy is also associated with several other comorbidities, including cognitive deficits, which are very common in patients with epilepsy (Suleymanova 2021). At present, most cases of epilepsy are treated or controlled with anti-epileptic drugs (AEDs), which, as have been shown, have limitations in performance, safety, and efficacy (Fattorusso et al. 2021). Sodium valproate (VAL) is one of the most common drugs used to treat epilepsy. It is also used in the treatment of bipolar diseases. As mentioned, AEDs have side effects, of which VAL is no exception and can cause memory loss and behavioral ramifications (Duman et al. 2019; Muralidharan et al. 2020).

Although there are many studies on how comorbidities with epilepsy develop, there is little information on how epileptic seizure causes memory impairment associated with learning (Holmes 2015). In addition to memory impairment, studies have indicated that generalized seizures are associated with increased oxidative stress (OS) and the production of reactive oxygen species (ROS). There is ample evidence that OS plays a pivotal role in promoting seizures and epilepsy, causing membrane lipid peroxidation and depletion of antioxidant enzyme levels (Olowe et al. 2020).

Nowadays, bioactive products in plants have become a popular field for researchers to identify more effective treatments with fewer side effects. They are also inexpensive due to their abundance in most places. The genus *Thymus* (of Lamiaceae family) has more than 300 species and is one of the largest types of aromatic plants that have important healing properties (Capatina et al. 2020). Among them, *Thymus vulgaris*, thanks to its long history of use as a medicinal healer, is the best known. In traditional medicine, *T. vulgaris* is used as an herbal tea and decoction in the treatment of rheumatism, cold, asthma and headache (Banerjee et al. 2019; Gedikoğlu et al. 2019; Komaki et al. 2016). In the last two decades, researchers have made great efforts to identify the properties and compounds of *T. vulgaris* including research conducted on zebrafish to identify the effect on cognitive function in a model of Alzheimer's disease. This study showed the positive effect of thyme on cognitive function due to its antioxidant properties (Capatina et al. 2020). Other research also points to the anti-anxiety and anti-stress effects of *T. vulgaris*. A recent study unveiled that *T. vulgaris* has anticonvulsant properties in mice (Skalicka-Woźniak et al. 2018).

This study was designed and tested to identify the neuroprotective properties of *T. vulgaris* alone or in combination with VAL in epilepsy-induced or pentylenetetrazole (PTZ) rats following memory deficit, and to investigate the possible mechanisms that *T. vulgaris* may suggest.

Material and methods

Plant Collection and Extraction

Fresh leaves of *T. vulgaris* were collected from Medicinal Plant Garden at the Shahid Beheshti University. Leaves of *T. vulgaris* were authenticated by a botanist, at the School of Life Sciences Herbarium, Shahid Beheshti University, and the voucher number SBU.SLSH.98.032 was assigned. The leaves were air-dried for 14 days and powdered. An amount of 200 g of the powdered leaves was soaked with 2 L of 70% ethanol for 72 h, filtered and concentrated using a rotary evaporator (EYEL A, Japan) under reduced pressure and temperature (50 °C). The *T. vulgaris* extract (TVE), was further dried and preserved in a desiccator containing activated silica until it was ready for use. The yield obtained was 6.4% w/w. The extract was reconstituted for use in the experiments by gently triturating to prepare a solution of it with normal saline as the vehicle.

Animals

Locally bred male Wistar rats (200-250 g) were used in the present study. These rodents were kept in standard cages in the animal room under controlled

conditions (ambient temperature 22 ± 2 °C and 12 h light/dark cycle). Standard food for rats (Pars Animal Feed Co., Iran) as well as water were made available to the animals in an unlimited manner. All the experiments were performed between 9 and 12 A.M. to reduce the effect of the light cycle on the susceptibility to seizures. Working with animals and the implementations of the experiments were completely done in accordance with the international ethical principles (European Community guidelines (EEC Directive of 1986; 86/609/EEC)). The research protocol was also approved by the University's Animal Ethics Committee.

Experimental Design and Treatment Protocol

Pentylenetetrazole (PTZ) was prepared from Sigma Company as a crystalline, white powder. Sodium valproate (VAL) (ampoule 400 mg / 4 ml) was prepared by Rahakin Company, Tehran, Iran. Twenty-five rats were randomly divided into six groups. Seizures were inducted by intraperitoneal (i.p.) injection of PTZ (60 mg/kg) dissolved in normal saline (Kumar et al. 2018). TVE was injected i.p. at dose of 100 mg/kg (Komaki et al. 2016). VAL was injected into rats i.p. at a dose of 100 mg/kg (Pahuja et al. 2013). The volume of injections in all animals was considered constant at 0.5 ml. The test protocol used to evaluate the effect of TVE and VAL alone or in combination on the behavioral activities was as follows:

- Group I (control group): Rats that received only normal saline.
- Group II (PTZ group): Rats that received normal saline half an hour before the PTZ injection.
- Group III (VAL group): Rats that received sodium valproate (100 mg/kg, i.p.) half an hour before the PTZ injection.
- Group IV (TVE group): Rats that received TVE (100 mg/kg, i.p.) half an hour before the PTZ injection.
- Group V (TVE + VAL group): Rats receiving sodium valproate (100 mg/kg, i.p.) and TVE (100 mg/kg, i.p.) half an hour before the PTZ injection.

Behavioral Evaluation Based on Racine Average Score

The motor behavior of the animals in each group was recorded and stored by a computer-connected camera for half an hour after the PTZ injection and was examined by researcher in a double-blind manner. Based on The Racine scale in animals the six-stage stereotypical behavioral manifestations that were displayed after PTZ injections and the latency and number of myoclonic jerks and generalized tonic-clonic seizure (GTCS) latency and duration were evaluated (**Table 1**) (Hosseini et al. 2021).

Table 1. *Modified Racine's scale for pentylenetetrazole (PTZ)-induced seizure in rats.*

Score	Behavioral manifestation
0	No behavioral sign
1	Ear and facial twitching
2	Head nodding and myoclonic jerks
3	Unilateral forelimb clonus with lorditic posture
4	Bilateral forelimb clonus with rearing and falling
5	Generalized tonic-clonic seizure (GTCS) with loss of postural tone

Evaluation of Passive Avoidance Memory

The rate of memory recovery in animals was assessed by a shuttle box (Borj Sanaat Co., Iran) (Hosseini et al. 2021). The device consists of two dark and light compartments separated by a guillotine door. The floor of this chamber has steel rods that can transmit electric shock to the limbs of living entities. Briefly, on the first day of the acquisition phase, each rat was placed separately in a clear compartment. After 30 s of habituation, the guillotine door was opened and initial latency (IL) was measured to enter the dark chamber. The rats that showed IL for more than 60 s were excluded from further analysis. When the rats entered the dark area, the guillotine door would quickly be closed and an electric foot shock (75 V, 0.2 mA, 50 Hz) was applied to them for 3 s. The animal would be transferred to its cage 30 s after the electric shock and this operation was repeated 5 min later. The rats were shocked every time they put all four limbs in the darkness. The training would end when the animal stayed in a bright room for 120 consecutive seconds. The number of shocks (SN) was measured until acquisition. Twenty-four hours later, like before, retention latency (RL) as well as total light compartment (TLC) was measured after seizure induction, but no electric shock was applied. Retention time was measured in 300 s.

Animal Euthanasia and Serum Extraction

After behavioral tests, the animals underwent deep anesthesia with ketamine and xylazine, and their thoracic blood was collected after cardiac puncture by a sterile syringe. The blood was allowed to clot for half an hour at room temperature and then the serums were separated by a centrifuge at 3000 rpm for 15 min and stored at -20 °C.

Measurement of Oxidative Stress Markers

In order to measure catalase (CAT), superoxide dismutase (SOD), and nitric oxide (NO) oxidative stress indices, animal serum samples, conventional kits (Novin Navand Salamat Co., Iran) available in the market, and microplate reader (BioteTek ELx808, USA) were used. The activity of CAT, SOD, and NO was measured according to the kit instructions at a wavelength of 550, 405, and 570 nm and was reported in mU/ml, mU/ml, and µmol/l, respectively.

Statistical Analysis of Data

The results of the present study were shown as median (min, max). The normality of the data was tested by Shapiro-Wilk test. If the data were normal, then one-way ANOVA and Tukey's post hoc test were used to examine the differences between the groups. If the hypothesis of normality of the data was rejected, then non-parametric Kruskal-Wallis test and Dunn's test post hoc test were used to examine the differences between the groups. All statistical analyzes were performed by GraphPad Prism software. Moreover, in all analyses, the value of *P* was set at less than 0.05.

Results

The Effect of Different Therapies on The Activity of PTZ-Induced Seizure

The effect of different therapies on the manifestations of PTZ-induced convulsive behavior is displayed in **Figure 1**(**A-D**). Regarding the myoclonic jerk latency, statistical analysis revealed a significant increase in the VAL and TVE + VAL group vis-à-vis PTZ group. TVE group did not show a significant difference in the myoclonic jerk latency compared to the PTZ group (**Figure 1A**). As exhibited in **Figure 1B**, the number of myoclonic jerks in different groups was affec-

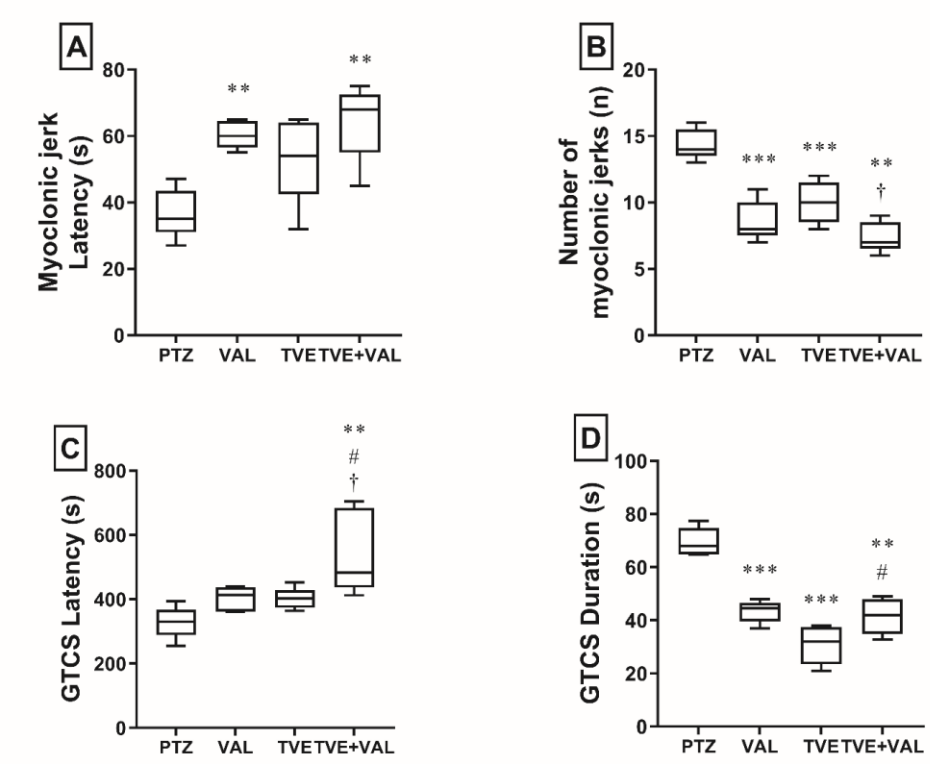

Figure 1. The effect of different treatments after PTZ challenge (60 mg/kg, i.p.) on latency of myoclonic jerk (**A**), number of myoclonic jerks (**B**), GTCS latency (**C**), and GTCS duration (**D**) in male Wistar rats. In a box plot, the distance between two upper and lower box line represents the interquartile range. The central line represents the median value (n = 5 rats per group). *p < 0.05; **p < 0.01; ***p < 0.001 significant difference between VAL, TVE or TVE + VAL group with PTZ group. #p < 0.05 significant difference between TVE + VAL group with VAL group. †p < 0.05 significant difference between TVE + VAL group with TVE group. *TVE*: *Thymus vulgaris* extract, *PTZ*: pentylenetetrazole, *GTCS*: generalized tonic clonic seizure.

ted; the effect had a significant decrease in the TVE, VAL and TVE + VAL groups compared to the PTZ group. Regarding the GTCS latency factor, a significant increase was witnessed in the TVE + VAL group compared to the PTZ group (**Figure 1C**). In the scrutiny of factors, GTCS duration revealed a significant decrease in the TVE, VAL, and TVE + VAL groups in comparison to the PTZ group (**Figure 1D**).

The Effect of Different Treatments on Passive Avoidance Memory

There was no statistically significant difference between IL and SN in different treatment groups (**Figure**s **2A** and **B**). However, RL in the PTZ group showed a significant decrease compared to the control group, indicating memory impairment. The VAL, TVE and TVE + VAL groups increased the RL level significantly compared to the PTZ group (**Figure 2C**). Regarding the TLC factor, the PTZ group exhibited a significant decrease compared to the control group. Only TVE and TVE + VAL groups statistically significantly increased TLC compared to PTZ group. However, this increase was not statistically noticeable in VAL group compared to PTZ group (**Figure 2D**).

The Effect of Different Therapies on Oxidative Stress Markers

As shown in **Figure 3A**, there was a significant decrease in serum CAT levels in the PTZ group compared to the control group. Moreover, only TVE + VAL treatment caused a significant increase in the serum level of CAT compared to the PTZ group. Further statistical analysis also revealed that there was a significant difference in the CAT serum levels between TVE or VAL *vs.* TVE + VAL groups. Statistical analysis of SOD serum level in PTZ group displayed a significant decrease compared to the control group. Conversely, in the TVE and TVE + VAL groups, there was no significant difference observed *vis-à-vis* the control group. Further statistical analysis signaled that there was a significant difference in terms of increasing serum SOD levels in the TVE and TVE + VAL groups *vs.* PTZ group (**Figure 3B**).

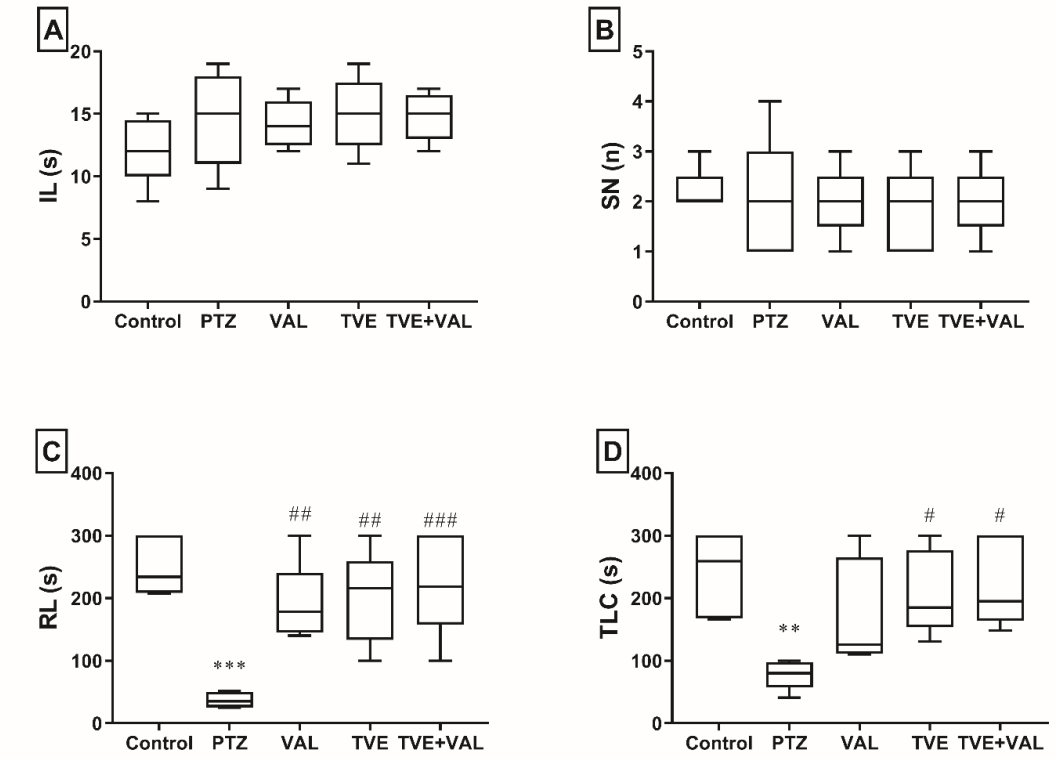

Figure 2. The effect of different treatments after PTZ challenge (60 mg/kg, i.p.) on IL (**A**), SN (**B**), RL (**C**), and TLC (**D**) in male Wistar rats. In a box plot, the distance between two upper and lower box line represents the interquartile range. The central line represents the median value (n = 5 rats per group). **p < 0.01; ***p < 0.001 significant difference between PTZ group with control group. #p < 0.05; ##p < 0.01; ###p < 0.001 significant difference between VAL, TVE or TVE + VAL group with PTZ group. *TVE*: *Thymus vulgaris* extract, *PTZ*: pentylenetetrazole, *IL*: initial latency, *SN*: shock number, *RL*: retention latency, *TLC*: total light compartment.

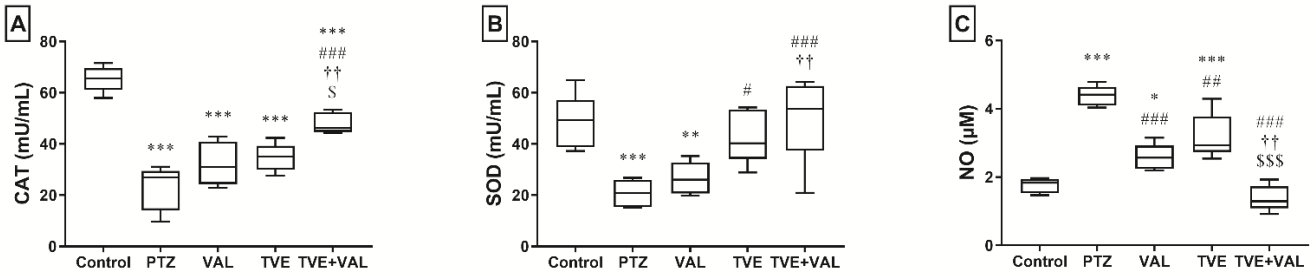

Figure 3. The effect of different treatments after PTZ challenge (60 mg/kg, i.p.) on serum level of NO (**A**), CAT (**B**), and SOD (**C**) in male Wistar rats. In a box plot, the distance between two upper and lower box line represents the interquartile range. The central line represents the median value (n = 5 rats per group). *p < 0.05; **p < 0.01; ***p < 0.001 significant difference between PTZ, VAL, TVE or TVE + VAL group with control group. #p < 0.05; ##p < 0.01; ###p < 0.001 significant difference between VAL, TVE or TVE + VAL group with PTZ group. ††p < 0.01 significant difference between TVE group with VAL group. $p < 0.05; $$$p < 0.001 significant difference between TVE + VAL group with TVE group. *TVE*: *Thymus vulgaris* extract, *CAT*: catalase, *SOD*: superoxide dismutase, *NO*: nitric oxide, *PTZ*: pentylenetetrazole.

There was a significant increase in serum NO levels in the PTZ group compared to the control group. Moreover, VAL, TVE, and TVE + VAL groups caused a significant decrease in the serum level of NO compared to the PTZ group. Further statistical analysis also revealed that there was a significant difference in the NO serum levels between the TVE or VAL group *vs.* TVE + VAL group (**Figure 3C**).

Discussion

In the present study, the effect of TVE alone or in combination with VAL on seizures and PTZ-induced passive avoidance memory deficits in rats was investigated. The results revealed that TVE alone or in combination with VAL have a statistically significant effect on improving the behavioral seizure manifestation compared to the PTZ group. The PA test also showed that PTZ reduced RL and TLC resulting in memory impairment in animals receiving PTZ. The results of this study are in line with reports that have been documented on memory impairment due to PTZ-induced seizures (Aghaie et al. 2021; Hosseini et al. 2021). With its effect on RL and TLC and its significant increase compared to the PTZ group, TVE reverses the effect of PTZ-induced memory impairment in rats, indicating its protective role against seizures and seizure-induced memory impairment. While no studies have been reported on the effect of TVE on memory impairment by convulsant agents so far, there is a report on the anticonvulsant and anti-plasmid conjugation effects of *T. vulgaris* in mouse model of electroconvulsions. This study shows that *T. vulgaris* has protective activity against seizures which is in line with result of current study (Skalicka-Woźniak et al. 2018). Also, there are some reports showed that thymol, main monoterpene phenol found in thyme essential oil, affect the learning and memory in animal models of high-fat diet-induced cognitive deficits (Asadbegi et al. 2017; FangFang et al. 2017), and further investigation showed that this effect of thymol is mediated by activating nuclear factor erythroid 2–related factor 2 (Nrf2)/ Heme oxygenase-1 (HO-1) signaling pathway which is the regulates many antioxidant enzyme level in the cells (FangFang et al. 2017). Therefore, the positive effects of TVE on seizures as well as the improvement of memory impairment caused by seizures which were observed in the present study can be partially attributed to the active constituent like thymol in the TVE.

Various mechanisms have been proposed for how PTZ injection causes seizures as well as the associated memory impairment. One of the most important factors in the development of seizures as well as the resulting behavioral changes is the OS and the deviation of ROS from its normal level (Olowe et al. 2020). ROS, which comprise highly active oxygen-containing molecules, are common oxidizing compounds that attack and oxidize various molecules to produce secondary oxidized products. The mitochondria damaged by OS in pyramidal neurons of Alzheimer's Disease are subjected to neurodegeneration (Moreira et al. 2010). Thus, inhibition of the mitochondrial complexes leads to diminished ATP production and resulted in impaired energy metabolism. Several lines of evidence support that NO impairs

mitochondrial/cellular respiration and other functions by inhibiting the activities of several key enzymes, particularly cytochrome c oxidase, and thereby causing ATP depletion (Cui et al. 2011). A number of enzymes play a quintessential antioxidant role against the attack of ROS. One of the most important of these enzymes is CAT. CAT is a common enzyme in mammalian and non-mammalian cells that catalyzes the breakdown of hydrogen peroxide into water and oxygen and can convert millions of hydrogen peroxide (H_2O_2) molecules into water and oxygen every second. H_2O_2 is one of the productive compounds of ROS and is the product of the body's natural aerobic metabolism. However, this by-product is toxic to eukaryotic cells and can cause DNA damage, oxidation of lipid and proteins, and lead to mutation or even cell death. To prevent damage to cells and tissues, hydrogen peroxide is converted to oxygen and water by the CAT enzyme (Furger 2021; Gwozdzinski et al. 2021). One of the most important antioxidant mechanisms of the body against the attack of ROS is the presence and activity of the SOD enzyme. SOD enzymes are metalloproteins. These enzymes catalyze the dismutation reaction of superoxide anion (O_2^-) to oxygen and hydrogen peroxide. The presence of a sufficient amount of SOD in cells and tissues keeps the concentration of O_2^- at a very low level. SOD activity in cells and extracellular environments is critical to prevent OS-related diseases (Gwozdzinski et al. 2021; Wang et al. 2021). NO is a molecular mediator of many physiological processes such as vasodilation, inflammation, thrombosis, immunity and neurotransmission. NO is made by the NO synthase (NOS) enzyme from L-arginine, oxygen, and NADPH and participates in vascular homeostasis by inhibiting vascular smooth muscle contraction, platelet aggregation, and leukocyte adhesion to endothelium. People with atherosclerosis, diabetes, and high blood pressure often manifest NO pathway disorders (Ghimire et al. 2017). In the present study, the results showed a decrease in the CAT and SOD serum levels and an increase in NO following the PTZ injection. The present study, similar to various prior studies, showed that the injection of PTZ in animal models increases OS and decreases antioxidant levels (Hosseini et al. 2021; Kawakami et al. 2021; Kumar et al. 2018). Also, data from the present study showed that the TVE affected the serum level of CAT and SOD and counteracted the effects of PTZ by increasing it and decrease the serum level of NO. The direct effect of TVE on CAT and NO have not been reported, but there are some reports it has antioxidant activities and it can affect ROS formation (Banerjee et al. 2019; Capatina et al. 2020; Gedikoğlu et al. 2019). In a recently reported study, *T. vulgaris* essential oil decreased lipid peroxidation and increased SOD, glutathione (GSH) and glutathione peroxidase (GPx) in zebrafish model of cognitive dysfunction (Capatina et al. 2020). Therefore, the positive effects of TVE on seizures as well as the improvement of memory impairment caused by seizures which were observed in the present study can be partially ascribed to the antioxidant properties of TVE.

The present study for the first time examined the anticonvulsant effects of TVE, as well as its possible antioxidant properties. The elicited results could pave the way for future studies. Despite this strength, the present study had a limitation: this study did not investigate the pathway and inflammatory cytokines and also measure the antioxidant level in the brain tissue due to the small number of samples and time constraints. This issue can be addressed in future research designs.

Overall, the present study provided evidence for the potential neuroprotective effects of the TVE alone or in combination with VAL. In addition, it was shown that TVE can be protective against induced OS during seizures. Therefore, a treatment strategy that could address the potential therapeutic effect of TVE with VAL in the treatment of seizures as well as memory impairment associated with seizures calls for further research. Additional study designs are also needed to fully elucidate the mechanisms of anticonvulsant function as well as safety in their chronic use.

Declaration

Ethics approval and consent to participate

The research protocol was also approved by the University's Animal Ethics Committee.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

None received.

Authors' contributions

AH performed the research, analyzed and interpreted the data regarding the project, and was a major contributor in writing the manuscript. VA performed the research, analyzed and interpreted the data regarding the project, and was a major contributor in writing the manuscript. FA performed the research, analyzed and interpreted the data regarding the project, and was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

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