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RESEARCH

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# Metabolites of *Trichoderma harzianum* re-balance T-reg/Th-17 cytokine axis in epileptic rats

Mohamed A. Alblihed[1*]

## Abstract

**Background** Recurrent seizure is synchronous neuronal network hyperexcitation. Even though many anti-epileptic drugs (AEDs) are available, but have several drawbacks, including multiple drug interactions, suboptimal response rates, significant adverse effects, and a narrow therapeutic index. *Trichoderma* is a biocontrol agent which maintains stress-related gene expression to adapt to unstable environmental conditions. The current study sought to delineate the ameliorative effects of metabolites of *Trichoderma harzianum* (*T. harzianum*) culture filtrate (ThCF) by assessing the functions of T regulatory cells and rebalancing oxidative stress.

**Methods** Experimental rats were divided into control, epileptic, valproic acid-treated, and *T. harzianum* cultured filtrate (ThCF). Lipid peroxidation, nitric oxide, and antioxidant defense enzymes were estimated. Moreover, interleukins-6, -10, -17, tumor necrosis factor (TNF-α), and transforming growth factor (TGF-β) were estimated using ELISA kits, in addition to T-reg markers; cytotoxic T-lymphocyte-associated protein 4 (CTLA4), and forkhead box P3 (FOXP3) were estimated by qRT-PCR.

**Results** Data revealed that the *T. harzianum* cultured filtrate (ThCF) retarded the lipid oxidation rate and has antioxidant activities, as well as increased levels of GPx, CAT, and SOD. Moreover, ThCF re-balances T-reg/Th-17 cytokines, restoring the pro/anti-inflammatory cytokines and Treg markers, e.g., FOXP3 and CTLA-4, to their normal level.

**Conclusion** *Trichoderma harzianum* has a potent antioxidant activity with high capacity to scavenge ROS and down-regulate all T-reg markers. Therefore, the present data are directed toward the characterization of new active constituents of secondary metabolites of *T. harzianum* with significant therapeutic functions in several diseases. The promising findings may suggest more clinical and experimental scenarios to reduce AED's drawbacks and side effects.

**Keywords** Epilepsy, *Trichoderma harzianum*, Lipid peroxidation, Antioxidants, T-regulatory cells

## 1 Background

Epilepsy is one of the most common chronic central nervous system illnesses. Different illnesses feature caused by epilepsy and afflict about 1% of the general population. Around 80% of the world's 50 million epileptics live in countries with low and medium incomes. About 70% of epileptic individuals are anticipated to be seizure-free, if properly diagnosed and treated. However, in low-income nations, around 75% of patients do not receive the necessary care; in some cases, this percentage might reach 90% [1]. Even though many anti-epileptic drugs (AEDs) are available, they have several drawbacks, including multiple drug interactions, suboptimal response rates, significant adverse effects, and a narrow therapeutic index. Some biological control agents (BCAs) are represented by

*Correspondence:
Mohamed A. Alblihed
mabulihd@tu.edu.sa
[1] Department of Medical Microbiology, College of Medicine, Taif University, P.O. Box 11099, 21944 Taif, Saudi Arabia

fungal species of Trichoderma, e.g., *Trichoderma viride* (*T. viride*), *Trichoderma harzianum* (*T. harzianum*), and *Trichoderma virens* (*T. virens*) [2].

*Trichoderma* sp. as endophytic fungi have bioactive compounds with wide applications as immunomodulatory, antimicrobial, antiviral, and antioxidant activities. They contain many chemical classes, e.g., flavonoids, phenolic acid, terpenoids, steroids, alkaloids, glycosides, quinones, and xanthones [3]. Moreover, secondary metabolites of *Trichoderma* spp., e.g., peptaibols perform many biological functions, e.g., uncoupling of oxidative phosphorylation, immunosuppression, inhibition of the mitochondrial ATPase, and inhibition of platelet aggregation [4].

Harzianolides, peptaibols, and certain volatile compounds are secondary metabolites produced by *Trichoderma* spp. [5]. In addition, pyrones derivatives of *Trichoderma* metabolites can release γ-aminobutyric acid and acetylcholine and share in the pathogen resistance mechanisms of more plants [6]. Moreover, Katoch, Singh [7] reviewed that *Trichoderma* produced varieties of peptaibol with potential bio- and pharmacological activities, e.g., wound-healing, antifungal, antibacterial, anticancer, immunosuppressive, anti-mycoplasmic, and anti-trypanosomal activities.

*T. harzianum* is a saprophytic fungus used as a biological control agent. Previously, Isham, Tibodeau [8] documented many biologic activities for thiodioxopiperazines, e.g., antiviral, antimicrobial, antiparasitic, and anti-inflammatory effects, and immunosuppressive and antineoplastic activity. A potent component endochitinase has a significantly greater antifungal activity is encoded by *T. harzianum.* [9]. Chitinases or endochitinase is one of the lytic enzymes produced from *T. harzianum* [10]. Moreover, chitooligosaccharides (COSs) as biodegradation products of chitosan can perform many biological activities [11]. In addition, Li [12] identified and characterized some non-volatile metabolites from different culture strains of *T. harzianum,* e.g., terpenoids, harzianolide, and peptaibols. Whereas, they confirmed that peptaibols generated catecholamine production from bovine adrenal medullary cells under the control of Ca2+, and some of them have anti-tumor activities and affect the cell cycle at the S phase. On the other hand, T regulatory cells (Treg) have a critical role during many of the immune homeostasis pathways. T reg may inhibit the pathogen-specific immunity in different infectious diseases, such as those caused by CNS-infecting neurotropic viruses [13].

Based on the prementioned expatiations, the current study aimed to delineate the ameliorative effects of secondary metabolites of *Trichoderma harzianum* culture filtrate (ThCF) in comparison with valproic acid as AED via assessment of the functions of T-regulatory cells and evaluation of the oxidative stress status.

## 2 Methods

### 2.1 Animals

A total of twenty-four adult male Sprague Dawley rats weighing between 150 and 180 g were procured from the animal facility at King Fahd Specialist Medical Centre in Jeddah, Saudi Arabia. These rats were individually housed in metabolic cages within a controlled setting, maintaining a temperature of 23 ± 1 °C and humidity of 55 ± 5%. The rats followed a 12-h light/dark cycle, and they had unrestricted access to food and water. All procedures were performed according to the guidelines for the care and use of laboratory animals and approved by the animal research ethics committee of Taif University, which was under an approval number: 44-172.

### 2.2 Drug and chemicals

Acros Organics (New Jersey, USA) supplied the Pilocarpine hydrochloride (99%) (PILO). Methylscopolamine ($C_{18}H_{24}NO_4$·Br, CAT no. 155-41-9,) and diazepam ($C_{16}H_{13}ClN_2O$, CAT no. 439-14-5) were acquired from CaymanChem CO. (Nanjing, China). Depakine Chrono®500 mg, which produced by Sanofi Aventis CO, was served as the source of valproic acid (AED code: N03AG01).

### 2.3 Preparation of the fungal cultural filtrates of Trichoderma isolates

The fungal organism used in this study (*Trichoderma harzianum*) was generously donated from the Section of Mycology and Plant Pathology, Department of Botany, Faculty of Science. After employing the single spore technique to purify the colonies, the identified fungi were obtained. These fungal cultures were preserved by creating a spore suspension in 20% glycerol and storing them at − 80 °C. To reactivate the cultures, they were placed on potato dextrose agar (PDA) [14].

### 2.4 Induction of epilepsy

Epilepsy was induced in an experimental setting following the method outlined by Turski, Ikonomidou [15]. Prior to administering a 300 mg/kg injection of pilocarpine hydrochloride, the experimental rats received an intraperitoneal (i.p.) injection 1 mg/kg of methylscopolamine for a duration of 30 min. Subsequently, the rats' behavior was assessed to determine the presence of seizure activity based on specific criteria. These criteria included the manifestation of epileptic seizure symptoms such as sluggishness, salivation, tremors, and convulsions. Model rats exhibited such behaviors for 120 min following the administration of pilocarpine

hydrochloride, while the control group displayed normal behavior. The rats experienced seizure episodes characterized by continuous generalized seizure activity without periods of normal behavior within each episode. After 1 h of continuous seizure episodes, the attacks occurred at intervals of 2–5 min. Diazepam (4 mg/kg, i.p) was administered every 20 min as required to terminate the seizures. Control rats underwent the same protocol, except they were injected with phosphate-buffered saline (PBS, pH 7.4; 0.2 ml/rat) instead of pilocarpine, followed by diazepam after 1 h. Throughout the study, the experimental rats were divided into four groups consisting of six rats each as follows:

*Control animals (C)* was provided with a standard diet and had unrestricted access to sterile water. They were administered with phosphate-buffered saline (PBS, pH 7.4, 0.2 ml/rat) orally, using intragastric intubation at intervals similar to the treated groups.

*Epileptic group (EP)* was given an injection of pilocarpine hydrochloride at a dose of 300 mg/kg., as described previously [16].

*Valproic acid-treated epileptic group (EP-VPA)* was injected with 300 mg/kg of PILO hydrochloride as described previously [17] and then orally fed with 500 mg/kg VPA dissolved in PBS (pH 7.4; 0.2 ml/rat) using intragastric intubation. VPA was performed twice/week at intervals parallel to other groups for four consecutive weeks [18].

*The fourth group* was epileptic and treated with ThCF (EP-ThCF;). The last group was treated with 300 mg/kg of pilocarpine hydrochloride injection as described previously, then orally fed with *T. harzianum* cultured filtrate (ThCF) using intragastric intubation at a dose of 400 mg/kg b.wt/day [19]; continued for 4 weeks.

### 2.5 Blood; tissue sampling and biochemical measurements

At the end of the experiment, all rats were euthanized following the established procedures for the handling and use of laboratory animals. Blood samples were promptly collected into sterile vacutainer tubes, allowed to coagulate, and then subjected to centrifugation at 3500×*g* for 15 min to obtain plasma or serum. These prepared samples were transferred to sterilized tubes and stored at − 20 °C until they were required for various biochemical and cytokine analyses. Additionally, to assess oxidative stress indicators, the hippocampus was extracted from the brain and placed on plates filled with ice. The hippocampal tissue sections were homogenized in chilled PBS (pH 7.4) using a Potter–Elvehjem homogenizer (Braun, Melsungen, Germany) with 1000 g for eight up-and-down strokes. Following filtration, the homogenate was spun in a Beckman TJ-6 centrifuge (Beckman Instruments; Munich, Germany) at 600×*g* for 10 min at 4 °C. Until the conclusion of numerous experiments, the clear supernatant was maintained at − 80 °C. The amount of total protein in the supernatant (/gm tissue) was calculated [20]. Before being employed for RNA extraction and RT-PCR analysis, some slices of brain tissues were stored at − 70 °C in sterilized Eppendorf tubes.

### 2.6 Evaluation of the oxidative stress

Prooxidant and antioxidant activities were carried out in the brain homogenate tissues in accordance with the assay kits' obtained production guide from MyBioSource Co. According to the approach of Uchiyama and Mihara [21], thiobarbituric acid reactive substances (TBARS), which were assessed as malondialdehyde (MDA) in tissue samples, were used to estimate the presence of lipid peroxidation products in brain homogenate. Nitric oxide was measured according to Montgomery and Dymock [22]; glutathione peroxidase activity [23]; catalase [24] and superoxide dismutase (SOD) activity was estimated kinetically [25].

### 2.7 ELISA and RNA isolation and qRT-PCR

Levels of different cytokines, e.g., IL-6; IL-10; IL-17; TNF-α, and TGF-β (pg/mg protein tissues), were estimated using ELISA Kit purchased from My Bio-source according to the manufacturer's protocol in triplicates. On the other hand, T regulatory cell markers, e.g., Forkhead box protein P3 (FOXP3) and cytotoxic T-lymphocyte antigen-4 (CTLA-4), were estimated by quantitative Real-Time PCR. SYBR Green, I was analyzed using an applied Bio-System with software version 3.1 (StepOne™, USA). The qPCR assay sets the target primer sequence at an annealing temperature of 60 °C for one min. Denaturation temperature at 95 C for one min and extension temp at 72 °C for one min (in one cycle) repeated for 40 cycles. The relative quantification of gene expression was performed using the comparative threshold (CT) method [26, 27]. Changes in mRNA expression level were calculated following normalization to GAPDH. All experiments were performed in triplicate independently. PCR (RT–PCR) used the rat-specific primer sequences as follows: FOXP3: F;5-TCATCCGCTGGGCCATCCTG-3; R;5-GTGGAAACCTCACTTCTTGGTC-3; CTLA4: F;5-GGACGCAGATTTATGTCATTGATC-3 and R;5-CCA AGCTAACTGCGACAAGGA-3; and GAPDH: F;5-GTG AAGGTCGGAGTCAACG-3 and R;5-CAATGCCAG CCCCAGCG-3.

### 2.8 Immunohistological tissue staining

The primary and secondary antibodies were purchased from Abcam (Cambridge, MA), which included anti-FoxP3 (Cat. #: AB75763) as primary antibody and Goat

Anti-Rabbit IgG H&L (DyLight 488 pre-adsorbed, AB96899) as secondary antibodies. Immunohistochemistry (Formalin/PFA-fixed paraffin-embedded sections) of rat brain tissue staining FOXP3 using antibody CAT # ab75763 with final conc. 1/100, DAB staining (in brown) and hematoxylin QS as a counterstain (in blue). Using ImageJ software is used to analyze the histoarchitecture of the FOXP3 positive cells. Six animals were used in each of the experimental groups to gather quantitative data; each individual had 3 to 4 high power fields to be examined, and the values were averaged to represent the animal.

### 2.9 Statistical analysis

To compare different groups, the data were analyzed using the Tukey–Kramer post hoc analysis approach. The mean and standard deviation (SD) of the results were reported. The Statistical Package for Social Science (SPSS) version 20 software (IBM Corp., 2011) was employed to analyze the obtained results. Subset for alpha = 0.05 and the means for groups in homogeneous subsets are displayed the subset of alpha = 0.05, and all values were represented as Mean ± Std and $n = 6$ animals/group. Means that within the same parameter and not sharing a common superscript symbol(s), are differ significantly at $P < 0.05$.

## 3 Results

Figure 1A–E illustrates that all prooxidants, e.g., lipid peroxidation and nitric acid, were elevated in the pilocarpine-induced epileptic group and recorded 108.33 ± 9.34 nmol/g. tissue and 56.08 ± 8.24 mmol/g. tissue, respectively), and both treatments with VPA or ThCF ameliorated levels of LPO (42.00 ± 4.50 and 48.88 ± 8.84, respectively) in addition to the reduced level of NO (10.56 ± 4.10 and 12.91 ± 4.19, respectively) near to normal levels 16.63 ± 2.42 mmol/l. On the other hand, EP-VPA recorded a significant improvement ($P < 0.001$) in hippocampal activity for all antioxidant enzymes; CAT, SOD, and GPx (6.58 ± 0.71, 26.64 ± 2.91 and 26.53 ± 3.45 U/g. tissue, respectively). This came in parallel with the oral inoculation with ThCF, which significantly elevated all antioxidant enzymatic activity, recording 4.60 ± 0.67 ($F$ value = 90.565; $P < 0.001$); 25.99 ± 3.61 (41.16, < 0.0001), and 39.17 ± 12.67 (18.445, 0.001) U/g. Tissue for CAT, SOD, and GPx, respectively (see, Table S1 in Additional file 1, which showed the changes in antioxidant markers and inflammatory cytokines).

Regarding IL-6, a cytokine with pleiotropic properties is generated as a response to tissue damage and infections. Figure 2B illustrated that IL-6 recorded a higher significant increase ($P < 0.001$) in epileptic animals (144.60 ± 7.54 Pg/mg protein tissue) as compared to controls (36.07 ± 3.14 Pg/mg protein tissue) (Fig. 2A). Significantly, treatment with valproic acid (EP-VPA) and *T. harzianum* culture filtrate (EP-ThCF) rebalanced IL-6 levels and recorded (58.33 ± 3.03 and 58.09 ± 9.64 Pg/mg protein tissue), respectively.

The present data showed that IL-10 was decreased significantly ($P < 0.001$) in homogenate tissues of epileptic rats (Fig. 2B) and recorded 98.12 ± 10.66 pg/mg protein tissue) as compared to controls (217.90 ± 6.03 pg /mg protein tissue).

On the contrary hand, VPA-treated (EP-VPA) animals displayed a significant elevation in IL-10 levels ($P < 0.0001$) in the hippocampus (188.43 ± 13.96 pg/mg protein tissue) parallel to oral inoculation with *T. harzianum* culture filtrate (EP-ThCF), which was significantly elevated ($P > 0.001$) and recorded 193.22 ± 11.87 Pg/mg protein tissue. Non-treated epileptic group (EP) recorded a very highly significant elevation ($P > 0.001$) in IL-17 level (Fig. 2C) in its hippocampal homogenate tissue (112.14 ± 6.61 pg/mg protein tissue) in comparison with the control group (37.33 ± 5.12 pg/mg protein tissue). Significantly, treatments with VPA or ThCF reduced levels ($P > 0.05$) of IL-17 and recorded 55.11 ± 5.35, and 60.56 ± 8.43 pg/mg protein tissue, respectively.

Current results illustrated (Fig. 2D) that TNF-α showed a significant increase ($P < 0.05$) in epileptic rats (91.40 ± 4.73 pg/mg protein tissue) if compared to the control groups (19.93 ± 4.28 pg/mg protein tissue). The present results showed a significant reduction ($P > 0.05$) in TNF-α's level in hippocampal homogenate tissue in EP-VPA (37.12 ± 3.81 pg/mg protein tissue). Furthermore, the oral treatment with ThCF ameliorated the TNF- α, which decreased to 34.08 ± 4.07 pg/mg protein tissue ($P > 0.05$).

Figure 3A illustrated a dramatically significant elevation ($P > 0.001$) in TGF-β in the pilocarpine-induced, which recorded 201.11 ± 13.43 pg/mg protein tissue; in comparison with that recorded in controls or EP-VPA or EP-ThCF (57.71 ± 3.36, 85.03 ± 13.10, 71.87 ± 10.32 pg/mg protein tissue, respectively).

FOXP3 plays a critical role in regulating the gene expression of regulatory T (Treg) cells and contributes to numerous potential suppressive effects. The present data (Fig. 3B) showed that FOXP3 gene expression recorded the highest level ($P < 0.001$) in EP (6.18 ± 0.69 relatives to controls 1.05 ± 0.08). While treatments with VPA or ThCF recorded a significant decrease (2.70 ± 0.23 and 2.74 ± 0.51, respectively). To confirm the data of FOXP3 gene expression, protein markers of Treg, FOXP3 via immunohistochemistry (IHC) staining showed that the epileptic hippocampus regions; *Cornu Ammonis* (CA1; CA2 & CA3) and dentate gyrus

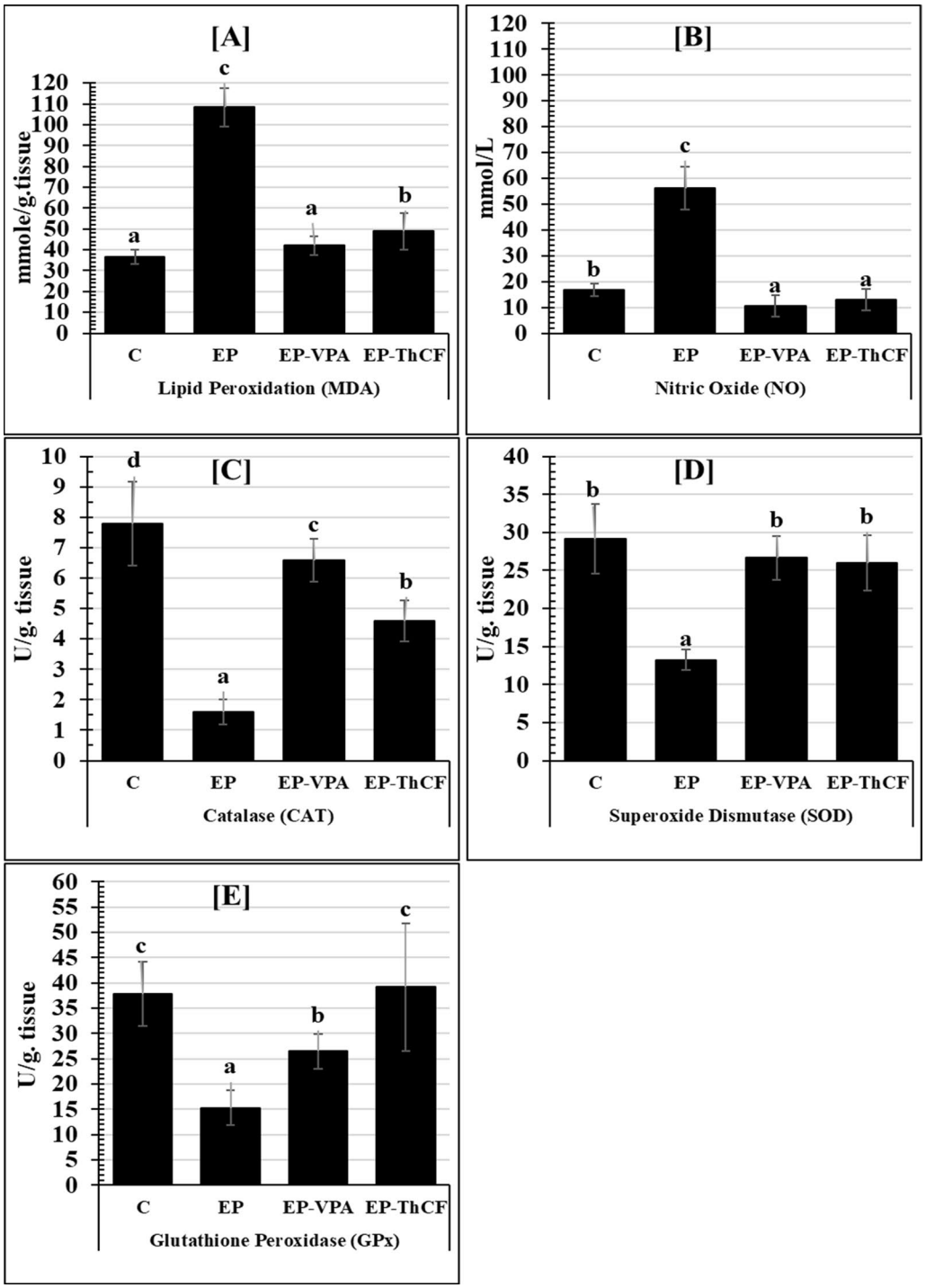


**Fig. 1** Biochemical estimation of some pro/and antioxidant biomarkers in hippocampal homogenate tissue of different groups: control (C), epileptic (EP), VPA-treated (EP-VPA), and epileptic treated with *T. harzianum* culture filtrate (E-ThCF). Values were represented as Mean ± SD and $n = 6$ animals. Means that within the same parameter and not sharing a common superscript symbol(s) differ significantly at $P < 0.05$

(DG), recorded 10.833 ± 1.472, 10.167 ± 1.47; 8.500 ± 1.05 and 14.500 ± 2.74 FOXP-3 positive cells, respectively, brains. While treatments with VPA or ThCF recorded a significant decrease (Table 1 and Fig. 4). On the other hand, the current data (Fig. 3C) recorded a significant elevation ($P < 0.0001$) of mRNA gene expression of

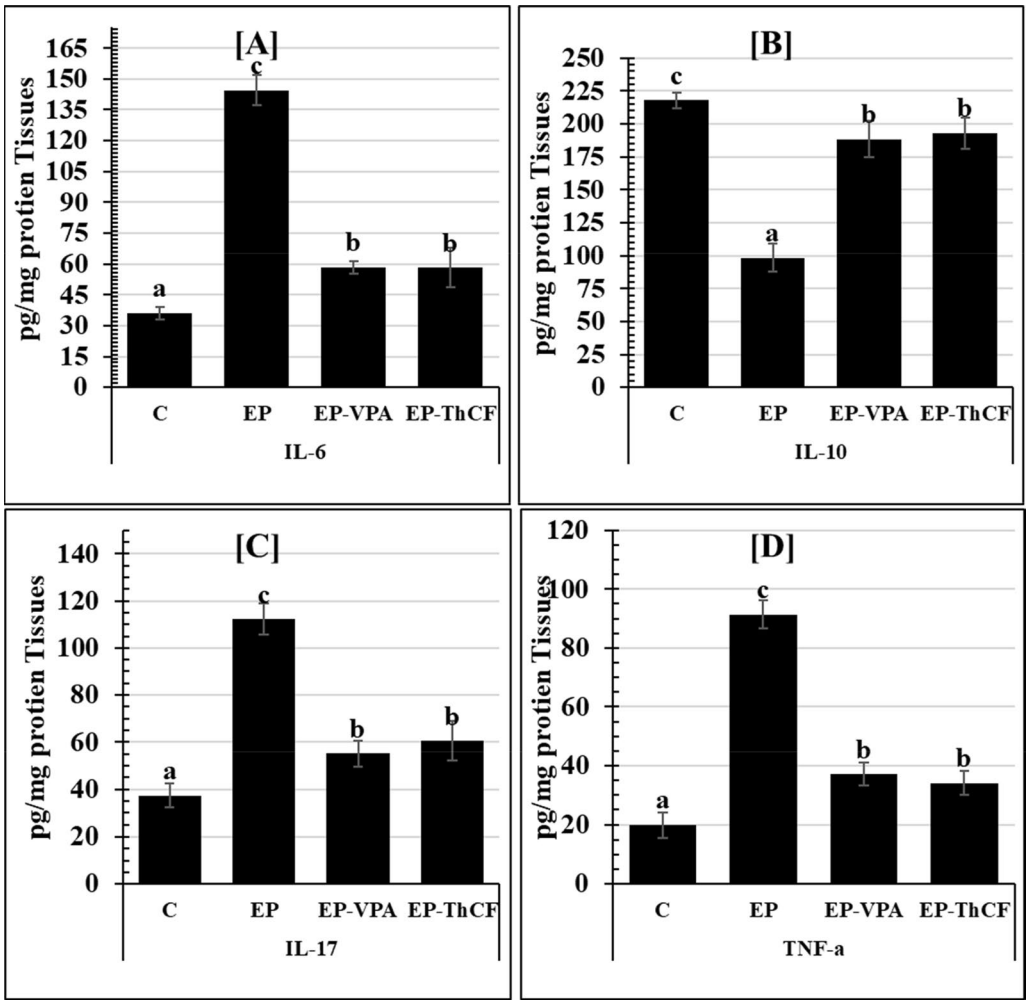


**Fig. 2** Changes in pro/and anti-inflammatory cytokines: **A** IL-6, **B** IL-10, **C** IL-17, and **D** TNF-α by ELISA in hippocampal homogenate tissues of different groups: control (C), epileptic (EP), VPA-treated (EP-VPA) and *T. harzianum* culture filtrate (E-ThCF). Values were represented as Mean ± SD and $n = 6$ animals. Means that within the same parameter and not sharing a common superscript symbol(s) differ significantly at $P < 0.05$

CTLA4 in EP (5.30 ± 0.75 relatives to control 1.01 ± 0.01). However, gene expression of mRNA of CTLA4 after treatment with VPA or ThCF showed a significant decrease ($P < 0.05$) (2.57 ± 0.35 and 2.39 ± 0.49, respectively) in contrast to EP (5.30 ± 0.75).

## 4 Discussion

There is a scientific shortage of clinical correlation regarding whether the therapeutic effect of antiepileptic drugs (AEDs) is mediated by their reducing proconvulsant cytokines or increasing release of protective cytokines. Therefore, the current study sought to delineate the ameliorative effects of secondary metabolites of the fungus *Trichoderma harzianum* culture filtrate (ThCF) in comparison with valproic acid as one of the AEDs through assessment of oxidative status and evaluation of the functions of T regulatory cells.

The data revealed that lipid peroxidation and nitric acid were elevated in pilocarpine-induced epileptic rats. Previously, convulsions and seizures are accompanied by high levels of free fatty acids (FFAs) in epileptic brain tissues [28]. Lipid peroxidation and the released arachidonic acid lead to cellular damage to plasma and mitochondrial membranes [29]. In addition, activated microglia during the development of epilepsy release cytotoxic and inflammatory substances such as nitric oxide (NO), reactive oxygen species, arachidonic acid metabolites, and prostaglandins [30]. Nitric oxide synthase (NOS) consists of three isoforms: endothelial NOS (eNOS), inducible NOS (iNOS), and neuronal NOS (nNOS).

Recently, neuroprotection processes in neuropathological conditions may be caused by the suppression of nNOS [31]. During the different pathological situations and neuropathological conditions, severe damage occurs

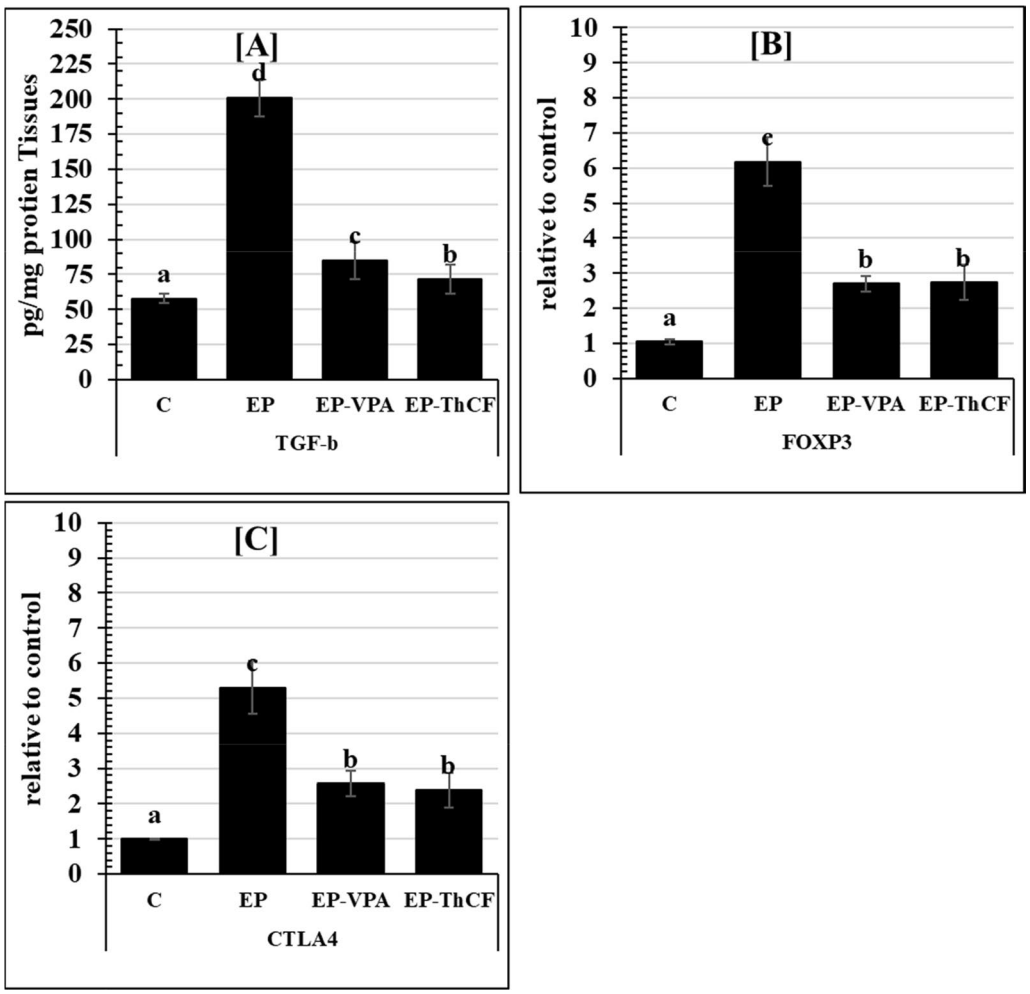


**Fig. 3** Changes in the mRNA gene expression of nuclear transcription factors of T regulatory cells in hippocampal homogenate tissues **A** TGF-b; **B** FOXP3 and **C** CTL4 of different groups: control (C), epileptic (EP), VPA-treated (EP-VPA) and *T. harzianum* culture filtrate (E-ThCF). Values were represented as Mean ± SD and $n = 6$ animals. Means that within the same parameter and not sharing a common superscript symbol(s) differ significantly at $P < 0.05$

**Table 1** Immunohistochemical analysis for FOXP3 positive cells in hippocampal region of different groups: Control (C), epileptic (EP), Valproic acid-treated (EP-VPA), and Culture Filtrate of *Trichoderma harzianum*–treated (EP-ThCF)

| Animal Groups | Hippocampus Region | | | |
|---|---|---|---|---|
| | ***Cornu Ammonis-1* (CA1)** | ***Cornu Ammonis-2* (CA2)** | ***Cornu Ammonis-3* (CA3)** | **Dentate Gyrus (DG)** |
| Control | $2.000 \pm 0.632^{a}$ | $4.500 \pm 1.049^{a}$ | $4.667 \pm 1.506^{a}$ | $2.667 \pm 0.516^{a}$ |
| EP | $10.833 \pm 1.472^{c}$ | $10.167 \pm 1.472^{b}$ | $8.500 \pm 1.049^{b}$ | $14.500 \pm 2.739^{b}$ |
| EP-VPA | $7.167 \pm 1.169^{b}$ | $3.333 \pm 1.966^{a}$ | $3.667 \pm 2.251^{a}$ | $4.833 \pm 0.983^{a}$ |
| EP-ThCF | $2.500 \pm 1.643^{a}$ | $3.833 \pm 3.125^{a}$ | $4.000 \pm 1.789^{a}$ | $4.833 \pm 4.167^{a}$ |
| *F* value | 63.208 | 14.316 | 10.291 | 25.771 |
| *P* value < | 0.000 | 0.000 | 0.000 | 0.000 |

The data were analyzed using the Tukey–Kramer method for post hoc analysis to compare various groups with each other. The means for groups in homogeneous subsets are displayed the subset of alpha = 0.05. And all values were represented as Mean ± Std and $n = 6$ animals/group. Means that within the same parameter and not sharing a common superscript symbol(s) differ significantly at $P < 0.05$. Using ImageJ software is used to analyze the histoarchitecture of the FOXP3 positive cells. Six animals were used in each of the experimental groups to gather quantitative data; each individual had 3–4 high power fields to be examined, and the values were averaged to represent the animal

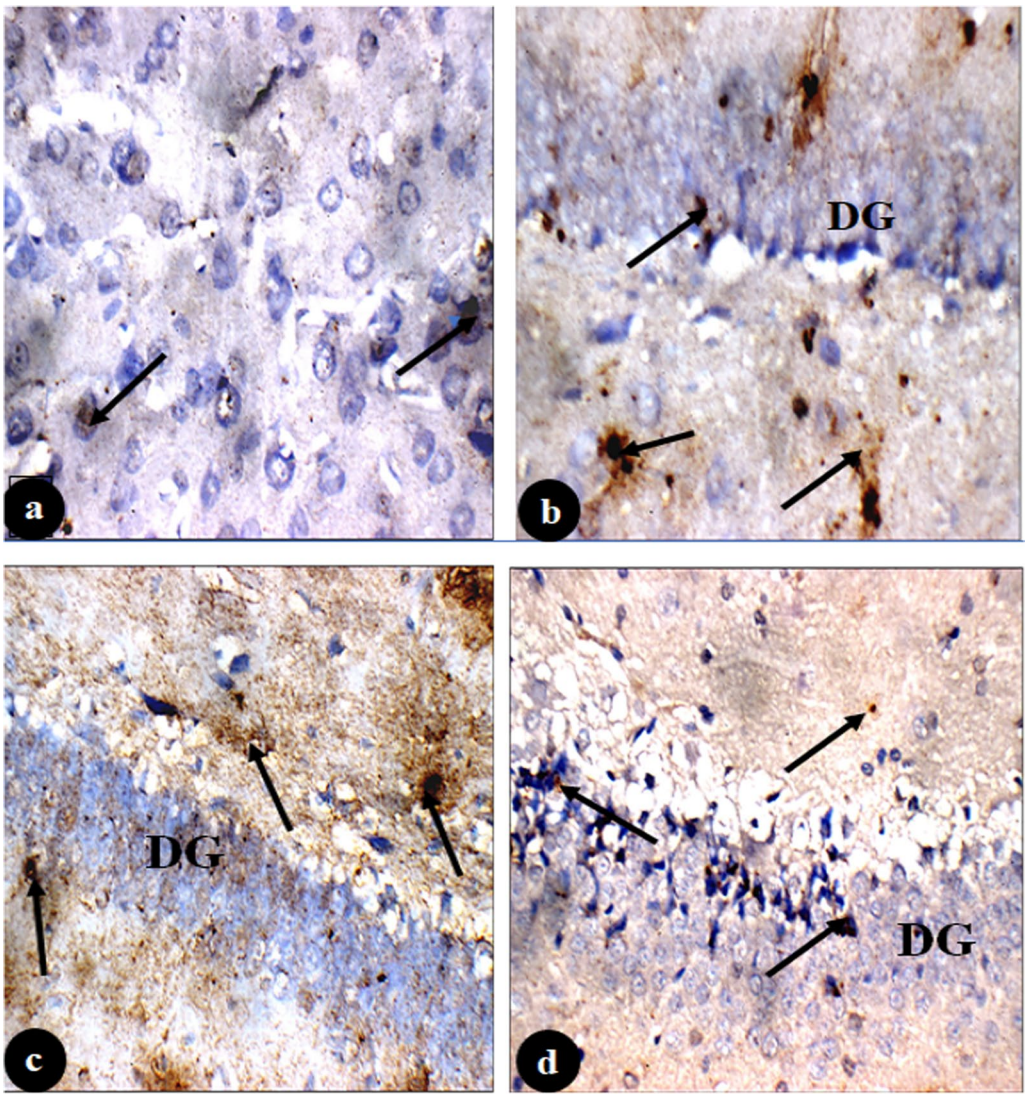


**Fig. 4** Immunohistochemistry photographs (Formalin/PFA-fixed paraffin-embedded sections) of rat brain tissue staining anti-FOXP3 using antibody CAT # ab75763 with final conc. 1/100, DAB staining (in brown) and hematoxylin QS as a counterstain (in blue). Photographs with black arrows showed positive FOXP3 bearing cells in brain tissues from different groups **a** control brain with 3 positive cells for FOXP3 (arrow, ×400); **b** epileptic DG's brain with positive cells for FOXP3 (arrow) in DG (×400), **c** VPA-treated DG with positive cells for FOXP3 (arrow, ×400) and **d** DG region of *T. harzianum* culture filtrate treated group with the positive cells for FOXP3 (arrow) in DG (×400)

to the cellular components, e.g., DNA, proteins, and lipids. These caused a generation of different free radicals and free radical scavengers [32]. Moreover, lipid peroxidation leads to peroxidative injury in the neural cellular membrane and is correlated with the processing of paroxysmal epileptiform activity [33].

Distinctively, the antioxidative enzymatic system was impaired during the present results. SOD activity was decreased in the frontal cortex, and activity of GPX was decreased in the pons-medulla, hippocampus, cortex, and cerebellum of epileptic brains. Also, the recorded less activity of antioxidant enzymes agreed with the decreased levels of Zn- and Cu-SOD activity recorded in epileptic mice [28].

On the other hand, the effect of many AEDs on immune response showed that phenobarbital and valproic acid (VPA) can retarded the T lymphocyte response either in humoral or cytotoxic mice, according to Sonmez, Serin [34]. They noticed that topiramate (TPM) and valproic acid (VPA) reduced the release and levels of some cytokines; IL-10, IL-1a, IL-1b, IL-6, and TNF-α, as well as low levels of serum immunoglobulin, in patients with idiopathic generalized and partial epilepsy's blood levels of interleukin.

Regarding retarded levels of prooxidant markers such as lipid peroxidation and nitric oxide after treatment with culture filtrate of *T. harzianum* may be due to the effects of harziane-type diterpene derivatives released by *Trichoderma* sp. [35]. Moreover, Laokuldilok, Potivas [36] reviewed that chitooligosaccharides (COSs), one of *T. harzianum* metabolites, have antioxidant activity and retard the lipid oxidation rate when used as a food preservative ingredient in food products.

The current antioxidant scavenging properties recorded after treatment with culture filtrate of *T. harzianum* may be due to the biological activities of thiodioxopiperazines produced by *T. harzianum*. In addition, platelet-activating factor (PAF), as one of the most potent lipid messengers, is produced by *T. harzianum* [37]. Plasma PAF-AH is associated with plasma lipoproteins and scavenges the oxidized phospholipids during various pathological processes, e.g., disorganization of membrane structure and PAF-like proinflammatory action. Recently, the ethanolic extracts of the fungus *T. stromaticum* downregulate the inflammatory cascade reactions, reduce the cerebral expression of VCAM-1 and ICAM-1 and ameliorate ECM, thereby preserving the integrity of the blood–brain barrier [38]. Moreover, the recorded antioxidant activity of ThCF may be due to the presence of silver nanoparticles (AgNPs), one of the most bioactive metabolites produced by *T. harzianum*. AgNPs showed significant antioxidant properties, as ferric reducing antioxidant power (FRAP) was observed by measuring the ferric reducing antioxidant power repeatedly using FRAP, and diphenylpicrylhydrazyl (DPPH) assays [39].

Mastouri, Björkman [40] approved that the protective capacity enhanced by *T. harzianum* T22 colonization supports many protective mechanisms via their high capacity to scavenge ROS and reuse the oxidized forms of ascorbate and glutathione pathways. It was noticed that the enzymes peroxidase, phenylalanine ammonia-lyase, polyphenol oxidase, superoxide dismutase, glutathione reductase catalase, and ascorbate peroxidase are upregulated and increased by *Trichoderma harzianum* and hence improve the ability to reducing damaging ROS in plants [40]. The protection against reactive oxygen damage was correlated with high expression of antioxidant enzymes and stimulated more scavenging or removing damaged features after ROS, especially $O_2^-$ and $H_2O_2$.

Recently, the neuroglia inflammation diminished by the upregulation of many pro-inflammatory mediators; pro-IL-1β, IL-6, TNF-α, and many chemokines [41]. Moreover, activated microglia by ATP, ROS, or glutamate in the microenvironment will produce many chemical mediators for inflammation processes in CNS, e.g., IL-1a and TNF-a [42]. A suppressive type of CD4+T helper lymphocytes, regulatory T cells (Tregs) are crucial for controlling immune reactions. The CTLA4 and FOXP3 expression identifies the most well-characterized Tregs. Significantly, the present data showed that mRNA expression of TNF-a, IL-17, TGF-b, CTLA4, and FOXP3 was up-regulated in epileptic groups.

Significantly, IL-6 and IL-17 in cerebrospinal fluid were noticed in epileptic individuals [43]. Recently, it was recorded that mRNA levels of IL-17a, IL-6, IFN-γ, and IFN-λ3 cytokines were correlated with many types of epilepsy; XLE, TLE and IGE [44]. Therefore, a cascade of subsequent inflammatory events that may attract cells of the adaptive immune system occurs after an increase in IL-1, IL-6, and TNF-α in astrocytes and microglia [45]. Previous research has indicated that IL-6 is a pleiotropic cytokine that is expressed and increased in the sera of numerous neurological conditions including epilepsy, meningitis and Alzheimer's disease [46]. Previously, IL-6 was suggested to protect against injury in the brain in case of acute epileptic seizures. Long-term overexpression of IL-6 may worsen epilepsy and even result in gliosis and a reduction in the number of neurons in the tissue [47].

In addition, IL-17 is shared in the progress of different neuroinflammatory disorders and correlated with disease severity [48]. IL-17a facilitates the migration of active Th17 lymphocytes across BBB (blood–brain barrier) [43]. Wang, Wang [44] proposed the possible role of IL-17 throughout the development of seizure indirectly. Moreover, injured endothelial cells and the blood–brain barrier (BBB) may release a set of regulatory cytokines, which activate T cells and trigger many immunological cascade reactions in the brain. Therefore, high-release levels of inflammatory chemicals will up-regulate in different stages of epilepsy [49].

It is well known that IL-2 can up-regulate many pro-survival proteins that share homeostasis of the peripheral T-reg [50]. Significantly, gliovirin, as one of the Epipolythiodiketopiperazine (ETP) alkaloids, can reduce IL-2 mRNA levels. Reducing IL-2 may drive thymic-Treg's downregulation via FOXP3 & STAT5 [51] and consider a critical messenger for TGF-β-induced peripheral T-reg and FOXP3 expression [52]. Therefore, the recorded downregulation of all T-reg markers after treatment with ThCF may result from its content of ETP-alkaloids that can reduce IL-2 mRNA levels.

Previously, TGF-β and IL-6 were reported to differentiate naive T cells into Th-17 cells [53]. Moreover, IL-1β, IL-6, TGF-β+, IL-23 and IL-21 induce IL-17 secretion via differentiation of Th17 [54]. In addition, IL-10 and TGF-β secreted by T-regs alter the production of inflammatory Ig-E antibodies to the non-inflammatory isotypes IgG4 and IgA, respectively [55]. TGF-β signaling was reported to be active in astrocytes during epileptogenesis

[56]. High levels of TGF-β lead to many transformations in astrocytes and inhibition of GABA-related genes and inflammatory signals [57]. Since TGF-β1 regulates cell function in the brain via Tregs FOXP3 and CTLA4, the present results revealed their high expression profiles in epileptic hippocampal tissue. TGF-beta can coordinate with other inflammatory cytokines (IL-6 and IL-21) to promote the development of Th-17 cells [58].

In disease states like cancer, fibrosis, and inflammation, TGFs are frequently chronically over-released. These high levels of TGFs stimulate disease processing by altering cell growth, migration, or phenotype. Pilocarpine-induced TGF-β1 up-regulation during epileptogenesis was prevented by treatment with VPA or ThCF. These findings suggested that under conditions of a dysfunctional BBB, TGF-β signaling represents a promising therapeutic target for the prevention of experimental epilepsy. Treg supplementation protects animals from neurological diseases by reducing inflammation in neuroglia and peripherally derived leukocyte infiltration [59].

## 5 Conclusions

The present data strongly support the vital role of secondary metabolites of *T. harzianum* in mitigating the side effects of antioxidant and anti-neuroinflammatory therapy. Therefore, *T. harzianum* exhibits potent antioxidant activity and effectively scavenges ROS, while downregulating T-reg markers. Additionally, this study suggested to identify new active constituents of *T. harzianum's* secondary metabolites with therapeutic potential in the clinical and experimental opportunities to reduce the drawbacks and side effects of AEDs.

**Abbreviations**

| | |
|---|---|
| AEDs | Anti-epileptic drugs |
| BBB | Blood–brain barrier |
| CAT | Catalase |
| CNS | Central nervous system |
| COSs | Chitooligosaccharides |
| CT | Comparative threshold |
| CTLA-4 | Cytotoxic T-lymphocyte-associated protein 4 |
| DPPH | Diphenylpicrylhydrazyl assays |
| ELISA | Enzyme-linked immunoassay |
| ETP | Epipolythiodiketopiperazine alkaloids |
| ECM | Extracellular matrix |
| XLE | Extra-temporal lobe epilepsy |
| FRAP | Ferric reducing antioxidant power assay |
| FOXP3 | Forkhead box P3 protein |
| GPX | Glutathione peroxidase |
| IGE | Idiopathic generalized epilepsy |
| ICAM-1 | Intercellular adhesion molecule 1 |
| IL-10 | Interleukin-10 |
| IL-17 | Interleukin-17 |
| IL-6 | Interleukin-6 |
| LPO | Lipid peroxidation |
| MDA | Malondialdehyde |
| NO | Nitric oxide |
| PBS | Phosphate buffer saline |
| PILO | Pilocarpine hydrochloride |
| PDA | Potato dextrose agar |
| qRT-PCR | Quantitative real-time polymerase chain reaction |
| AgNPs | Silver nanoparticles |
| SOD | Superoxide dismutase |
| T reg. | Regulatory T-lymphocytes |
| *T.* | *Trichoderma* |
| ThCF | *T. Harzianum* culture filtrate |
| TLE | Temporal lobe epilepsy |
| TBARS | Thiobarbituric acid reactive substances |
| TGF-β | Transforming growth factor-beta |
| TNF-α | Tumor necrosis factor-alpha |
| VPA | Valproic acid |
| VCAM-1 | Vascular cell adhesion molecule 1 |

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s43088-023-00425-1.

**Additional file 1. Table S1.** Changes in the biochemical estimation of some pro/and antioxidant biomarkers, pro/ and anti-inflammatory cytokines in the hippocampal homogenate tissue of different groups: control (C), epileptic (EP), VPA-treated (EP-VPA), and epileptic treated with *T. harzianum* culture filtrate (E-ThCF). Means for groups in homogeneous subsets are displayed the subset of alpha = 0.05. and all values were represented as Mean ± Std & n = 6 animals/group. Asterisks represent significant post-Hoc one-way ANOVA results of improvement over time, whereas n.s. means non-significant difference p>0.05; *means p<0.05; ** p<0.01 and *** <0.001. This scale is applied for seizure-related behavioral changes and their severity. Each number represents the latency and duration of the seizure severity. The data were analyzed using the Tukey-Kramer method for post-hoc analysis to compare various groups with each other.

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Not applicable.

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**Availability of data and materials**
The datasets (Tables and Figures) generated and analyzed during the current study are available from the corresponding author upon reasonable request. All data generated or analyzed during this study are included in the present article [and its Additional file 1 will be available from the corresponding author on reasonable request.

## Declarations

**Ethics approval and consent to participate**
All procedures were performed according to the guidelines for the care and use of laboratory animals and approved by the animal research ethics committee of Taif University, which was under an approval number: 44-172.

**Consent for publication**
Not applicable.

**Competing interests**
The author has no potential conflict of interest with any groups.

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