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Original Article

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In Vitro Antioxidant Activity and Anticonvulsant Properties on Zebrafish PTZ-Induced Seizure Model of a *Tilia viridis* Aqueous Extract

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Objectives: *Tilia viridis* (Bayer) Simonk. (Malvaceae) is widely distributed in Argentina and employed for its tranquilizing properties. Other species of the genus (*Tilia europaea* L., *Tilia cordata* Mill., *Tilia platyphyllos* Scop.) have been traditionally used for the treatment of epilepsy. Epilepsy affects approximately 65 million people worldwide and is characterized by an imbalance between excitatory and inhibitory processes in the brain, leading to unpredictable, unprovoked, recurrent seizures. Current pharmacological interventions often present mild to moderately severe side effects. Epilepsy has been associated with oxidative and nitrative stress as well as neuroinflammation. Herbal medicine therapies may offer new treatment options with multi-target antioxidant and anticonvulsant effects for patients whose seizures remain uncontrolled, potentially providing cost-effective solutions for individuals worldwide suffering from uncontrolled epilepsy.The aim of this study was to demonstrate the anticonvulsant activity of a standardized *T. viridis* aqueous extract (TE).
Methods: Study of the constituents of TE, TE's antioxidant and anticonvulsant activities and toxicity, and analysis of the possible relation between the potential activities and the compounds present in the extract. In order to demonstrate TE's anticonvulsant activity a zebrafish model was used. The study also assessed TE's toxicity and antioxidant activity. To standardize the extract, total polyphenols and flavonoids were quantified and specific flavonoids were identified and quantified using HPLC-MS/MS and HPLC-UV.
Results: TE exhibited anticonvulsant activity at low concentrations and demonstrated antioxidant effects by scavenging free radicals, exhibiting superoxide dismutase and peroxidase-like activities, as well as inhibiting lipoperoxidation. These actions can be attributed to the presence of polyphenols, particularly flavonoids.
Conclusion: TE holds promise as a complementary herbal medicine in the treatment of epilepsy and may also offer benefits for other neuropathies associated with oxidative stress, such as Parkinson's disease and Alzheimer's disease.

Keywords: epilepsy, flavonoids, infusion, natural products

INTRODUCTION

Epilepsy is a chronic and debilitating brain disease characterized by an imbalance of excitatory and inhibitory processes that lead to seizures [1]. Seizures are unpredictable, unprovoked, and recurrent. They are characterized by the sudden

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and transient onset of symptoms, such as uncontrollable movements, loss of consciousness or control, and confusion, caused by synchronous, irregular, and excessive neuronal activity [2]. The main goal of epilepsy treatment is to eliminate or reduce the frequency of seizures [3]. Currently, pharmacological intervention is the primary treatment approach, but only 70% of patients achieve seizure control with antiepileptic drugs [4]. In addition, mild-to-moderate side effects such as skin hypersensitivity, visual disturbances, or weight gain, have been reported [5]. Primary generalized epilepsies are often treated with valproic acid (VPA), which requires a high therapeutic dose and is associated with impaired abstract thinking and memory and increased risk of pancreatitis and liver failure [6].

Epilepsy is associated with oxidative and nitrosative stress and neuroinflammation, processes that are also linked to stroke, age-related dementia, and carcinogenesis [7]. The brain is vulnerable to the effects of free radicals, as it contains few enzymatic antioxidants, such as catalase or glutathione peroxidase [7]. Consequently, these free radicals can oxidize neurotransmitters, such as serotonin, dopamine, or noradrenaline, disrupting their function. Neuronal membranes, which are rich in polyunsaturated fatty acids, are susceptible to lipid peroxidation, resulting in reduced membrane fluidity and altered transport [7]. Research should focus on drugs with fewer adverse effects that can be used long-term to prevent or treat epilepsy. Medicinal plants have potential as they contain novel antiseptic compounds with antioxidant and antispasmodic activity [8].

Tilia viridis (Bayer) Simonk. is a plant widely used in Argentina for its sedative properties. Researchers have examined the antioxidant activity of this plant's aqueous extracts [9]. Species of this genus are traditionally used for the treatment of epilepsy. *Tilia platyphyllos* Scop. was used by the Mayan civilization [10], dating back to the 16th and 17th centuries. The ethnopharmacological use of *Tilia cordata* Mill. [11] and *Tilia europaea* L. against epilepsy has also been reported [12], and Tilia species have traditionally been used to treat digestive disorders such as gastroenteritis and enterocolitis. Their therapeutic activity is primarily attributed to their anxiolytic properties [13].

This study investigated the potential anticonvulsant effects and toxicity of a standardized 5% aqueous *T. viridis* extract (TE) using a zebrafish model. The pentylenetetrazole (PTZ)-based zebrafish swim model, which mimics generalized tonic-clonic seizures and epileptiform brain activity, is a valuable model for studying antiepileptic drugs [14]. The neurophysiology of the zebrafish is similar to that of mammals. We also evaluated the antioxidant properties of TE, which were standardized for total polyphenol and flavonoid content, and identified and quantified specific flavonoids and caffeoyl derivatives by HPLC MS/MS and HPLC-UV.

MATERIALS AND METHODS

1. Plant material and extract preparation

We collected *Tilia viridis* (Bayer) Simonk (Malvaceae) inflorescences in Buenos Aires in January. Drs. Gustavo Giberti and Hernán Gerónimo Bach authenticated the plant material, and a voucher specimen (BAF 17414) was deposited at the Museum of Pharmacobotany of the Faculty of Pharmacy and Biochemistry of the University of Buenos Aires. The extract, an infusion of 5% (w/v), was prepared according to the Argentine National Pharmacopoeia, edition VII, as follows: 50 g of dried inflorescences were mixed with distilled boiling water (1 L) for 20 min. The extract was then filtered and freeze-dried. The final yield, expressed as a percentage of the original plant material, was 22.86 ± 0.24%.

2. Phytochemical studies

1) Quantification of polyphenols and flavonoids

We determined the total polyphenol content by the Folin-Ciocalteu method using a UV-Vis spectrophotometer (Jasco 630, Tokyo, Japan). In brief, 5 mL of Folin-Ciocalteu reagent, 10% (v/v), was added to 1.0 mL of TE (dissolved in water). Next, 4 mL of Na_2CO_3 solution (7.5% w/v) was added after 3-8 min. Approximately 60 min later, the absorbance was measured at 765 nm. We used a standard curve of gallic acid (10-50 μg/mL; $r^2 = 0.9996$) to determine TE's polyphenol concentration. The results were expressed as %w/w [15].

We quantified the total flavonoids in TE following Chang et al.'s methodologies [16]. We used a standard curve of quercetin, and added 1.5 mL of distilled water, 0.1 mL of 1M potassium acetate, 0.1 mL of 10% (w/v) aluminum chloride, and 2.8 mL of distilled water to 0.5 mL of TE. We then measured the absorbance after incubation for 30 min at room temperature. The results were expressed as %w/w.

2) Identification of flavonoids and phenolic acids by HPLC-MS/MS

We used HPLC-MS/MS to identify flavonoids and phenolic

acids in TE. We used an UltiMate 3000 coupled to a TSQ Quantum Access MAX Triple Quadrupole mass spectrometer with an ESI ionization source (Massachusetts, USA). The calibration conditions were as follows: the spray voltage was 3.5 kV; the vaporizer temperature was 233℃; the capillary temperature was 314℃; the sheath gas pressure was 10 units, and the auxiliary gas was 45 units. We utilized both single ion monitoring (SIM) and selective reaction monitoring (SRM) methods. We used the positive mode for flavonoid glycosides and the negative mode for phenolic acids. We used a Hypersil C18 (150 mm × 4.6 mm and 5 μm particle diameter) column. The mobile phase consisted of A: water: 0.1% formic acid and B: methanol: 0.1% formic acid, and was run in gradient form, 15% B - 40% B for 25 min; B 40% for 25 min, 40% B - 85% B for 10 min, 100% B for 5 min, and B 100% for 10 min. The flow rate was 1.0 mL/min.

3) Identification and quantification of epicatechin and tiliroside

We determined the epicatechin content in TE according to the method described by the Institute for Nutraceutical Advancement, as used by Zokti et al. [17], using a Varian Pro Star HPLC-UV device. We used a Rheodyne injection valve (20 μL) and set the oven at 30℃. We selected 280 nm as the wavelength for the photodiode array detector and an Agilent Zorbax Eclipse XDB-C18 (250 mm × 4.6 mm and 5 μm particle diameter) as the column. The mobile phases were composed of A: H_2O with 0.12% phosphoric acid and B: acetonitrile, and the flow rate was 1.0 mL/min [17]. The elution gradient ranged from 15%-25% B over 12 min, and this mobile phase composition was maintained until the 20th min. Subsequently, B was reduced to 15% by the 22nd min and was constant until the 30th min. We performed the identification and quantification by comparing the retention times (rt) and peak areas obtained with a commercial epicatechin standard (Sigma) with those of the TE sample. We dissolved TE in high-purity quality water (Milli-Q) and the epicatechin standard in methanol. We prepared the working dilutions with Milli-Q. The acetonitrile, methanol (J.T. Baker), and phosphoric acid (Carlo Erba Reagents) were HPLC grade.

We determined the tiliroside content in TE using the same equipment, temperature, and column as previously described [18]. The flow rate: 1.2 mL/min; Detector: 325 nm; Mobile phase: A: water: acetic acid (98:2) and B: methanol: acetic acid (98:2). The elution gradient: from 15% to 40% B over 30 min, from 40% to 75% in the next 10 min, from 75% to 85% in 5 min, finally 100% B in the following 5 min. TE and tiliroside standard (Extrasynthese) were dissolved in methanol: water (70:30).

3. Pharmacological study

1) Zebrafish husbandry and embryo collection

We kept adult wild-type zebrafish (Danio rerio) in glass aquaria at 28 ± 1℃ with a 14/10 h light/dark cycle. The water in the aquaria was constantly aerated and maintained at a pH of 7.0-8.0. We used TetraMinPRO® dry food (three times a day) and live brine shrimp (*Artemia persimilis*) VitaFish, Argentina (once a day) for the fish feeding. We obtained the zebrafish embryos by natural pair mating. For embryo production, we paired three females and two males in plastic net traps the night before spawning to protect the eggs from cannibalism. We selected eight to twelve-month-old sexually mature males and females. The first light stimulus after the dark cycle triggered spawning. After fertilization, we preserved the collected embryos in E3 medium (NaCl 0.29 g/l, KCl 0.012 g/l, $CaCl_2$ 0.036 g/l, and $MgSO_4$ 0.039 g/l in deionized water and 50 ppb methylene blue, a fungal growth inhibitor). We selected fertilized eggs in good condition for further treatment. We determined egg characteristics with a stereomicroscope (Leica Zoom 2000, Wetzlar, Germany). In this study, embryos refer to zebrafish before hatching (zero to three days post-fertilization (dpf)), while larvae refer to animals after hatching (over three dpf) [19].

2) Ethics statement

We conducted all animal studies in strict accordance with the National Institutes of Health guidelines for animal care and husbandry. All protocols were approved by the Institutional Animal Care Committee of the Universidad Nacional de Quilmes (CE-UNQ 2/2014) (Buenos Aires, Argentina).

3) Anticonvulsant activity evaluation

In a 96-well plate that was maintained at 28 ± 1℃ containing E3 medium, we transferred three unhatched embryos per well one dpf. We stimulated zebrafish larvae at five dpf to exhibit epileptic behavior with the proconvulsant PTZ [14]. We exposed five dpf larvae to PTZ (0.25 to 25 mM). We then quantified spontaneous swimming activity. Subsequently, we selected the PTZ that resulted in a significant difference in spontaneous movements compared to untreated larvae for further experimentation with VPA (100 mM) and TE as anticonvulsants. The PTZ control group was only exposed to PTZ.

To investigate the anticonvulsant effect of TE, we incubated five dpf zebrafish larvae in E3 media supplemented with TE (0.1, 1, 10, 50, 100, 250, and 500 μg/mL) or VPA 1 h before PTZ exposure. We measured swimming activity after the PTZ was added.

4) Measurement of spontaneous swimming activity

We assessed spontaneous swimming activity by measuring cumulative spontaneous movements during a 15-min tracking period, 10 min and 1 h after PTZ treatment. For this purpose, the refraction of light as it passes through the zebrafish body was detected by an infrared microbeam [14, 19]. In brief, we illuminated zebrafish with two infrared microbeams per each well of a 94-well plate. The larvae moving through the light beam caused a transient signal fluctuation, which was detected by a phototransistor array. This output was digitized by a multichannel ADC system (WMicrotracker, Designplus SRL). We continuously calculated signal activity events, defined as the number of times the larvae traversed the infrared microbeams, in real-time by capturing these slight fluctuations. We considered swimming activity the total number of these events over 15 min.

5) In vivo toxicity assessment on zebrafish larvae

At one dpf, we transferred three unhatched embryos to each well of a 96-well plate containing E3 medium at 28 ± 1℃. We treated zebrafish larvae at five dpf as previously described, either in the presence or absence of PTZ (5 mM) and VPA (100 mM) or TE (0.1 to 500 μg/mL). After 24 h, we examined each larva for survival, posture, edema, signs of necrosis, morphology, and swim bladder health. The median lethal concentration (LC_{50}) for TE was determined by linear regression and extrapolation of the 50% mortality [20].

4. *In vitro* antioxidant activities

1) DPPH free radical scavenging activity

We assessed antioxidant activity by measuring the ability to scavenge diphenyl-2-picrylhydrazyl (DPPH) [21]. In brief, we diluted TE with Milli-Q to produce solutions (0.001-1,000 μg/mL). We used Vitamin C as an antioxidant reference standard. First, we added 100 μL of each sample solution to a vial. Then, we added 400 μL of a 100 mM Tris-hydrochloride buffer and 500 μL of a 500 μM DPPH solution in absolute ethanol. The samples were then incubated in the dark for 20 min, and the absorbance was measured at 517 nm. We prepared a DPPH control using 100 mM of Tris hydrochloride buffer. We used the equation: Radical scavenging activity (%) = [(control Abs. - sample Abs.)/control Abs.]*100 to calculate the results.

2) Inhibition of lipid peroxidation: TBARS determination of egg yolk

We mixed 25 μL of diluted TE (0.1 to 2000 μg/mL) with 100 μL of distilled water and 125 μL of a 10% (v/v) egg yolk solution prepared in 1.15% (w/v) potassium chloride. We then added 375 μL of a 20% acetic acid solution and 375 μL of a 1% (w/v) thiobarbituric acid in 1.1% (w/v) sodium lauryl sulfate solution. We heated the mixture in a water bath at 95℃ for 90 min. We then added 1.250 mL of butanol and shook each tube for 10 s. After centrifugation (3,000 rpm, 10 min), we measured the butanol layer's absorbance at 532 nm. The results were calculated as: Inhibition of lipid peroxidation (%) = [(A0-As)/A0]*100, where A0 is the oxidation control's absorbance and As is the sample's absorbance [22].

3) Superoxide dismutase-like activity

We determined superoxide dismutase (SOD)-like activity by assessing TE's ability to inhibit the auto-oxidation of epinephrine to adrenochrome in the presence of atmospheric oxygen at a high pH [23]. We first mixed 50 μL of TE (0.01-2,000 μg/mL), 910 μL of phosphate buffer at pH 10.7, and 40 μL of 2 mM epinephrine (Sigma, St Louis, MO, USA). We measured the absorbance at 480 nm every 10 s for 5 min. The antioxidant activity was calculated as Inhibition of epinephrine autooxidation (%) = [(ΔAbs/min epinephrine-ΔAbs/min sample)/ΔAbs/min epinephrine]*100. Then we calculated the activity in U/mL assuming that 1 U inhibits the autooxidation of epinephrine by 50%.

4) Peroxidase-like activity

We incubated 200 μL TE (100 to 1,000 μg/mL) with 775 μL 3,3'-diaminobenzidine (DAB) (5×10^{-4} M) and 25μL H_2O_2 0.030 M, with DAB without H_2O_2 as a blank. The final volume in the reaction tube was 1,000 μL. We recorded the changes in absorbance for 5 min at 10s intervals at 465 nm and calculated the Δabsorbance/min. We constructed a calibration curve of peroxidase concentration versus Δabsorbance/min using horseradish peroxidase (linear relationship of 1.95×10^{-3} to 2.5×10^{-5} U/mL). We determined the peroxidase activity in triplicate, interpolating from the standard curve [24].

We calculated the IC_{50} (inhibitory concentration at 50%) for the DPPH or phospholipid peroxidation assays and the EC_{50} (effective concentration at 50%) for the SOD and peroxidase-like activity assays using the concentration-response curves. These calculations are based on the right triangle principle: IC_{50} or EC_{50} = D-[(A-50% max response)*X]/Y. A is the immediately higher response of 50% of the maximum response; B is the immediately lower response of 50% of the maximum response; D is the logarithm of the concentration corresponding to response A; C is the logarithm of the concentration corresponding to response B; X = D-C; and Y = A-B [25].

Table 1. **Quantification of total polyphenols and flavonoids by spectrophotometry and epicatechin and tiliroside by HPLC-UV in TE**

Polyphenols (%w/w)	Flavonoids (%w/w)	Epicatechin (%w/w)	Tiliroside (%w/w)
10.74 ± 0.79	1.94 ± 0.13	0.156 ± 0.003	0.119 ± 0.003

5. Statistical analysis

Statistical analyses were performed using GraphPad Prism 8 software (Inc., USA). In all analyses, we assumed statistical significance if the p-value was less than 0.05 ($p < 0.05$), indicated by *; 0.01, indicated by **; 0.001, indicated by ***; and 0.0001, indicated by ****.

RESULTS

1. Phytochemical analysis

TE's total flavonoids and polyphenols content is detailed in Table 1. We identified and quantified tiliroside (rt: 39.5 min) and epicatechin (rt: 7.2 min) by HPLC-UV (Table 1; Fig. 1). We used HPLC-MS/MS to identify and quantify the other polyphenolic compounds (Table 2). For more information, see Supplementary Material.

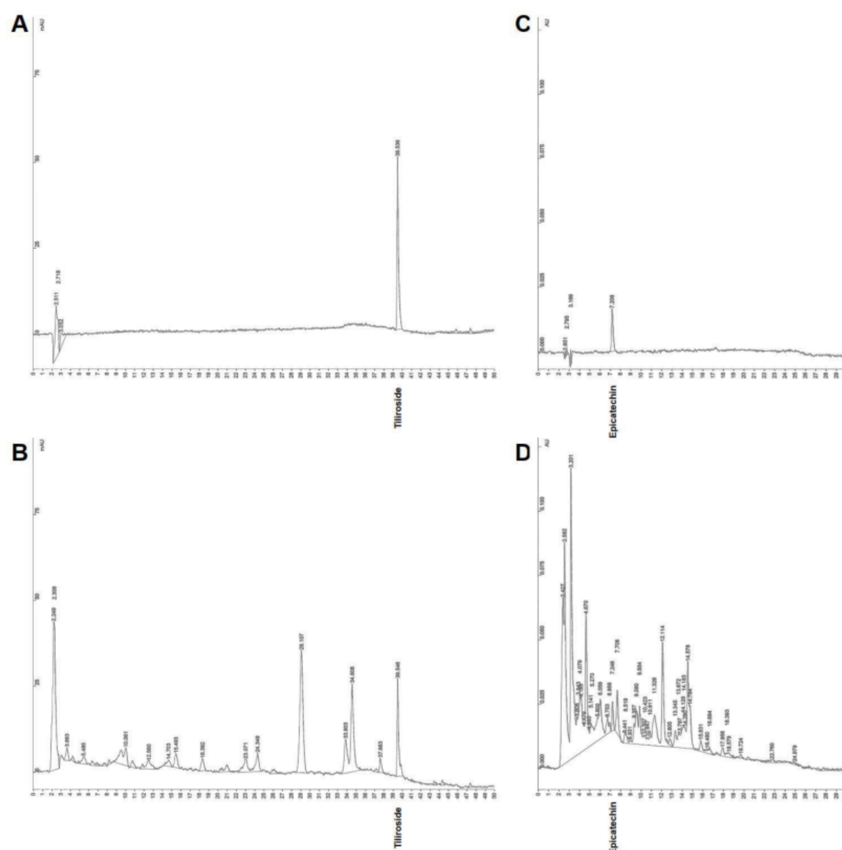

Figure 1. HPLC-UV chromatogram of TE. (A) Tiliroside at 325 nm; (B) TE at 325 nm; (C) Epicatechin at 280 nm; (D) TE at 280 nm. Representative chromatograms are shown based on three determinations.

2. Measurement of spontaneous swimming activity: TE's anticonvulsant effects

We found that 5 mM was the PTZ concentration that induced epileptiform behavior. This resulted in erratic swimming characterized by involuntary and rapid circular movements, especially along the sides of the well. This concentration led to a 300% increase in swimming activity compared to untreated larvae, both 10 min and 1 h after administration. Although 25 mM PTZ also resulted in increased movement after 10 min, it exhibited high toxicity, with some larvae dead after 1 h. Therefore, we selected 5 mM for subsequent experiments (Fig. 2).

Table 2. Quantification of caffeoyl derivatives and flavonoids by HPLC-MS/MS

Compound	Concentration (%w/w)
Caffeic acid	0.0037
Caffeic acid isomer (as caffeic acid)	0.051
Chlorogenic acid	0.0051
Chlorogenic acid isomer (as chlorogenic acid)	0.00082
Q-3-O-xyloside	0.014
Q-3-O-arabinopyranoside	0.0069
Q-3-O-glucoside	0.15
Q-3-O-rhamnoside	0.11
Q-rham1 (as Q-3-O-r)	0.19
Q-rham2 (as Q-3-O-r)	0.69
Same MW as rutin diglicoside (as rutin)	0.2
Rutin	0.02
Procyanidin B2	0.25

Under basal conditions, TE showed different effects on the larvae's swimming activity. At lower concentrations (0.1 and 1 μg/mL), TE did not significantly change the swimming behavior. However, at higher concentrations (10, 50, and 100 μg/mL), TE significantly increased swimming activity (Fig. 3A, B). We observed a significant induction of swimming activity at 10 and 50 μg/mL after 10 min, but not after 1 h post-exposure. In contrast, 100 μg/mL significantly induced swimming activity at both 10 min and 1 h (Fig. 3A, B). The highest concentration, 500 μg/mL, induced 100% larvae mortality.

In the presence of PTZ 5 mM, the larvae treated with VPA showed similar swimming behavior to the control group (Fig. 3C, D). TE at concentrations of 0.1 and 1 μg/mL significantly counteracted the convulsive effect of PTZ after 10 min, with 1 μg/mL being the only dose that significantly reversed the PTZ effect after 1 h. TE at a concentration of 10 μg/mL reduced the swimming activity induced by PTZ, although the effect was not statistically significant (Fig. 3C, D).

3. Larval toxicity

We assessed mortality after 10 min, 1 h, and 24 h. We observed no mortality in the TE treatments after 10 min and 1 h or in the control larvae after 24 h. PTZ was toxic after 24 h

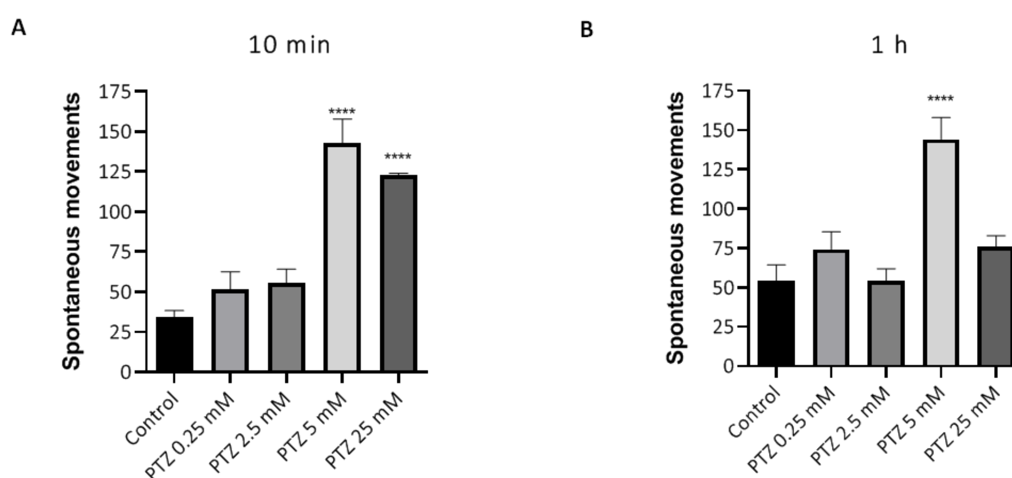

Figure 2. Influence of PTZ on spontaneous movements of larvae. Larvae were exposed to different concentrations of PTZ (0.25 to 25 mM). Swimming activity was quantified as the cumulative spontaneous movements during a 15-min tracking period. Results obtained 10 min (A) and 1 h (B) after PTZ treatment. Results were presented as spontaneous movements and expressed as mean values ± SEM from two or more experiments, with at least 24 larvae tested under each condition. ****p < 0.0001 significant differences compared to control, as determined by ANOVA + Dunnett's test.

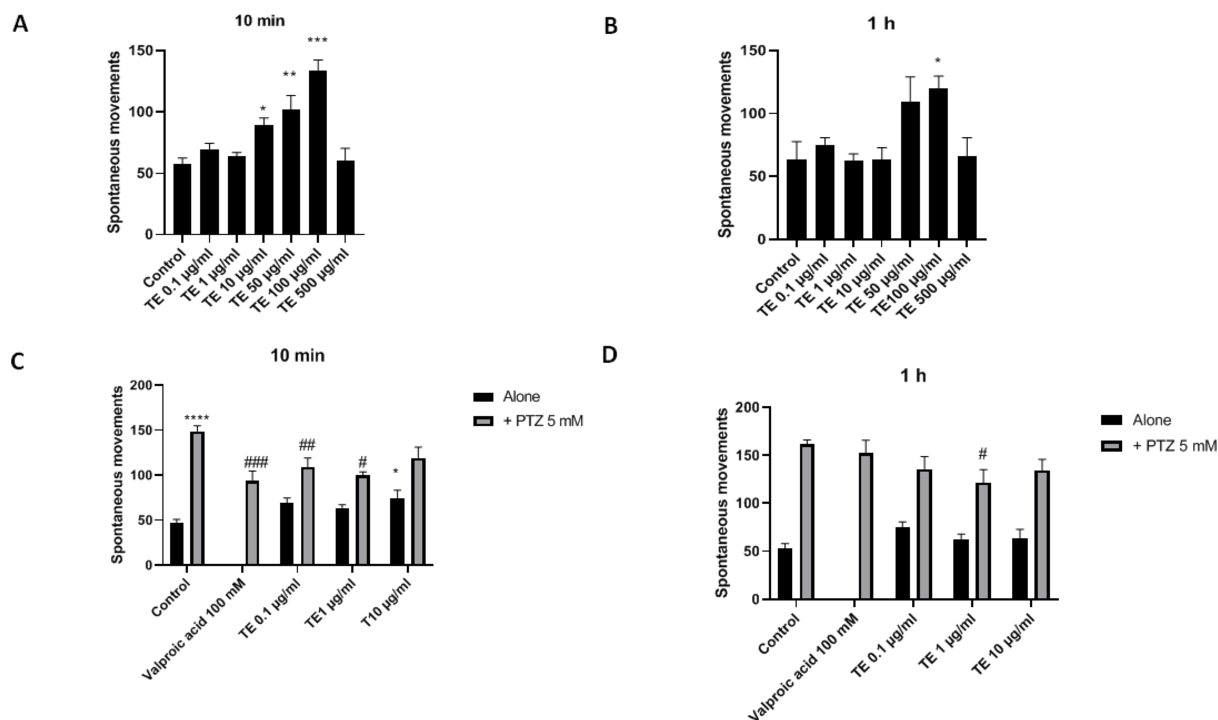

Figure 3. Influence of TE on spontaneous movements of larvae. (A, B) Effect of TE on basal condition (without PTZ) at 10 min and 1 h, respectively. (C, D) Effect of TE in the presence of PTZ (5 mM) at 10 min and 1 h, respectively. Larvae were exposed to various concentrations of TE (0.1 to 500 μg/mL), 1 h before PTZ treatment. Swimming activity was quantified as the cumulative spontaneous movements during a 15-min tracking period at 10 min and 1 h after treatment with PTZ. Results were presented as spontaneous movements and expressed as mean values ± SEM from two or more experiments, with at least 24 larvae tested under each condition. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 indicate significant differences compared to the basal condition, as determined by ANOVA + Dunnett's test. #p < 0.05; ##p < 0.01; ###p < 0.001 indicate significant differences compared to PTZ, as determined by ANOVA + Dunnett's test.

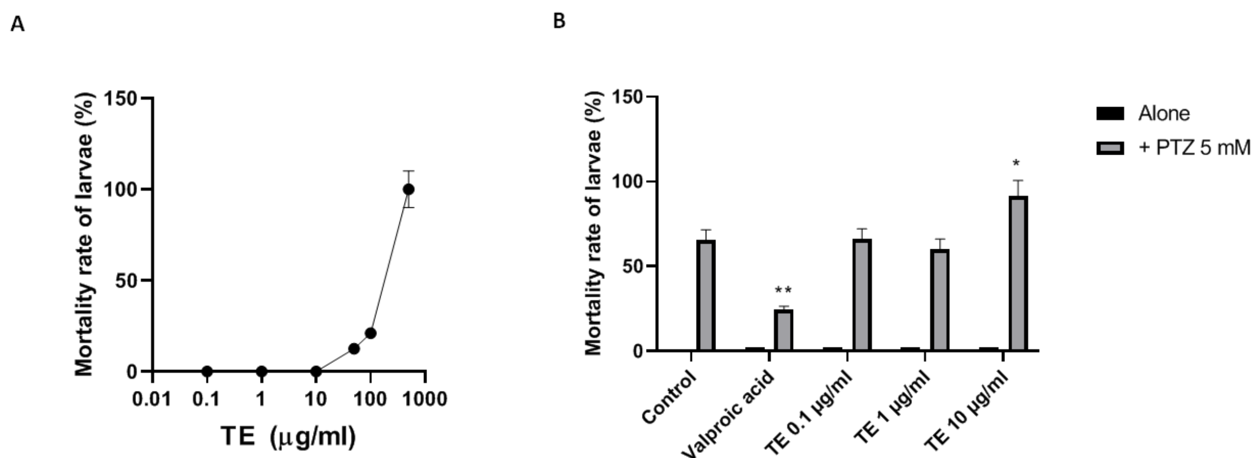

Figure 4. Larval mortality rate. (A) Impact of TE on larval mortality rate. (B) Impact of VPA (100 mM) and TE in the presence of PTZ (5 mM). The mortality rate was assessed 24 h after treatment, and deceased larvae were registered. Results were presented as percentage of larval mortality rate and expressed as mean ± SEM from two or more experiments, with at least 24 larvae tested under each condition. *p < 0.05; **p < 0.01 indicate significant differences compared to PTZ (5 mM) alone, as determined by ANOVA + Dunnett's test.

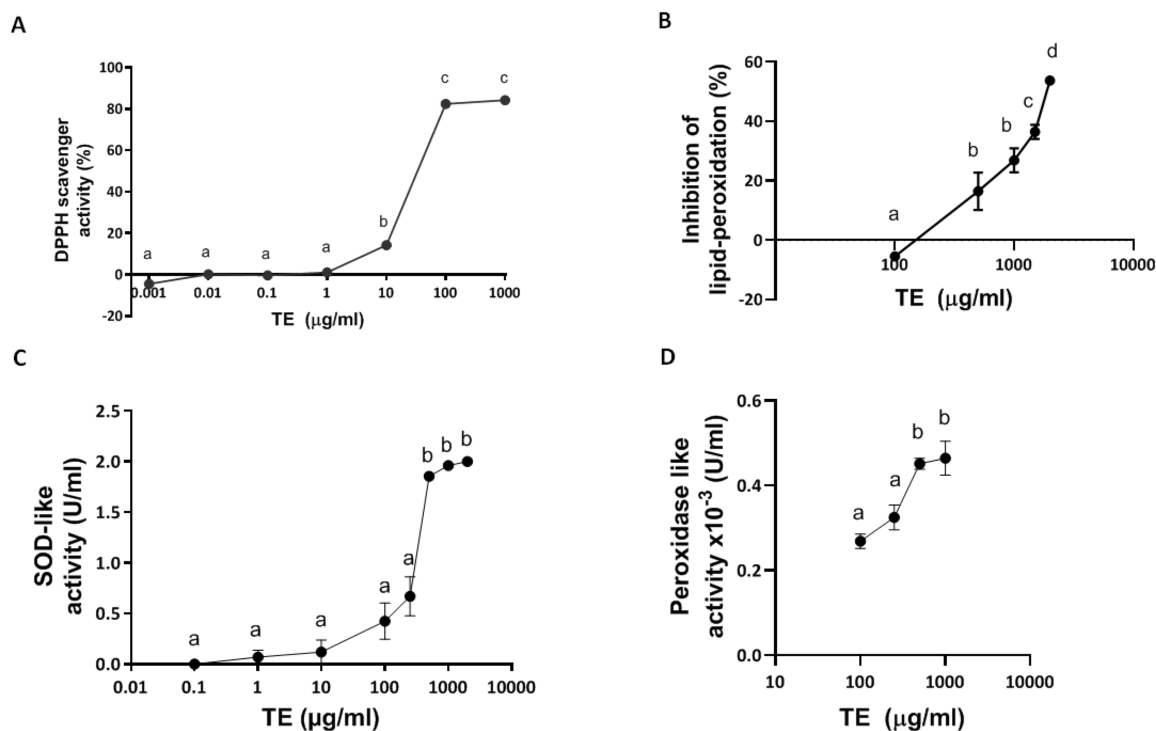

Figure 5. *In vitro* antioxidant activity of TE. (A) DPPH scavenger activity. (B) Inhibition of lipid peroxidation. (C) SOD-like activity. (D) Peroxidase-like activity. (A, B) results are presented as percentages compared to the control condition and expressed as mean ± SEM of three experiments made in triplicate. (C, D) results are presented as U/mL and expressed as mean ± SEM of three experiments made in triplicate. a, b, c and d, indicate significant differences between values as determined by ANOVA + Tukey's test.

and demonstrated a 62.5% larvae mortality rate. Under basal conditions without PTZ, TE induced toxicity at concentrations higher than 10 µg/mL after 24 h, e.g., 50 µg/mL resulted in a mortality rate of 12.5%, 100 µg/mL resulted in a mortality rate of 20.8%, and 500 µg/mL resulted in 100% mortality (Fig. 4A). The LC_{50} was 247.98 µg/mL. In the presence of PTZ, VPA reduced PTZ-induced mortality by approximately 61%. However, TE did not prevent PTZ-induced mortality at low concentrations and enhanced PTZ-induced mortality at 10 µg/mL (Fig. 4B).

4. *In vitro* antioxidant activity

TE scavenged the DPPH radical, inhibited lipid peroxidation (Fig. 5A, B), and exhibited SOD- and peroxidase-like activities (Fig. 5C, D) in a concentration-response curve. The concentrations required for DPPH scavenging and SOD-like activity were lower than those required to inhibit lipid peroxidation and exhibit peroxidase-like activity. The IC_{50} values were 25.18 ± 0.71 µg/mL for DPPH scavenging activity and 977.23 ± 1.00 µg/mL for lipid peroxidation inhibition. The EC_{50} value for SOD-like activity was 297.85 µg/mL. We could not determine the EC_{50} value for peroxidase-like activity, as it was below the lowest concentration tested.

DISCUSSION

In this study, we determined the antioxidant and anticonvulsant activities of TE. TE effectively eliminated free radicals, prevented the peroxidation of egg phospholipids, and acted as SOD and peroxidase enzymes. Importantly, TE shows high efficacy in scavenging DPPH radicals and exerting SOD-like activity. TE also showed an anticonvulsant effect, especially at low concentrations. The main mechanism by which PTZ triggers seizures is thought to be its inhibitory effect on the γ-aminobutyric acid A (GABAergic) system [3]. This effect is the result of its selective blocking action on the chloride-ionosphere complex at the GABA-A receptor. This type of seizure can be prevented by drugs that enhance GABA-A receptor-mediated inhibitory neurotransmission, such as benzodiazepines and phenobarbital [26].

The anticonvulsant effect of other Tilia species, such as *T.*

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americana and *Tilia platyphyllos*, was demonstrated in a PTZ-induced seizure model in mice [27, 28]. Tilia extracts exhibit anxiolytic effects. For example, *T. Americana* showed anxiolytic and sedative effects, observed in experimental models and human studies [29] by acting as a benzodiazepine agonist on GABA-A receptors, which in turn facilitates GABA transmission. The interaction of Tilia extracts with benzodiazepine sites at GABA-A receptors has been demonstrated for *Tilia cordata* and *Tilia tomentosa* extracts [27, 30]. Although this study did not examine the mechanism of TE's anticonvulsant effect, it may interact with GABA receptors [31].

Many free radicals are formed in epilepsy, which can potentially lead to oxidation and impairment of neurotransmitters such as serotonin, dopamine, or noradrenaline. Neuronal membranes may become susceptible to lipid peroxidation, leading to reduced membrane fluidity and altered membrane transport, which contributes to neurodegeneration, neurogenesis, neuronal circuit reorganization, hyperexcitability, and reduced seizure threshold [32]. Similar processes have been observed in various experimental models of epilepsy, such as the PTZ model.

One study involving VPA monotherapy in a group of 32 pediatric patients demonstrated an increase in the activity of enzymes with antioxidant properties, such as SOD, glutathione peroxidase, glutathione reductase, and catalase, and a decrease in oxidative stress markers, such as malondialdehyde [33]. The antioxidant effect of TE could play a crucial role in reducing the oxidative stress associated with epilepsy, potentially providing neuroprotection. Consequently, TE's combined antiseizure and antioxidant activities may enhance the effectiveness of antiseizure therapy and/or limit epileptogenesis.

The antioxidant and anticonvulsive properties of natural products have been previously demonstrated. For instance, the roots and aerial parts from *Astragalus mongholicus* can effectively scavenge hydroxyl radicals, reduce lipoperoxidation, and increase the latency of PTZ-induced seizures in mice while decreasing seizure duration [34]. Similarly, *T. Americana* has exhibited both anticonvulsant and antioxidant effects [27]. TE contains polyphenols and flavonoids. Flavonoids show antiepileptic activity by modulating the GABA-A-Cl-channel complex, given their structural similarity to benzodiazepines [35].

Several studies have highlighted the potential of natural drugs in epilepsy treatment through the regulation of oxidative stress. Additionally, proanthocyanidins mitigate PTZ-induced epileptic seizures in mice through their antioxidant activity [36]. TE's anticonvulsant activity may be attributed to polyphenols, particularly flavonoids. Certain flavonoids, like amentoflavone, possess anxiolytic and anticonvulsant properties and exhibit biphasic activity. These compounds as act as antagonists at nanomolar concentrations and act as negative allosteric modulators of GABA at the $\alpha1\beta2\gamma21$ site at higher concentrations (μM) [37]. A similar pattern was observed with the anticonvulsant flavone hispidulin, which, at low concentrations (0.8-5 μM), exerts a positive modulatory effect on the receptor subunits $\alpha1,2,3,5,6\beta2\gamma2S$, by enhancing current flow. At higher concentrations (> 30 mM), it has the opposite effect [38]. This observation may explain TE's excitatory activity at high concentrations, even under basal conditions. Flavonoids have intricate impacts GABAergic receptors at the benzodiazepine coupling site.

Zebrafish toxicity tests and safety evaluations have been recognized by the Food and Drug Administration to assess new pharmacological agents. Zebrafish embryos and larvae models can be used to test for substances present in low quantities, as they are significantly more susceptible to toxins than adult zebrafish. TE exhibited an LC_{50} of 247.98 μg/mL, which is consistent with findings from other plants. For instance, the dried fruit of *Carpesium abrotanoides* L., carpesii fructus, demonstrated an LC_{50} value of 230.40 μg/mL [39]. According to OECD guidelines for assessing the toxicity of crude extracts/fractions in fish embryos (FET), the toxicity levels are categorized as: dangerous (10 mg/L < LC_{50} < 100 mg/L), toxic (1 mg/L < LC_{50} < 10 mg/L), and carcinogenic (LC_{50} < 1 mg/L) [40]. Considering these criteria, TE can be classified as a non-dangerous drug.

The increased mortality of larvae that we observed with TE 10 μg/mL during PTZ 5 mM stimulation may result from TE's stimulatory activity. The anticonvulsant effects of TE may be attributed to its ability to reduce oxidative damage, in addition to its interaction with GABAergic neurotransmission. Moreover, TE is an antioxidant and a potential therapeutic agent for other neurological conditions associated with oxidative stress.

CONCLUSION

This research highlights the potential of low-concentration TE as a coadjuvant for epilepsy therapies due to its anticonvulsant and antioxidant properties. TE may also offer benefits for Parkinson's disease, Alzheimer's disease, and other neuropathies associated with oxidative stress.

AUTHORS' CONTRIBUTIONS

Elina Malén Saint Martin: investigation, formal analysis (zebrafish experiments, phytochemical studies, determination of total polyphenols, determination of total flavonoids, quantification of CBD and epicatechin and antioxidant assays); Ayelén Sosa: investigation, formal analysis (zebrafish experiments); Carolina Martínez: investigation, supervision methodology (zebrafish experiments), review of the manuscript; Jimena Prieto: investigation, supervision methodology (zebrafish experiments), review of the manuscript; Carla Marrassini: Investigation, supervision and formal analysis (antioxidants assays and quantification of total polyphenols and flavonoids); Cecilia Dobrecky: investigation, methodology (HPLC MS/MS assays, quantifications of Tilia compounds); Maria Rosario Alonso: Investigation, supervision of methodology (HPLC-UV quantification of CBD, tiliroside, and epicatechin). Review and editing manuscript; Claudia Anesini: conceptualization, project administration, funding acquisition, supervision of the results, and writing, reviewing, and editing the manuscript.

ETHICAL APPROVAL

All animal studies were conducted in strict accordance with the National Institutes of Health guidelines for animal care and husbandry. The Institutional Animal Care Committee of the Universidad Nacional de Quilmes (CE-UNQ 2/2014) (Buenos Aires, Argentina) approved all study protocols.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest in this work.

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SUPPLEMENTARY MATERIALS

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