Original Research

Effects of Leontice leontopetalum and Bongardia chrysogonum on oxidative stress and neuroprotection in PTZ kindling epilepsy in rats

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Abstract: We investigated the effects of Leontice leontopetalum and Bongardia chrysogonum on apoptosis, gamma-aminobutyric acid (GABA_A) receptor positive cell number, cyclin-B1 and bcl-2 levels and oxidative stress in pentylenetetrazol (PTZ) kindling in rats. Kindling was produced by subconvulsant doses of PTZ treatments in rats. Wistar albino rats were divided into 4 groups; Control, PTZ treated (PTZ), PTZ+L. leontopetalum extract treated (PTZ+LLE) and PTZ+B. chrysogonum extract treated (PTZ+BCE) groups. Extracts were given a dose (200 mg/kg) 2h before each PTZ injection. PTZ treatment significantly decreased the glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) activities and bcl-2 levels and increased the total oxidant status (TOS), malondialdehyde (MDA), cyclin-B1, oxidative stress index (OSI) and number of neurons that expressed GABA_A receptors when compared to the control. LLE and BCE possessed antioxidant activity in the brain and ameliorated PTZ induced oxidative stress, decreased cyclin-B1, increased bcl-2 levels, and kept the GABA_A receptor number similar to that of the control despite the PTZ application.

Key words: Bcl-2; Brain; Bongardia chrysogonum; Cyclin-B1; GABA_A; kindling model; Leontice leontopetalum; Oxidative stress; Pentylenetetrazol.

Introduction

Epilepsy is the second most prevalent neurological problem worldwide (1). Despite current antiepileptic drug treatment, one-third of epileptic patients have drug resistant epilepsy (1). Temporal lobe epilepsy (TLE) is an epileptic disorder that has one of the worst prognosis (2). Kindling is an experimental model of TLE (3). Repeated application of sub-convulsive electrical or chemical stimuli, such as pentylenetetrazol (PTZ), causes progressive intensification of seizures (4). Due to chronic convulsive activity, neuronal degeneration and death occurs, which are reported both in clinical situations and experimental animal studies (5).

There is increasing evidence regarding the link between the cell cycle and neuronal death (4). Immunohistochemistry studies have shown cyclin B expression in the hippocampus of TLE patients (6) and PTZ-kindled rats (cyclin-B1) (4).

Oxidative stress is another factor contributing to seizure related neuronal death (7). Oxidative stress causes cellular injury and may cause cell death through the oxidation of biomolecules (2). The current literature research has focused on the possible interrelationship among oxidative stress, mitochondrial dysfunction, cell injury, and epilepsy (8). Hence, a compound that would decrease the reactive oxidative injury in the brain due to the seizures may be functional for treatment of epilepsy (9).

Medicinal herbs provide molecules that may be candidates for epilepsy medication (10). The roots of Leontice leontopetalum have been used as a cure for epilepsy in Turkish folk medicine (11). The plant has antioxidant and anti-cholinesterase activities (12). Bongardia chrysogonum has also been used in Turkish folk medicine for epilepsy (13). Although L. leontopetalum and B. chrysogonum have been used as medicinal plants for treatment of epilepsy in humans, there is no available research regarding the effect of L. leontopetalum aqueous root extract on any chronic models of epilepsy in experimental models. In a very recently study, in vitro antioxidant and free-radical scavenging properties and antioxidant activity (in mice) of B. chrysogonum root extract was reported (14). Therefore, the aim of this study was to determine the anticonvulsant, antioxidant, and neuroprotective effects of L. leontopetalum and B. chrysogonum, which belong to the same family, extracts and associated seizure-induced neuronal loss and alterations in the gamma-aminobutyric acid (GABA_A) receptor number in a PTZ kindling model of epilepsy in rats.

Materials and Methods

Plant collection

L. leontopetalum plant were collected from Tuşba, in the province of Van, Turkey, a voucher specimen was
prepared in the Van YYU VANF herbarium under number 114872. *B. chrysogonum* was collected from the Van YYU campus and a voucher specimen was prepared under number 165062 at the same herbarium.

**Preparation of the extract**
The roots of *L. leontopetalum* and *B. chrysogonum* were separately dried for 1 month, and following pounding, they were sieved through a 1 mm mesh. The powder obtained was dissolved in distilled water and macerated in a water bath for 50 min at 70 °C with continuous stirring. Following 10 min of cooling, it was filtered through a filter paper and the root aqueous extract was prepared.

**Animals and treatments**
Male Wistar albino rats, aged 2 months, were obtained from the Van Yuzuncu Yil University, Experimental Animals Facility. The animals were kept in a light/dark photoperiod of 12:12 and under stable heating conditions (21 °C). Standard rat pellet and tap water was given ad libitum. The study was approved by the Ethics Committee of the Van Yuzuncu Yil University.

The animals were divided into 4 groups (n = 6): (1) the control group: saline (SF) (0.5 ml, orally (po), (2) the pentylenetetrazol (PTZ) group: SF (0.5 ml, po) + PTZ (35 mg/kg, intraperitoneally (ip)), (3) PTZ + *L. leontopetalum* extract (LLE), (PTZ+LLE) group: LLE (200 mg/kg, po) + PTZ (35 mg/kg, ip), and (4) PTZ + *B. chrysogonum* extract (BCE) (PTZ+BCE) group: BCE (200 mg/kg, po) + PTZ (35 mg/kg, ip). SF, LLE, or BCE were given to rats orally via a gauge every other day (Monday, Wednesday, and Friday) 2h before each PTZ injection. Rats in the control group treated only saline. PTZ kindling was produced by injecting a subconvulsant dose of PTZ, 35 mg/kg (a total of 18 injections, every other day). PTZ was administered 75 mg/kg, as a challenge dose in 19th injection day. Seizure intensity of individual bands (23).

**Antioxidative Parameters**

**Tissue preparation**
The frozen brain tissue samples were thawed and homogenized using an ultrasonic homogenizer in glass porcelain for 5 min in ice-cold media. Next, they were centrifuged at 10000g, 30 min at 4 °C. Supernatant fractions were used for the determination of antioxidant defense enzymes and oxidative stress parameters.

**Analysis of the oxidative stress**
Serum total antioxidant status (TAS) (16) and total oxidant status (TOS) (17) were determined spectrophotometrically using the Rel assay diagnostics kit (Mega Tip, Gaziantep, Turkey). Using those findings, the oxidative stress index (OSI) was calculated (18). Brain lipid peroxidation was determined by measuring the malondialdehyde (MDA) levels (19). Superoxide dismutase (SOD) activity was determined by using the method of Spitz and Oberley (20). Glutathione peroxidase (GSHPx) enzyme activity was determined using the method by Paglia and Valentine (21). Measurement of the nitric oxide (NO) was performed using the colorimetric nitric oxide assay kit (Relassay®, Gaziantep, TR), which uses Griess reagents to convert nitrite into a deep purple azo compound.

**Western blot analysis**
Tissues obtained from the brain and hippocampus were homogenized using steel beads in a homogenization buffer (2 mM Tris-HCl pH 7.2, 150 mM NaCl, 10% glycerol, 0.2% NP-40 additionally protease inhibitors which inhibits cysteine, serine, aspartic, and metallo-proteases.). Following centrifugation (Centric 200R) occurred at 4 °C for 10 min at 14000 RPM. The final supernatant was used as the total protein. Protein quantities were measured using the method described by Bradford (22). All the samples equated to the same total protein amount using the homogenization buffer as a diluent. 30 μg of total protein from each sample was loaded onto a 4-12% SDS-PAGE gel for separation. The separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA). After incubation in 5% skim milk powder containing TBS-T (20 mM Tris HCl, 137 mM NaCl and 0.1% Tween-20, pH 7.6) for 2 h at room temperature to block nonspecific binding, the PVDF membrane was reacted for 16 h at 4 °C with primary antibodies against Bcl-2 (ProteinTech, 1:1000), B -Tubilin (ProteinTech, 1:1000), and Cyclin B1 (ProteinTech 1:1000), washed in TBS-T, and incubated for 1 h at room temperature with corresponding horseradish peroxidase labelled anti-rabbit secondary antibody (Cell Signaling, 1:5000). As the final step after three times rinses with TBS-T, the ECL developer solution was applied to the membranes to visualize by using an imaging system (Chemidoc MP, Biorad). The Image J analysis software was used to determine the integrated intensity of individual bands (23).

**Sample preparation and histological evaluations**
Specimens were fixed in 10% neutral buffered formalin (NBF) and embedded in paraffin. Paraffin sections of 4 μm were stained using GABA<sub>A</sub> receptor alpha immunohistochemistry and the TUNEL method for apoptosis, and examined under an Olympus BX53F light microscope (Olympus, Tokyo, Japan).

**GABA<sub>A</sub> Receptor alpha immunohistochemistry**
Rabbit polyclonal to GABA<sub>A</sub> Receptor alpha 1 (Abcam, Cat: ab33299) was used as the primary antico. Staining was performed in the presence of appropriate positive controls using the Ventana Benchmark XT immunohistochemistry automatic staining system and Ventana Ultraview Dab Detection Kit. Positive staining cell counting was done with images taken from 5 selected similar fields (at 200X magnification).

**TUNEL Assay**
DNA fragmentation was analyzed using the TUNEL kit (ApopTag ® Plus Peroxidase In Situ Apoptosis Detection Kit), following the manufacturer’s guidelines. At least 10 areas of each section were analyzed at 200X magnification with a light microscope Olympus BX53F (Olympus, Tokyo, Japan).
Statistical analysis

Statistical significance was established using the Kruskal-Wallis and Tukey tests. A value of \( p < 0.05 \) was considered statistically significant. Data are presented as the mean ± SEM. Statistical Package for the Social Sciences software, version 24, was used for the statistical evaluations.

Figure 1. Effect of LLE or BCE administration on the development of PTZ-induced kindling. Data are presented as the mean ± SEM. Different letters show significant differences between the groups (\( p < 0.05 \)).

Table 1. Effects of LLE, BCE, and/or PTZ on cyclin-B1 and bcl-2 levels of the whole brain and hippocampus tissue.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Cyclin-B1 (whole brain)</th>
<th>Cyclin-B1 (hippocampus)</th>
<th>Bcl-2 (whole brain)</th>
<th>Bcl-2 (hippocampus)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.4836 ± 0.7334 (^a)</td>
<td>9.1869 ± 0.7957 (^a)</td>
<td>35.6290 ± 1.1781 (^a)</td>
<td>47.0616 ± 1.7758 (^a)</td>
</tr>
<tr>
<td>PTZ+LLE</td>
<td>13.1103 ± 0.7184 (^a)</td>
<td>14.5900 ± 1.2882 (^a)</td>
<td>28.9698 ± 0.9072 (^a)</td>
<td>32.2976 ± 1.1581 (^a)</td>
</tr>
<tr>
<td>PTZ+BCE</td>
<td>30.2407 ± 1.6594 (^a)</td>
<td>32.3570 ± 1.0864 (^a)</td>
<td>21.2438 ± 0.8639 (^a)</td>
<td>13.5422 ± 1.1020 (^a)</td>
</tr>
<tr>
<td>PTZ</td>
<td>48.1650 ± 2.3837 (^a)</td>
<td>43.8667 ± 0.4618 (^a)</td>
<td>14.1583 ± 0.8626 (^a)</td>
<td>7.1000 ± 0.8234 (^a)</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± SEM. Different letters show significant differences between the groups (\( p < 0.01 \)).

Results

Repeated administration of PTZ (35 mg/kg), resulted in increasing convulsive activity in the PTZ group (Figure 1). LLE treatment (200 mg/kg) significantly decreased the seizure scores with the 4th, 9th, 12th, 16th, and 18th injections (\( p < 0.05 \)) compared to that in the PTZ group (Figure 1). BCE administration significantly decreased the seizure scores with the 12th, 16th, and 18th injections (\( p < 0.05 \)) compared to that in the PTZ group (Figure 1).

The expression levels of cyclin-B1 protein in the whole brain (Figures 2A, B) and hippocampus (Figures 2D, E) were significantly increased (Table 1) in the PTZ, PTZ+BCE, and PTZ+LLE groups when compared to the control (\( p < 0.01 \)). Compared to the PTZ group, the expression level of cyclin-B1 protein in the PTZ+LLE and PTZ+BCE groups significantly decreased in the whole brain (\( p < 0.01 \)) and hippocampus (\( p < 0.01 \)) (Figures 2B, E). The expression of cyclin-B1 protein in the PTZ+LLE group was significantly decreased compared to that in the PTZ+BCE group in the hippocampus (\( p < 0.01 \)) and whole brain (\( p < 0.01 \)).

As shown in Figures 2A and C (in the whole brain), and 2D and F (in the hippocampus), the bcl-2 expression levels were significantly increased in the other groups when compared to the control (Table 1) (\( p < 0.01 \)). When compared to the PTZ group, the bcl-2 expression levels in the PTZ+BCE and PTZ+LLE groups were increased in the whole brain (\( p < 0.01 \)) and hippocampus (\( p < 0.01 \)). The bcl-2 expression levels were significantly increased by LLE pretreatment compared to the PTZ+BCE group in the hippocampus (\( p < 0.01 \)) and in whole brain (\( p < 0.01 \)).

As shown in Figure 3, PTZ treatment significantly increased the MDA levels, in the brain tissue when compared to the Control (\( p < 0.01 \)) (Table 2). Both pretreatment with LLE or BCE reversed (\( p < 0.01 \)) the PTZ-induced increase in the MDA level (Figure 3A, Table 2). PTZ treatment decreased the SOD levels in the brain tissue when compared to the control (\( p < 0.01 \)) (Figure 3B, Table 2). Both pretreatment with LLE or BCE significantly increased the SOD levels when compared to the PTZ group (\( p < 0.01 \)) (Figure 3B, Table 2). Neither
Effects of LLE and BCE in PTZ kindling epilepsy

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Table 2. Effects of LLE, BCE, and/or PTZ on oxidative stress.

<table>
<thead>
<tr>
<th>Groups</th>
<th>TAS (micromol H₂O₂/Eq/L)</th>
<th>TOS (micromol H₂O₂/Eq/L)</th>
<th>OSI (arbitrary Unit)</th>
<th>MDA (nmol/mg-tissue)</th>
<th>SOD (U/mg-tissue)</th>
<th>GSH-Px (EU/g-tissue)</th>
<th>NO (µmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.857 ± 0.360</td>
<td>5.505 ± 0.521</td>
<td>0.021 ± 0.003</td>
<td>1.8515 ± 0.046</td>
<td>9.5290 ± 0.0981</td>
<td>0.0140 ± 0.0008</td>
<td>192.17 ± 27.18</td>
</tr>
<tr>
<td>PTZ+LLE</td>
<td>2.342 ± 0.034</td>
<td>5.189 ± 1.295</td>
<td>0.022 ± 0.005</td>
<td>1.6665 ± 0.0266</td>
<td>8.305 ± 0.3834</td>
<td>0.0118 ± 0.0018</td>
<td>251.54 ± 47.07</td>
</tr>
<tr>
<td>PTZ+BCE</td>
<td>2.121 ± 0.143</td>
<td>5.782 ± 0.406</td>
<td>0.028 ± 0.003</td>
<td>1.6826 ± 0.0593</td>
<td>8.2990 ± 0.5399</td>
<td>0.0066 ± 0.0007</td>
<td>284.44 ± 60.54</td>
</tr>
<tr>
<td>PTZ</td>
<td>2.218 ± 0.053</td>
<td>21.060 ± 5.250</td>
<td>0.096 ± 0.025</td>
<td>4.2132 ± 0.1591</td>
<td>5.1547 ± 0.0956</td>
<td>0.0062 ± 0.0012</td>
<td>278.89 ± 21.97</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± SEM. Different letters show significant differences between the groups (p < 0.01).

Figure 4. Effects of LLE, BCE, and/or PTZ pretreatment on the (A) TOS levels of the serum and (B) OSI values. Different letters show significant differences between the groups (p < 0.01). Data are presented as the mean ± SEM.

Table 3. Effects of LLE, BCE, and/or PTZ pretreatment on the number of GABA_A receptor in whole brain and hippocampus.

<table>
<thead>
<tr>
<th>Groups</th>
<th>GABA_A Receptor Positive Cells (hippocampus)</th>
<th>GABA_A Receptor Positive Cells (whole brain)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>19.67 ± 1.96</td>
<td>25.83 ± 1.78</td>
</tr>
<tr>
<td>PTZ+BCE</td>
<td>17.33 ± 1.86</td>
<td>24.00 ± 0.73</td>
</tr>
<tr>
<td>PTZ+LLE</td>
<td>18.86 ± 0.96</td>
<td>29.57 ± 1.69</td>
</tr>
<tr>
<td>PTZ</td>
<td>32.17 ± 3.07</td>
<td>38.50 ± 5.50</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± SEM. Different letters show significant differences between the groups (p < 0.05).

Discussion

We investigated the effects of L. leontopetalum and B. chrysogonum on oxidative stress markers, cyclin-B1 and bcl-2 levels, and the number of neurons that expressed GABA_A receptors. Results suggest a possible anticonvulsant effect of LLE and BCE against PTZ-induced kindling. It has been reported that lupanine, saponin, and tannin alkaloids, which have known anticonvulsant activities (24-26), are present in L. leontopetalum.

The PTZ, LLE, nor BCE treatments changed the NO levels in the brain when compared to the control (Table 2). PTZ treatment decreased the GSH-Px levels in the brain tissue when compared to the control (p < 0.01). LLE administration reversed the PTZ induced decrease in the GSH-Px levels (p < 0.01). BCE administration did not change the PTZ-induced decrease in the GSH-Px level when compared to the PTZ group (Figure 3C, Table 2).

TOS levels were increased by PTZ and restored by the LLE and BCE pretreatments, OSI values were increased by PTZ and restored by the LLE and BCE pretreatments (Table 2, Figure 4). A significant increase was also found in the number of neurons that expressed GABA_A receptors in the PTZ group when compared to the control, in both the hippocampus (Figure 5A) and whole brain (Figure 5B) (p < 0.05) (Table 3). Differences between the number of neurons that expressed GABA_A receptors in the whole brain and hippocampus were not significant between the control, PTZ+LLE, and PTZ+BCE groups (Table 3, Figures 5A, 5B, 6, 7).

Using the TUNEL assay, there was no statistically significant difference in the number of apoptotic neurons in the brain among the control, PTZ, PTZ+BCE, and PTZ+LLE groups (data not shown).
Neuronal cell death in the central nervous system is closely linked to a cell division process (28). We investigated the effects of *L. leontopetalum* and *B. chrysogonium* on the cyclin-B1 levels of the whole brain and hippocampus. We found a significant increase in the cyclin-B1 levels of the PTZ, PTZ+BCE, and PTZ+LLE groups when compared to the control. Consistent with our results, it was reported that neuronal cyclin B expression occurred in TLE patients (6) and in PTZ-kindled rats (4). In addition, Neuronal death that was accompanied by cyclin b1 expression was reported in PTZ kindled rats (4). Similarly, our results may indicate that PTZ caused neuronal loss via an aberrant cell cycle in the whole brain and hippocampus. In the present study, cyclin-B1 levels were significantly low in the PTZ+LLE and PTZ+BCE groups when compared to the PTZ group. These results suggest an attenuating effect of the extract administrations on PTZ induced cyclin-B1 expression.

Bcl-2 is a protein that can inhibit apoptosis (29). In the present study, we also observed a significant decrease in the bcl-2 levels of both the hippocampus and whole brain in the PTZ group. In the whole brain and hippocampus, both the BCE and, especially, LLE pretreatments significantly increased the bcl-2 levels when compared to the PTZ group. Consistent with our results, Ullah et al. (30) reported that epileptic seizures decreased bcl-2 levels in the rat brain. An increase in bcl-2 levels inhibits apoptosis (31). The ameliorating effects of LLE and BCE on bcl-2 levels is promising because the extracts may decrease seizure-triggered apoptotic neuronal cell death. A significant increase in the anti-apoptotic bcl-2 levels and a significant decrease in the cell cycle marker cyclin-B1 levels were observed with the BCE and LLE pretreatments in the whole brain and hippocampus when compared to the PTZ group, which may be due to the neuroprotective properties of the extracts.

We also investigated the effects of LLE and BCE on the oxidative stress markers. Several rodent studies have demonstrated that MDA levels were significantly increased with PTZ (9, 32). In the present study, we found a significant increase in the MDA levels in the PTZ group when compared to the control. We also found that pretreatment with LLE and BCE resulted in a significant decrease in the MDA levels in the rat brain. It was reported that Valproate (an antiepileptic drug) decreased brain MDA levels when compared to a PTZ-kindled group (33). It was reported that significant increase in brain MDA levels results in free-radical formation and oxidative stress (32). Our results were consistent with the study by Rahmati et al. (32), which indicated that oxidative stress may be a factor involved in the epilepsy pathophysiology of PTZ kindling. Both LLE and BCE have exerted a protective effect against lipid peroxidation.

Oxidative stress caused by extreme free-radical release is probably involved in epileptogenesis (2). The brain has antioxidant protection enzymes (such as SOD and GSH) that protect it from oxidative injury (34). The antioxidant enzyme GSH-Px prevents the production of hydroxyl radicals (33). We also investigated the GSH-Px and SOD levels and found that GSH-Px activity was significantly decreased in the PTZ group when compared to the control. Consistent with our results, it was reported that PTZ kindling leads to a significant decrease in GSH-Px levels (33). The decreased levels of GSH-Px may be attributed to the GSH-Px was depleted during the struggle against oxidative stress (33). We also found that pretreatment with LLE ameliorated the GSH-Px levels. GSH-Px detoxifies the excessive reactive oxygen species (ROS) ; hence, GSH-Px may hinder lipid peroxidation (35). Indeed, our results indicated that the MDA values were also ameliorated in the LLE-treated group. Pretreatment with LLE may protect the antioxidant enzyme activity of GSH-Px, thus preventing the generation of free-radicals and extreme ROS production and lipid peroxidation. We also found that PTZ administration caused a significant decrease of brain SOD activity in the PTZ group and pretreatment with LLE and BCE significantly increased the SOD activity when compared to the PTZ group. Consistent with our results, a significant decrease was reported in the SOD activity in the PTZ-kindled group (33, 36). Ilhan et al. (33) reported that valproate prevented a PTZ kindling-induced reduction in SOD activity. SOD is an antioxidant enzyme (33). SOD catalyzes the reaction which superoxide is converted to hydrogen peroxide (36). It was concluded that LLE and BCE pretreatment might protect the antioxidant enzyme activity of SOD.

We investigated the serum TAS, TOS levels, and calculated the OSI values. Neither PTZ, LLE, nor BCE altered the TAS levels of the serum. We found that PTZ kindling significantly increased the serum TOS levels and OSI when compared to the control. Both the LLE and BCE pretreatments decreased the TOS and OSI values (the same level as the control) when compared to the PTZ group. Consistent with our findings, a study by Tutanc et al. (37) reported increased TOS and OSI levels in epileptic children.

We also investigated the number of neurons that expressed GABA<sub>A</sub> receptors in the whole brain and hippocampus. We found a statistically significant increase in the number of neurons that expressed GABA<sub>A</sub> receptors in the hippocampus and whole brain of the PTZ group when compared to the control, PTZ+LLE, and PTZ+BCE groups. An upregulation of GABA<sub>A</sub> receptor subtypes in TLE patients was reported (38). It was also reported that an acute injection of PTZ results in temporary changes in the mRNA levels of GABA<sub>A</sub> receptors, but not the number of receptors (39), while chronic administration of PTZ significantly increased the number of neurons that expressed GABA<sub>A</sub> receptors (40). Surviving neurons might increase the number of GABA<sub>A</sub> receptors for combating the repeated waves of excitation, thus augmenting their response to the inhibitor neurotransmitter (38). In conclusion, both LLE and BCE have antioxidant activity and both may produce their effects mainly through their antioxidant properties. LLE and BCE pretreatment kept the number of GABA<sub>A</sub> receptors close to that of the control, decreased the cyclin-B1 levels, and increased the bcl-2 levels in both the hippocampus and whole brain, despite the PTZ application. Since we used only a 200 mg/kg dose of the extracts, more studies are required to determine whether LLE and BCE alter the investigated parameters in a dose-dependent fashion.
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Conflict of interest
None declared.

References

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