

## Neuroprotective and Nitric Oxide–Modulating Effects of D-Limonene in a Penicillin-Induced Epilepsy Model in Rats

Elif Azize Özşahin Delibaş,<sup>1</sup> Inayet Güntürk,<sup>2</sup> Şeyma Özsoy<sup>3</sup>

<sup>1</sup>Department of Nutrition and Dietetics, Tokat Gaziosmapaşa University, Faculty of Health Sciences, Tokat, Türkiye

<sup>2</sup>Department of Nutrition and Dietetics, Osmaniye Korkut Ata University, Faculty of Health Sciences, Osmaniye, Türkiye

<sup>3</sup>Department of Physiology, Tokat Gaziosmapaşa University, Faculty of Medicine, Tokat, Türkiye

### ABSTRACT

**Objective:** Epilepsy involves dysregulated inflammatory pathways and oxidative stress (OS). Nitric oxide (NO), an endogenous vasodilator, may exert neurotoxic effects under OS. D-limonene, a monoterpene with antioxidant and anti-inflammatory properties, can modulate these processes. This study addressed the neuroprotective effects of D-limonene by assessing its influence on NO levels in serum and brain tissue and its interaction with sodium valproate (VPA) in a penicillin-induced epilepsy model in rats.

**Materials and Methods:** Thirty-five male Wistar albino rats (12–16 weeks, 200±50 g) were clustered into 5 groups (n=7): control (penicillin 500 IU, 2.5 µL, i.c.); D-limonene 50 mg/kg + penicillin 500 IU; D-limonene 100 mg/kg + penicillin 500 IU; VPA 300 mg/kg + penicillin 500 IU; and combination (D-limonene 100 mg/kg + VPA 300 mg/kg + penicillin 500 IU). Treatments were administered intraperitoneally. NO levels were determined using a commercial colorimetric assay kit. Data were analyzed using one-way ANOVA followed by Sidak's multiple comparisons test and are presented as mean±SD, with statistical significance set at p<0.05.

**Results:** Serum NO peaked in the penicillin group (568.0±84.95 µmol/L) and decreased dose-dependently with D-limonene (490.5±86.12; 347.6±25.39 µmol/L). VPA further reduced NO (259.0±20.04 µmol/L), whereas the combination modestly increased it (390.6±74.95 µmol/L). Tissue NO showed a similar trend, with the lowest level observed with VPA (120.4±12.60 µmol/L) and partially restored by combination treatment (168.8±20.85 µmol/L).

**Conclusion:** D-limonene reduced NO levels dose-dependently. VPA had a stronger inhibitory effect, whereas their combination attenuated this inhibition, suggesting that D-limonene may modulate NO metabolism and confer neuroprotection in experimental epilepsy.

**Keywords:** D-limonene, epilepsy, neuroprotection, nitric oxide, oxidative stress, sodium valproate.



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#### Address for correspondence:

Elif Azize Özşahin Delibaş,  
Department of Nutrition and Dietetics, Tokat Gaziosmapaşa University, Faculty of Health Sciences, Tokat, Türkiye  
**Phone:** +90 505 238 37 17  
**E-mail:** elif.delibas@gop.edu.tr

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## INTRODUCTION

Epilepsy is a complex neurological disorder characterized by spontaneous and recurrent seizures arising from abnormal neuronal hyperexcitability and hypersynchronization.<sup>1</sup> The cellular and



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molecular mechanisms underlying the disease have been extensively investigated through clinical observations and experimental animal models. During seizures, increased neuronal activity and metabolic demand lead to mitochondrial dysfunction and excessive production of reactive oxygen and nitrogen species (ROS and RNS). This process overwhelms the brain's antioxidant defense capacity, resulting in oxidative and nitrosative stress (OS and NS), which contributes to neuronal damage and the progression of epileptogenesis. Furthermore, findings from status epilepticus models, including elevated lipid peroxidation, decreased antioxidant enzyme activity, and disruptions in mitochondrial membrane integrity, provide strong evidence supporting these mechanisms.<sup>2</sup>

Nitric oxide (NO) plays a pivotal role as both a physiological neuromodulator and a pathological effector when overproduced during seizure-induced neuronal hyperexcitability. Excessive NO production via inducible nitric oxide synthase (iNOS) leads to the formation of peroxynitrite, a highly reactive oxidant that exacerbates mitochondrial dysfunction, protein nitration, and neuronal injury, ultimately increasing seizure susceptibility and contributing to epileptogenesis.<sup>3</sup>

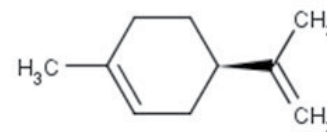
Current antiepileptic drugs (AEDs), including valproic acid (VPA) and carbamazepine, provide symptomatic control by targeting ion channels or synaptic transmission. However, their efficacy in addressing redox balance and neuroinflammation remains limited.<sup>4</sup> Furthermore, long-term AED use may induce hepatic oxidative stress (OS) or impair antioxidant enzyme activity.<sup>5</sup> Consequently, identifying natural compounds with antioxidant and anti-inflammatory properties represents a promising adjunctive approach to reducing seizure-related neuronal damage and improving outcomes.<sup>6,7</sup>

D-limonene is a naturally occurring monoterpene (Fig. 1) that is abundant in citrus essential oils and recognized for its wide range of biological activities. A growing body of evidence indicates its capacity to modulate oxidative and inflammatory processes within the nervous system, thereby contributing to neuronal protection. In preclinical studies, D-limonene has been associated with reduced oxidative damage and improved redox homeostasis, indicating its potential value as a natural compound in neurological disorders characterized by OS and NS, such as epilepsy.<sup>8</sup>

In view of the limitations of current antiepileptic pharmacotherapy and the need for safer, multi-target agents, exploring the potential of D-limonene to mitigate OS and regulate NO metabolism in epilepsy models may offer valuable translational insights. Therefore, this study aimed to examine the neuroprotective and NO-modulating effects of D-limonene in a penicillin-induced experimental epilepsy model in rats and to compare its efficacy with that of VPA.

## KEY MESSAGES

- D-limonene modulates nitric oxide levels in experimental epilepsy.
- D-limonene reduces nitrosative stress in a dose-dependent manner.
- Valproic acid produces stronger suppression of NO levels.
- Co-administration reveals a regulatory rather than additive interaction.
- Findings support D-limonene as a potential adjunct neuroprotective agent.



**Figure 1.** Chemical structure of D-limonene. D-limonene is a monocyclic monoterpene hydrocarbon widely distributed in citrus fruits and known for its antioxidant and neuroprotective properties.

## MATERIALS AND METHODS

### Study Design and Ethical Approval

The study protocol was approved by the Tokat Gaziosmanpaşa University Animal Experiments Local Ethics Committee (Approval Number: 51879863-35; Date: 07.03.2025). All experimental procedures were conducted in accordance with the principles outlined in the European Union Directive for the protection of animals used for scientific purposes (2010/63/EU).

### Experimental Animals and Housing

Thirty-five male Wistar albino rats aged 12–16 weeks and weighing approximately 200±50 g were used in the study. The animals were housed under controlled laboratory conditions at 23±2 °C with a 12-hour light/dark cycle and had free access to standard laboratory chow and water. Rats that did not meet the inclusion criteria were excluded from the study.

### Experimental Groups

The remaining animals were randomly allocated into five experimental groups (n=7 per group):

Group 1 (control + penicillin): received intracortical (i.c.) penicillin (500 IU in 2.5 µL) together with intraperitoneal (i.p.) saline (1 mL).<sup>9</sup>

Group 2: received D-limonene (50 mg/kg, i.p.) combined with penicillin (500 IU in 2.5  $\mu$ L, i.c.).<sup>10</sup>

Group 3: received D-limonene (100 mg/kg, i.p.) combined with penicillin (500 IU in 2.5  $\mu$ L, i.c.).<sup>10</sup>

Group 4 (positive control–VPA): received sodium valproate (VPA, 300 mg/kg, i.p.) together with penicillin (500 IU in 2.5  $\mu$ L, i.c.).<sup>11</sup>

Group 5: received D-limonene (100 mg/kg, i.p.) and sodium valproate (VPA, 300 mg/kg, i.p.) together with penicillin (500 IU in 2.5  $\mu$ L, i.c.).

### Drug Administration

Penicillin (Sigma-Aldrich, USA), D-limonene [(R)-(+)-limonene, 97%, Sigma-Aldrich; Cat. No. 183164-500ML], and sodium valproate (Sanofi, France) were used in this study. Penicillin was dissolved in physiological saline before administration. All chemicals were administered via the intraperitoneal (i.p.) route 30 minutes after penicillin injection. All drugs were freshly prepared immediately before administration and administered under sterile conditions. The selected doses were based on previously published studies demonstrating anticonvulsant and neuroprotective effects.<sup>9–11</sup>

### Electrocorticographic Recording Procedure

After anesthesia with urethane, the rats were positioned in a stereotaxic apparatus (Harvard Stereotaxic Instrument, USA). A rostrocaudal scalp incision (~3 cm) was made, and the soft tissue covering the left somatomotor cortex was carefully removed. The skull was thinned using a rotary drill.

Electrocorticographic (ECoG) recordings were obtained using two Ag/AgCl ball electrodes, and an Ag/AgCl clamp electrode served as the ground. The positive electrode was placed 1 mm anterior to the bregma and 2 mm lateral to the sagittal suture, whereas the negative electrode was positioned 5 mm posterior to the bregma and 2 mm lateral to the sagittal suture. A grounding electrode was attached to the right ear.

Body temperature was maintained at 37 °C using a homeothermic heating blanket connected to a rectal probe (Harvard Instruments, USA). Cortical electrophysiological signals were amplified and recorded using an MP150 data acquisition system with an EEG-100C amplifier (Bipac Systems, USA).

After intracortical penicillin administration, the development of epileptiform activity was confirmed by the appearance of characteristic spike discharges in the ECoG recordings. ECoG recordings were used solely to verify the successful induction of epileptiform activity, and quantitative ECoG parameters were not evaluated as outcome measures in the present study.

### Biochemical Analysis

Brain tissue samples were rinsed three times with cold saline and dried using filter paper. Fresh tissues were weighed using a precision balance and homogenized at a ratio of 1:9 (w/v) in phosphate-buffered saline (PBS; Sigma-Aldrich, P4417, Lot #SLCH5832; pH 7.2–7.6) on ice using a Teflon-tipped homogenizer (Bandelin, Germany).

The homogenates were centrifuged at 5000 $\times$ g for 5 minutes at 4 °C. Supernatants were collected and stored at –80 °C until analysis.

For biochemical analyses, plasma and tissue homogenates were thawed on ice, and all measurements were normalized to total protein content. Protein concentrations were determined using the Thermo Scientific™ Pierce™ BCA Protein Assay Kit (Catalog Nos. 23225 and 23227).

NO levels in plasma and tissue samples were quantified using a commercially available colorimetric assay kit (Elabscience, Catalog No. E-BC-K035-S) according to the manufacturer's instructions.

### Statistical Analysis

Statistical analyses were performed using SPSS software (version 26.0; IBM Corp., Armonk, NY, USA). Differences between groups were analyzed using one-way analysis of variance (ANOVA) followed by Sidak's multiple comparisons test. Data are presented as mean $\pm$ SD, and statistical significance was set at  $p < 0.05$ .

## RESULTS

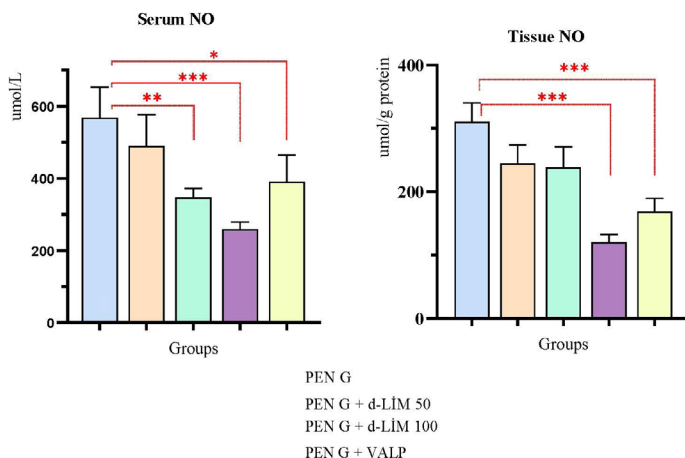
Biochemical analyses were performed to evaluate the dose-dependent effects of D-limonene (50 and 100 mg/kg, i.p.) and compare them with those of the reference anticonvulsant VPA (300 mg/kg, i.p.) on NO levels in penicillin-induced epileptic rats. VPA was used as a positive control because of its well-documented anticonvulsant efficacy. The results are presented as mean $\pm$ SD.

Table 1 presents the effects of D-limonene and VPA on serum and brain NO levels in penicillin-induced epileptic rats. The PEN-G group showed the highest NO concentrations in both serum and tissue, confirming enhanced nitrosative stress after penicillin administration. Treatment with D-limonene resulted in a dose-dependent decrease in NO levels, which was significant at 50 mg/kg ( $*p < 0.01$ ) and more pronounced at 100 mg/kg ( $**p < 0.001$ ). VPA administration caused the most substantial reduction in NO concentrations ( $***p < 0.0001$ ), whereas the combination of D-limonene (100 mg/kg) and VPA slightly increased NO levels compared with VPA alone but remained significantly lower than those in the PEN-G group (Fig. 2).

**Table 1.** Effects of D-limonene and VPA on serum and brain tissue NO levels in epileptic rats

Groups	Serum NO (µmol/L)	Brain tissue NO (µmol/g protein)
PEN-G	568.0±84.95	310.2±30.11
PEN-G + D-LIM 50	490.5±86.12 <sup>#</sup>	244.9±29.16 <sup>#*</sup>
PEN-G + D-LIM 100	347.6±25.39 <sup>**</sup>	238.5±32.19 <sup>#*</sup>
PEN-G + VPA	259.0±20.04 <sup>***</sup>	120.4±12.60 <sup>***</sup>
PEN-G + D-LIM 100 + VPA	390.6±74.95 <sup>*</sup>	168.8±20.85 <sup>***</sup>

PEN-G: Penicillin; D-LIM: D-limonene; VPA: Valproic acid; NO: Nitric oxide; SD: Standard deviation. Values are expressed as mean±SD (n=7 per group). Data were analyzed using one-way ANOVA followed by multiple comparisons tests. Overall one-way ANOVA indicated significant differences among groups for serum NO (p=0.0011) and brain tissue NO (p<0.0001). Statistical significance is indicated as follows: \*\*\*p<0.0001, \*\*p<0.001, and \*p<0.01 vs. the PEN-G group; #p<0.05 vs. the PEN-G + D-LIM 100 group; #\*p<0.001 vs. the PEN-G + VPA group; #p<0.01 vs. the PEN-G + D-LIM 100 + VPA group.



**Figure 2.** Effects of D-limonene and valproic acid (VPA) on serum and brain nitric oxide (NO) levels in penicillin-treated rats. Data are presented as mean±SD (n=7 per group). Data were analyzed using one-way ANOVA followed by Sidak’s multiple comparisons test. Overall ANOVA showed significant differences in serum (p=0.0011) and tissue NO levels (p<0.0001).

\*p<0.01, \*\*p<0.001, \*\*\*p<0.0001 vs. PEN-G.

A comparable pattern was observed in brain tissue. The highest tissue NO level was recorded in the PEN-G group (310.2±30.11 µmol/g protein), whereas VPA treatment yielded the lowest value (120.4±12.60 µmol/g protein; \*\*\*p<0.0001). Both doses of D-limonene significantly reduced NO levels compared with PEN-G, and the combined treatment produced intermediate levels, indicating partial attenuation of VPA’s inhibitory effect.

Furthermore, the intergroup comparisons denoted by # (p<0.001) and #\* (p<0.01) revealed that D-limonene, either alone or in combination with VPA, significantly modulated NO concentrations relative to the VPA and combination groups, respectively. Collectively, these findings demonstrate the distinct and dose-dependent effects of D-limonene and VPA on NO metabolism in serum and brain tissue.

## DISCUSSION

Epilepsy is a complex neurological disorder involving OS and neuroinflammation. Recurrent seizures can lead to increased metabolic demand and mitochondrial dysfunction, resulting in higher levels of ROS and RNS, which can disrupt neuronal homeostasis and redox equilibrium. This imbalance between pro-oxidant and antioxidant systems can lead to excitotoxicity.<sup>1,3</sup> As Basha et al.<sup>12</sup> suggest, this interplay is important for understanding oxidative and inflammatory cascades as therapeutic targets in epilepsy. Natural compounds, particularly terpenes and other constituents of essential oils (EOs), have attracted interest because of their ability to restore redox balance, inhibit microglial activation, and preserve mitochondrial integrity, thereby reducing epileptogenesis.<sup>12,13</sup>

EOs are biologically active hydrocarbons comprising oxygenated derivatives and complex mixtures. These natural compounds have notable properties and, as such, are used in cosmetics, hygiene products, and food preservation. Recent research has highlighted their antioxidant and neuroprotective properties, with investigations into their use in the treatment of neurological disorders. A large number of plant-derived EOs have been reported to cross the blood-brain barrier with minimal toxicity, indicating their potential as therapeutic agents for conditions such as epilepsy and depression.<sup>14</sup>

The findings indicate that the antioxidant and anti-inflammatory properties of D-limonene may provide neuroprotection against seizure-induced oxidative damage and neuronal hyperexcitability. Because of its lipophilic structure, D-limonene can easily penetrate cellular membranes. This property contributes to the preservation of mitochondrial integrity and the mitigation of ROS-mediated neuronal injury. As demonstrated in previous studies, the anticonvulsant properties of EOs are predominantly attributed to their capacity to modulate OS.<sup>15</sup> Similarly, Rani et al.<sup>13</sup> and Zhu et al.<sup>7</sup> emphasized that monoterpenes such as limonene, linalool, thymol, and eugenol possess notable antioxidant, anti-inflammatory, neuroprotective, and anticonvulsant properties that contribute to the maintenance of neuronal redox balance. In addition, plant-derived EOs rich in limonene and α-pinene have been reported to reduce seizure duration and mortality in pentylenetetrazol (PTZ)-induced animal models.<sup>15</sup> Collectively, these observations suggest that D-limonene mitigates epileptogenesis by

suppressing OS and inflammatory responses, offering a plausible mechanistic basis for its therapeutic potential in epilepsy.<sup>12,13</sup> In addition to its antioxidant properties, previous experimental studies indicate that D-limonene may regulate NO production by suppressing inflammatory signaling pathways. Specifically, inhibition of iNOS expression and reduction of pro-inflammatory cytokines such as IL-1 $\beta$  and TNF- $\alpha$  have been reported after limonene administration, suggesting that attenuation of neuroinflammation may contribute to the observed decrease in NO levels.<sup>8,16</sup>

Evidence shows that mitochondrial dysfunction, OS and NS, and neuroinflammation are interrelated and play key roles in the development of epilepsy. In drug-resistant epilepsy (DRE), mitochondrial impairment and redox imbalance can directly contribute to neuronal hyperexcitability and disease progression.<sup>17</sup> Disruptions in mitochondrial redox homeostasis also compromise energy metabolism, leading to recurrent seizures and epileptogenesis.<sup>18</sup> These findings suggest that OS is not merely an outcome but functions as a self-perpetuating “vicious cycle” that triggers neuronal damage in epilepsy. Consistent with this concept, experimental and clinical studies have demonstrated that excessive production of reactive oxygen and nitrogen species contributes to neuronal injury and seizure propagation in epilepsy models. Therefore, therapeutic strategies aimed at restoring redox balance and limiting nitrosative stress are considered important approaches for reducing seizure-related neuronal damage.<sup>1,17,18</sup>

In parallel, Alshehri et al.<sup>2</sup> demonstrated that status epilepticus (SE) involves several factors, including mitochondrial dysfunction, OS and NS, and inflammation. Li et al.<sup>4</sup> described epilepsy as a multifactorial disorder involving the interplay of OS, neuroinflammation, and ion channel dysfunction. These authors also suggested that natural antioxidant compounds may serve as adjunctive agents alongside AEDs to restore redox and neuroinflammatory balance.

The study by Banach et al.<sup>19</sup> revealed that NO plays an important neuromodulatory role in the pathophysiology of epilepsy. According to their findings, NO regulates neuronal excitability and synaptic transmission within the nervous system, exhibiting either proconvulsant or anticonvulsant effects depending on physiological conditions. This bidirectional profile suggests that NO may act as both a trigger and a modulator in epileptogenesis. Therefore, in the development of NO-targeted therapeutic strategies, careful optimization of variables such as dose, timing, and target region is crucial to achieving an effective balance between its dual actions. This study aimed to address the neuroprotective potential of D-limonene in an experimental penicillin-induced epilepsy model through the assessment of NO levels in brain tissue and serum.

Seo et al.<sup>20</sup> reported that D-limonene exhibited a remarkable anticonvulsant effect in a PTZ-induced epilepsy model. The study revealed that D-limonene decreased seizure severity in a dose-dependent manner by enhancing GABA synthesis and decreasing neuronal excitability. These findings are consistent with the NO-regulating effects of D-limonene observed in the present study, supporting its potential role as a protective mechanism during epileptogenesis. In the study by Tang et al.,<sup>16</sup> D-limonene was reported to reverse corticosterone-induced NO elevation and reduce iNOS expression, thereby restoring NO balance. These findings provide further evidence for the NO-regulating effects of D-limonene, as observed in the present study. In the present study, ECoG recordings were performed solely to verify the successful induction of epileptiform activity and were not analyzed as quantitative outcome parameters.

Jiang et al.<sup>21</sup> reported that D-limonene exerted a marked neuroprotective effect in an LPS-induced neuroinflammation model, primarily through the suppression of inflammatory responses. They also observed that the lower dose was more effective than the higher dose, suggesting a dose-response relationship linked to the pharmacokinetic profile of D-limonene. In line with these findings, the present study investigated two different doses of D-limonene to evaluate its dose-dependent effects. The results demonstrated that the 100 mg/kg dose had a stronger regulatory effect on NO levels, indicating that higher concentrations of D-limonene may enhance the control of oxidative and inflammatory mechanisms associated with epileptogenesis.

Eddin et al.<sup>8</sup> reported that D-limonene reduced NO levels in models of PTZ- and maximal electroshock (MES)-induced epilepsy and consequently alleviated OS and NS. These findings are consistent with the NO-suppressing and redox-stabilizing effects of D-limonene observed in the present study. Furthermore, Zhu et al.<sup>3</sup> highlighted the importance of neuronal iNOS-expressing cells in the development of temporal lobe epilepsy (TLE). A decrease in iNOS and NO production has been linked to abnormal changes in synaptic activity, highlighting the vital role of NO as a signaling molecule and a key player in network activity and stability. Importantly, NO has a dual role in the nervous system, acting as both a neuroprotective signaling molecule and a mediator of neuronal injury depending on its concentration and cellular context. This bidirectional role may help explain why the combination of D-limonene and VPA resulted in moderately higher NO levels compared with VPA alone in the present study, suggesting a potential modulatory effect of D-limonene on physiological NO signaling rather than a simple antagonistic interaction.<sup>3,19</sup>

Kadam et al.<sup>5</sup> reported that VPA induces OS and mitochondrial dysfunction through increased ROS formation and glutathione depletion. In agreement with these findings, the present study demonstrated that VPA markedly reduced NO levels, indicating a disruption in redox signaling linked to mitochondrial impairment. Moreover, D-limonene modulated this reduction in a dose-dependent manner, suggesting that its antioxidant and mitochondrial-protective properties may contribute to the restoration of physiological NO homeostasis and redox balance. Although the combination group showed numerically higher NO levels than the VPA group, the difference was not statistically significant; therefore, this finding does not support a definitive antagonistic effect of D-limonene on VPA but rather suggests a possible modulatory interaction that warrants further investigation.

Collectively, the evidence highlights a shared mechanism linking OS, mitochondrial dysfunction, and NO dysregulation in epileptogenesis. Within this framework, the present study demonstrates that D-limonene restores redox balance by modulating NO metabolism. By combining antioxidant, antinitrosative, and mitochondrial-protective actions, D-limonene emerges as a promising adjunctive neuroprotective candidate that may enhance the therapeutic efficacy of conventional antiepileptic drugs and alleviate their redox-related adverse effects. Taken together, the present findings, together with previous experimental evidence, suggest that D-limonene may exert neuroprotective effects in epilepsy through modulation of OS, suppression of neuroinflammatory pathways, and regulation of NO homeostasis.<sup>8,20,21</sup>

This study has several limitations that should be considered when interpreting the findings. First, the absence of a sham control group limits the evaluation of baseline NO levels independent of penicillin administration. Second, electrocorticographic recordings were used only to confirm the induction of epileptiform activity and were not analyzed as quantitative outcome parameters. In addition, seizure severity, behavioral assessments, and additional OS markers were not evaluated in the present study. Future studies including these parameters may provide a more comprehensive understanding of the neuroprotective effects of D-limonene in experimental epilepsy.

## CONCLUSION

This study revealed that D-limonene exerted a dose-dependent neuroprotective effect by reducing NO levels in a penicillin-induced epilepsy model. Although VPA produced stronger inhibition of NO synthesis, coadministration with D-limonene partially reversed this suppression, suggesting that D-limonene helps maintain physiological NO balance. Overall, these findings indicate that D-limonene's modulation of NO metabolism contributes to reduced OS and preservation of redox homeostasis, supporting its potential as a complementary agent alongside VPA in epilepsy management.

**Ethics Committee Approval:** Ethics committee approval was obtained from Tokat Gaziosmanpaşa University Animal Experiments Local Ethics Committee (Approval Number: 51879863-35; Date: 07.03.2025).

**Informed Consent:** Written informed consent was not required as the study did not require human participants.

**Conflict of Interest:** The authors have no conflicts of interest to declare.

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