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Research paper

The potential role of nitric oxide in the anticonvulsant effects of betulin in pentylenetetrazole (PTZ)-induced seizures in mice

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ABSTRACT

Epilepsy poses a significant challenge, especially for drug-resistant cases, necessitating novel treatment avenues. This study explores the potential interplay between nitric oxide (NO) and the anticonvulsant effects of betulin, a triterpene with promising neuroprotective properties. While betulin exhibits anticonvulsant effects, the specific involvement of NO remains inadequately understood, constituting a pivotal gap in current knowledge. One hundred NMRI mice were randomly assigned to diverse treatment groups, with seizures induced by pentylenetetrazol (PTZ). Parameters such as seizure threshold, nitrite levels, total antioxidant capacity (TAC), malondialdehyde (MDA) levels, and iNOS/nNOS gene expressions were assessed. Betulin significantly increased seizure thresholds and mitigated PTZ-induced NO levels. These findings suggest a potential modulation of NO-related pathways, emphasizing betulin's anti-inflammatory and antioxidant attributes. The study sheds light on betulin's multifaceted impact on oxidative stress, NO regulation, and iNOS/nNOS gene expressions. The ability of betulin to suppress iNOS/nNOS gene expressions, leading to reduce NO production, underscores its potential as an anticonvulsant.

1. Introduction

Epilepsy is a prevalent chronic neurological disorder characterized by recurrent and unprovoked seizures, affecting a substantial number of individuals worldwide (Beghi, 2019; Mumtaz et al., 2022). The quest for effective anticonvulsant treatments remains a pressing challenge, particularly for the approximately 20–30% of individuals with drug-resistant epilepsy (DRE) who do not achieve satisfactory seizure control with currently available medications (Dalic and Cook, 2016; Perucca et al., 2018). Addressing this unmet medical need requires the development of novel and effective drugs for epilepsy treatment.

To investigate the underlying mechanisms of seizures and identify potential therapeutic interventions, researchers have turned to the pentylenetetrazol (PTZ)-induced seizure model in mice, known for its close resemblance to seizures observed in humans (Yuen and Trocóniz, 2015; Socała and Wlaź, 2021).

In recent years, the role of nitric oxide (NO) in seizure pathophysiology has garnered increasing attention (Amini-Khoei et al., 2023). NO, a gaseous signaling molecule, plays diverse roles in various physiological processes, including neuronal signaling and synaptic plasticity (Hölscher, 1997). Its degradation results in the production of nitrate (NO_3^-) and nitrite (NO_2^-), which can be recycled in vivo to regenerate NO (Cossenza et al., 2014). NO is synthesized through the enzymatic conversion of the amino acid L-arginine (L-arg) by the enzyme nitric oxide synthase (NOS) (Eduardo, 2011). There are three genetically distinct isoforms of NOS: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS) (Zhou and Zhu, 2009). While nNOS and eNOS are constitutively expressed and responsible for the basal production of NO under normal physiological conditions, iNOS is induced in response to inflammatory mediators (Prieto-Martín et al., 2012). Within the central nervous system (CNS), NO acts as a critical modulator of neurotransmission, influencing glutamatergic neurotransmission and excitatory neurotransmitter release (Huang et al., 2003; Džoljić et al., 2015; Khaledi et al., 2023). The signaling of NO in the target cells involves binding with soluble guanylate cyclase (GC), leading to the production of cyclic guanosine monophosphate (cGMP) and subsequent modulation of downstream substrates (Panthi et al., 2018). Altered NO levels have been reported in experimental models of epilepsy and in patients with epilepsy (Shafaroodi et al., 2015; Eldin et al., 2016). Notably, NO exhibits context-dependent effects, with both proconvulsant and

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anticonvulsant properties, determined by specific conditions and experimental settings (Zamanian et al., 2020).

Betulin, a pentacyclic triterpene found in certain plants, has gained attention for its potential therapeutic properties (Hordyjewska et al., 2019). Previous studies have reported various pharmacological activities of betulin, including anti-inflammatory, antioxidant, and neuroprotective effects (Liu et al., 2020; Tuli et al., 2021). Additionally, betulin has demonstrated anticonvulsant properties in animal models of seizures (Muceniece et al., 2016). Specifically, research has revealed that betulin possesses unique characteristics related to the $GABA_A$ receptor, both in vivo and in vitro, displaying binding affinity to the $GABA_A$ receptor and eliciting anticonvulsant effects in mice (Muceniece et al., 2008). Nevertheless, the involvement of NO in its anticonvulsant mechanism remains inadequately elucidated, constituting a significant deficiency in our current understanding.

Given the known involvement of NO in seizure pathophysiology and the reported anticonvulsant effects of betulin, it is hypothesized that NO may play a role in the anticonvulsant effects of betulin. Therefore, the present study aims to investigate the potential role of nitric oxide in the anticonvulsant effects of betulin using PTZ-induced seizures in mice. By elucidating the underlying mechanisms involved, this research may contribute to our understanding of epilepsy pathophysiology and provide a basis for the development of new anticonvulsant therapies.

2. Materials and methods

2.1. Ethics

The experimental procedures described in this study were conducted in strict adherence to the ethical guidelines outlined in the Guide for the Care and Use of Laboratory Animals (8th edition, National Academies Press) as prescribed by the National Institutes of Health (NIH). Approval for the study protocol was obtained from the Ethics Committee of the Shahrekord University of Medical Sciences (Ethics code: IR.SKUMS.REC.1398.205). To ensure the welfare of the animals, meticulous efforts were made to minimize any potential suffering throughout the experimental procedures.

2.2. Animals

In this study, a total of 60 male Naval Medical Research Institute (NMRI) mice were used as experimental subjects. The mice were sourced from the Pasteur Institute in Tehran, Iran, and were selected based on their weight range of 25–30 g and age range of 8–12 weeks. Upon arrival at the laboratory, the mice were acclimated for a period of one week to the controlled experimental conditions. The acclimation process involved housing the mice in a controlled environment with a 12-hour light-dark cycle and maintaining a controlled temperature range of 24–22ºC. Throughout the acclimation and experimental period, the mice had ad libitum access to standard laboratory food and water.

2.3. Study design

Following the acclimation period, the mice were randomly assigned to ten groups, each consisting of 6 mice, as described below:

Group 1 (control group) received a solution of normal saline. Group 2–5 received betulin at different doses, including 100 mg/kg (Group 2), 200 mg/kg (Group 3), 300 mg/kg (Group 4), and 400 mg/kg (Group 5). Group 6 received diazepam at a dose of 10 mg/kg. Group 7 received L-NAME (nitric oxide synthetase inhibitor) at a dose of 10 mg/kg. Group 8 received L-arg at a dose of 100 mg/kg. Group 9 received an ineffective dose of betulin (100 mg/kg) along with L-NAME (10 mg/kg). Group 10 received an effective dose of betulin (400 mg/kg) along with L-arg (100 mg/kg). All drugs were administered intraperitoneally (i.p.) 45 minutes prior to the induction of seizures. Betulin was injected acutely and simultaneously with NO mediators via intraperitoneal route. Dose and time of administrations were selected based on our pilot study as well as previous studies (Włodarczyk et al., 2013; Amini-Khoei et al., 2022). After behavioral assessment, anesthesia was induced using diethyl ether. Blood samples were collected under deep anesthesia for measurement of serum nitrite levels, total antioxidant capacity (TAC), and malondialdehyde (MDA) levels. Additionally, the prefrontal cortex of the brain was dissected to assess nitrite levels, TAC, MDA levels, and the expression of iNOS and nNOS genes (Tavakoli et al., 2023). All chemicals were purchased from Sigma Aldrich. Diazepam was purchased of Daru Pakhsh, Iran.

2.4. Seizure induction and evaluation of seizure threshold

In this experiment, a seizure pump was utilized to deliver a continuous infusion of PTZ (0.5%) at a rate of 1 ml/min to the tail vein of mice, while the tail was immobilized using a 30-gauge needle. The mice were allowed to move freely during the infusion, without any form of restraint. The PTZ infusion was terminated immediately after the onset of clonic seizures (stage 5 of Racine's scale), which was used to verify successful induction of seizures. In this method, the determination of seizure threshold is influenced by the dosage of PTZ administered as well as the duration of exposure (Van Erum et al., 2019; Rahimi-Madiseh et al., 2022).

2.5. Determination of nitrite levels in serum and prefrontal cortex

Nitrite concentration in prefrontal cortex and serum samples was determined using the Griess reaction. Initially, 100 µl of each homogenized prefrontal cortex tissue sample or serum was mixed with 100 µl of Griess reagent. Following a 10-minute incubation period at room temperature, the absorbance at 540 nm was measured with a plate reader (Stat Fax- 2100, Awareness Technology, USA). Finally, the nitrite concentration of each sample was calculated using sodium nitrite standards curves (Kumar et al., 2013).

2.6. Determination of TAC in serum and prefrontal cortex

The TAC of serum and prefrontal cortex tissue was evaluated using the FRAP method. The ability of serum and homogenized brain tissue to reduce Fe^{3+} to Fe^{2+} in the presence of the TPTZ2 reagent was determined, resulting in the formation of a blue-colored TPTZ2-Fe^{2+} complex. The optical absorption at 593 nm was recorded to measure the total antioxidant capacity (Benzie and Strain, 1996).

2.7. Determination of MDA levels in serum and prefrontal cortex

The level of MDA, a marker of lipid peroxidation and oxidative stress, was measured in serum and prefrontal cortex samples (Gaweł et al., 2004). The samples were mixed with acetic acid, thiobarbituric acid, and SDS solution, followed by heating in a Bain-marie for 1 hour. After cooling and centrifugation, the optical absorbance at 532 nm was recorded to quantify the MDA levels (Jain et al., 2011).

2.8. Determination of iNOS and nNOS gene expressions in the prefrontal cortex

To identify and quantify iNOS and nNOS genes in the prefrontal cortex, real-time polymerase chain reaction (RT-PCR) was performed. Total RNA from the tissue was extracted using the RNX-plus isolation reagent according to the manufacturer's instructions. Subsequently, the PrimeScript RT reagent kit was used to reverse-transcribe one µg of RNA from each sample. The mRNA expression of target genes was then evaluated by RT-PCR using SYBR Premix Ex Taq technology and a light cycler device. The thermal cycling procedure consisted of an initial activation step for 30 s at 96 °C, followed by 45 cycles of denaturation for 5 s at 96 °C, and a combined annealing/extension step for 20 s at 60

°C. Additionally, a melting curve analysis was conducted to ensure that all primers generated a single PCR product. The B2M gene was used as a normalizer, and alterations in the expression of each desired mRNA were calculated using the $2^{-\Delta\Delta Ct}$ relative expression formula (Tavakoli et al., 2023). The primer sequences are presented in Table 1.

2.9. Data analysis

All data were expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using SPSS 22. One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used for multiple comparisons. p-values less than 0.05 were considered statistically significant.

3. Results

3.1. Effect of betulin on the seizure threshold

Fig. 1 presents the seizure threshold results obtained in this study. The groups treated with betulin at doses of 200 mg/kg, 300 mg/kg, and 400 mg/kg exhibited a significantly increased seizure threshold compared to the control group ($p < 0.001$). Similarly, the diazepam, L-NAME, and L-arg groups showed a significantly increased seizure threshold compared to the control group ($p < 0.001$). The group that received a subeffective dose of betulin (100 mg/kg) in combination with L-NAME demonstrated a significantly increased seizure threshold compared to the group that received the subeffective dose of betulin alone ($p < 0.05$). The group that received an effective dose of betulin (400 mg/kg) in combination with L-arg did not exhibit a significant increase in the seizure threshold compared to the group that received the effective dose of betulin alone.

3.2. Effect of betulin on nitrite levels in serum

The levels of nitrite, an indicator of nitric oxide activity, were measured in serum samples to assess the effect of betulin (Fig. 2). Results showed that betulin administration at doses of 100, 200, 300, and 400 mg/kg significantly decreased nitrite levels compared to the control group ($p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.001$, respectively). Furthermore, the group treated with DIAZ exhibited a significant decrease in nitrite levels compared to the control group ($p < 0.001$). Besides, the group treated with L-NAME showed a significant decrease in nitrite levels compared to the control group ($p < 0.01$). The combination of a subeffective dose of betulin (100 mg/kg) with L-NAME resulted in a significant decrease in nitrite levels compared to the group that received the subeffective dose of betulin alone ($p < 0.01$) and compared to the control group ($p < 0.001$). Conversely, the group that received an effective dose of betulin (400 mg/kg) along with L-arg exhibited a significant increase in nitrite levels compared to the group that received the effective dose of betulin alone ($p < 0.01$) and showed a significant decrease in nitrite levels compared to the control group ($p < 0.001$).

3.3. Effect of betulin on nitrite levels in the prefrontal cortex

The nitrite levels were also measured in the prefrontal samples to assess the effect of betulin (Fig. 3). Results revealed that betulin administration at doses of 300 and 400 mg/kg significantly decreased nitrite levels compared to the control group ($p < 0.001$). Furthermore,

Table 1
The primer sequences used in PCR amplification.

Reverse sequence	Forward sequence	Primers
AGGGGTGATACGCTTTACCTTTA	TCATCGACACCTGAAATCTAGGA	**B2M**
AGAATAGGAGGAGACGCT GT	GGCTGTGCTTTGATGGAG ATGA	**nNOS**
GGACATCAAAGGTCTCACAGGC	CCAACAGGAGAAGGGGACGAA	**iNOS**

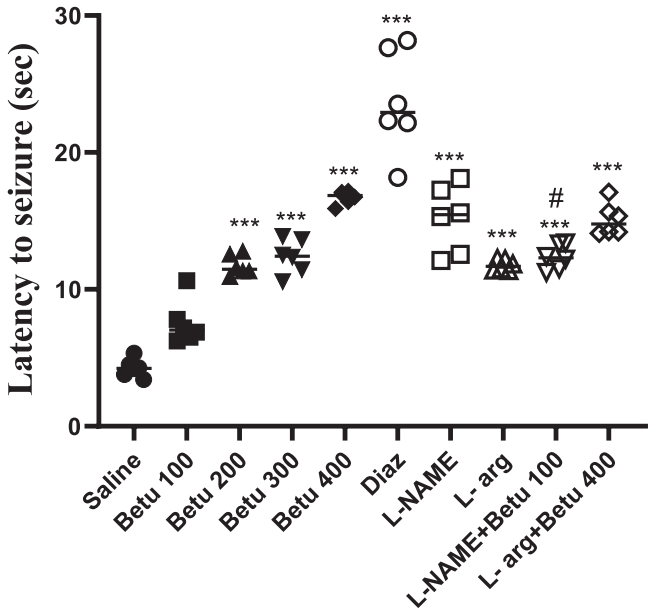

Fig. 1. The impact of betulin on the seizure threshold was examined by calculating values based on a sample of 6 mice, and the results were presented as the mean ± S.E.M. Statistical analysis involved the use of a one-way ANOVA followed by Tukey's post-test. Significance levels were denoted as ***p<0.001 in comparison to the control group treated with saline, and #p<0.05 in comparison to the group administered betulin at a dosage of 100 mg/kg. Betu: betulin; Diaz: diazepam.

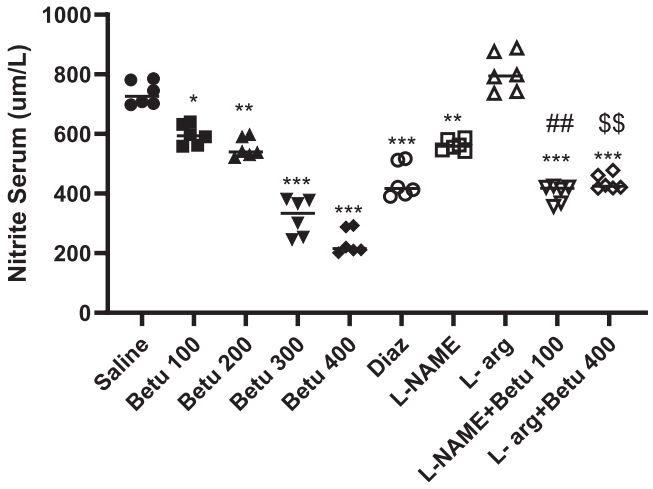

Fig. 2. The impact of betulin on serum nitrite levels was examined by calculating values based on a sample of 6 mice, and the results were presented as the mean ± S.E.M. Statistical analysis involved the use of a one-way ANOVA followed by Tukey's post-test. ***p<0.001, **p<0.01, and *p<0.05 in comparison to the control group treated with saline, ##p<0.01 in comparison to the group administered betulin at a dosage of 100 mg/kg, and $$p<0.01 in comparison to the group administered betulin at a dosage of 400 mg/kg. Betu: betulin; Diaz: diazepam.

the group treated with DIAZ exhibited a significant decrease in nitrite levels compared to the control group ($p < 0.001$). Besides, the group treated with L-NAME showed a significant decrease in nitrite levels compared to the control group ($p < 0.001$), while the group treated with L-arg showed no significant changes in the nitrite levels of the prefrontal cortex compared to the control group. The combination of a subeffective dose of betulin (100 mg/kg) with L-NAME resulted in a significant decrease in nitrite levels compared to the group that received the

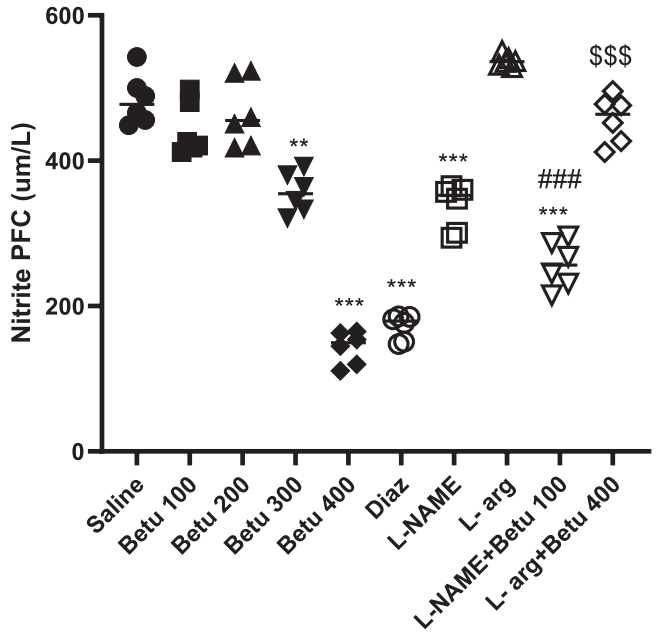

Fig. 3. The impact of betulin on PFC nitrite levels was examined by calculating values based on a sample of *6* mice, and the results were presented as the mean ± S.E.M. Statistical analysis involved the use of a one-way ANOVA followed by Tukey's post-test. ***p<0.001 in comparison to the control group treated with saline, ###p<0.01 in comparison to the group administered betulin at a dosage of 100 mg/kg, and $$$p<0.001 in comparison to the group administered betulin at a dosage of 400 mg/kg. Betu: betulin; Diaz: diazepam.

subeffective dose of betulin alone ($p < 0.001$) and compared to the control group ($p < 0.001$). Conversely, the group that received an effective dose of betulin (400 mg/kg) along with L-arg exhibited a significant increase in nitrite levels compared to the group that received the effective dose of betulin alone ($p < 0.001$), but did not show a significant change in nitrite levels compared to the control group.

3.4. Effect of betulin on TAC in serum

The TAC of serum samples from various experimental groups was assessed using the FRAP method (Fig. 4). Administration of betulin at a dose of 100 mg/kg showed a significant increase in TAC compared to the control group ($p < 0.01$). Similarly, betulin at doses of 200, 300, and 400 mg/kg resulted in significantly higher TAC levels compared to the control group ($p < 0.001$ for all comparisons). However, The administration of DIAZ or L-NAME did not result in a statistically significant alteration of TAC when compared to the control group ($p > 0.05$). Likewise, the co-administration of L-NAME with a subeffective dose of betulin (100 mg/kg) did not result in a significant alteration in TAC compared to the group that received 100 mg/kg of betulin alone ($P > 0.05$). Interestingly, the group treated with L-arg exhibited a significant decline in TAC of serum compared to the control group ($P < 0.05$). Furthermore, the co-administration of an effective dose of betulin (400 mg/kg) with L-arg resulted in a statistically significant reduction in TAC in comparison to the group receiving betulin alone at the same dose (400 mg/kg) ($p < 0.01$), while it led to a statistically significant elevation in TAC compared to the control group ($p < 0.01$).

3.5. Effect of betulin on TAC in the prefrontal cortex

Fig. 5 displays the TAC of the PFC. The findings demonstrated a significant increase in the TAC of the PFC with the administration of betulin at doses of 200, 300, and 400 mg/kg ($p < 0.001$). Conversely, the administration of DIAZ did not result in a significant change in the TAC of the PFC ($p > 0.05$). Similarly, L-arg administration did not have a

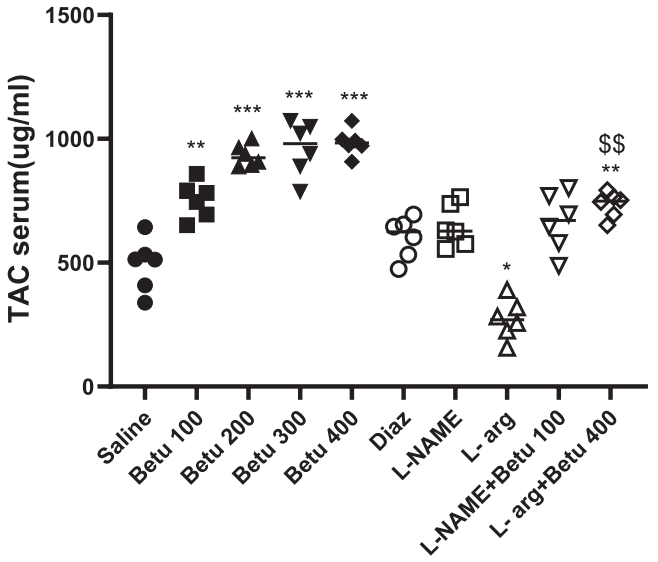

Fig. 4. The impact of betulin on serum's total antioxidant capacity (TAC) was examined by calculating values based on a sample of *6* mice, and the results were presented as the mean ± S.E.M. Statistical analysis involved the use of a one-way ANOVA followed by Tukey's post-test. ***p<0.001, **p<0.01, and *p<0.05 in comparison to the control group treated with saline, $$p<0.01 in comparison to the group administered betulin at a dosage of 400 mg/kg. Betu: betulin; Diaz: diazepam.

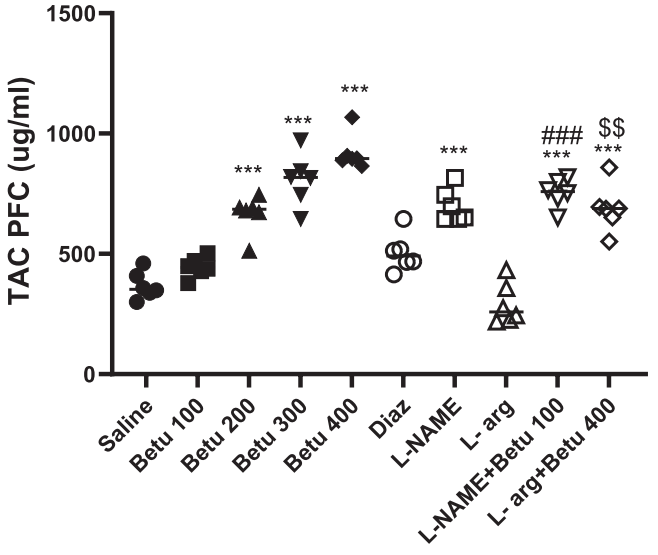

Fig. 5. The impact of betulin on brain's total antioxidant capacity (TAC) was examined by calculating values based on a sample of *6* mice, and the results were presented as the mean ± S.E.M. Statistical analysis involved the use of a one-way ANOVA followed by Tukey's post-test. ***p<0.001 in comparison to the control group treated with saline, ###p<0.001 in comparison to the group administered betulin at a dosage of 100 mg/kg, and $$$p<0.001 in comparison to the group administered betulin at a dosage of 400 mg/kg. Betu: betulin; Diaz: diazepam.

significant effect on the TAC of the PFC. However, when L-arg was combined with an effective dose of betulin (400 mg/kg), it led to a significant increase in the TAC of the PFC compared to the control group ($p < 0.001$), and a significant decrease in the TAC of the PFC compared to the group that received an effective dose of betulin alone ($p < 0.01$). Furthermore, the administration of L-NAME increased the TAC of the PFC significantly compared to the control group ($p < 0.001$). Similarly, the combination of L-NAME and a subeffective dose of betulin

significantly increased the TAC of the PFC compared to the control group and the group that received a subeffective dose of betulin alone (100 mg/kg) ($p < 0.001$).

3.6. Effect of betulin on MDA levels in serum

Fig. 6 shows the effect of betulin on the levels of MDA in serum. Administration of betulin at doses of 100, 200, 300, and 400 mg/kg demonstrated a significant reduction in MDA levels in serum ($p < 0.001$). Similarly, the administration of DIAZ, L-NAME, and L-arg also exhibited a significant decrease in MDA levels in serum ($p < 0.001$). Furthermore, when L-arg was combined with an effective dose of betulin (400 mg/kg), it resulted in a significant decrease in MDA levels in serum compared to the control group ($p < 0.001$); however, no significant changes were observed in MDA levels in serum compared to the group that received an effective dose of betulin (400 mg/kg) alone ($p > 0.05$). Additionally, the combination of L-NAME and a subeffective dose of betulin significantly decreased MDA levels in serum compared to the control group ($p < 0.001$); however, there were no significant differences in MDA levels in serum compared to the group that received a subeffective dose of betulin (100 mg/kg) ($p > 0.05$).

3.7. Effect of betulin on MDA levels in the prefrontal cortex

Fig. 7 illustrates the results of the assessment of MDA levels in the prefrontal cortex in response to different treatments. Betulin administration at a dose of 400 mg/kg resulted in a significant decrease in MDA levels in the PFC ($p < 0.001$). Additionally, when L-arg was combined with an effective dose of betulin (400 mg/kg), it led to a significant increase in MDA levels in the PFC compared to the group received 400 mg/kg of betulin alone ($p < 0.001$). No significant changes in MDA levels in the PFC were observed in the other experimental groups.

3.8. Effect of betulin on the iNOS and nNOS gene expressions in the prefrontal cortex

The administration of betulin at doses of 200 and 400 mg/kg significantly reduced the gene expression of nNOS in the PFC compared to the control group ($p < 0.01$). However, the doses of 100 and 300 mg/kg of betulin did not show a statistically significant effect on nNOS gene expression in the PFC when compared to the control group ($p > 0.05$). Moreover, the administration of L-NAME and L-NAME + subeffective dose of betulin (100 mg/kg) resulted in a significant decrease in nNOS gene expression in the PFC compared to the control group ($p < 0.01$). Conversely, the administration of L-arg showed a significant increase in nNOS gene expression in the PFC compared to the control group ($p < 0.05$). Notably, the combination of L-arg with an effective dose of betulin (400 mg/kg) caused a significant increase in nNOS gene expression compared to the group that received 400 mg/kg of betulin alone ($p < 0.001$). Additionally, the combination of a subeffective dose of betulin (100 mg/kg) with L-NAME resulted in a significant decrease in iNOS gene expression compared to the group that received the subeffective dose of betulin alone ($p < 0.05$) (Fig. 8a).

Furthermore, the administration of betulin at doses of 200 and 400 mg/kg resulted in a significant reduction in iNOS gene expression in the PFC compared to the control group ($p < 0.01$ and $p < 0.05$, respectively). However, the doses of 100 and 300 mg/kg of betulin did not demonstrate a significant effect on iNOS gene expression in the PFC when compared to the control group ($p > 0.05$). Similarly, the administration of L-NAME and L-NAME + subeffective dose of betulin (100 mg/kg) did not elicit a significant alteration in iNOS gene expression in the PFC compared to the control group ($p > 0.05$). On the contrary, the administration of L-arg showed a significant increase in iNOS gene expression in the PFC compared to the control group ($p < 0.001$). Additionally, DIAZ exhibited a remarkable decrease in iNOS gene expression compared to the control group ($p < 0.01$). Notably, when L-arg was combined with an effective dose of betulin (400 mg/kg), there was a significant increase in iNOS gene expression compared to the group that received 400 mg/kg of betulin alone ($p < 0.05$) (Fig. 8b).

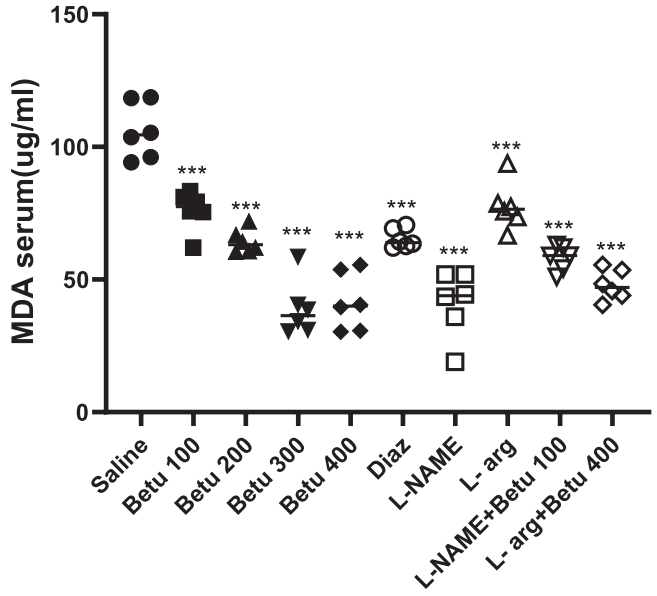

Fig. 6. The impact of betulin on serum malondialdehyde (MDA) levels was examined by calculating values based on a sample of *6* mice, and the results were presented as the mean ± S.E.M. Statistical analysis involved the use of a one-way ANOVA followed by Tukey's post-test. ***p<0.001 in comparison to the control group treated with saline. Betu: betulin; Diaz: diazepam.

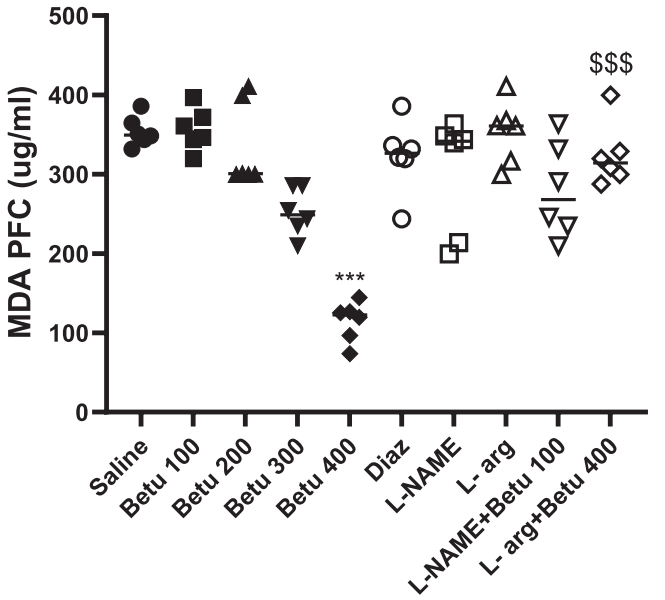

Fig. 7. The impact of betulin on brain malondialdehyde (MDA) levels was examined by calculating values based on a sample of *6* mice, and the results were presented as the mean ± S.E.M. Statistical analysis involved the use of a one-way ANOVA followed by Tukey's post-test. ***p<0.001 in comparison to the control group treated with saline, $$$p<0.01 in comparison to the group administered betulin at a dosage of 400 mg/kg. Betu: betulin; Diaz: diazepam.

4. Discussion

In this study, we investigated the potential role of NO in the anticonvulsant-like effects of betulin using a mouse model of PTZ-induced seizures. Our results demonstrate that betulin administration

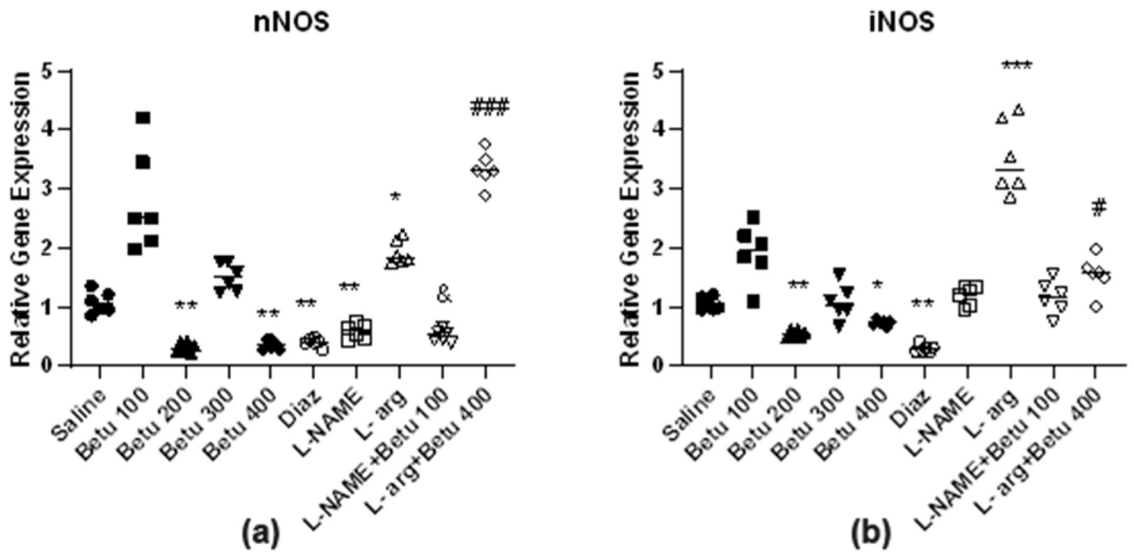

Fig. 8. The impact of betulin on the gene expression of nNOS (a) and iNOS (b) in the prefrontal cortex was examined by calculating values based on a sample of *6* mice, and the results were presented as the mean ± S.E.M. Statistical analysis involved the use of a one-way ANOVA followed by Tukey's post-test. ***p<0.001, **p<0.01, and *p<0.05 in comparison to the control group treated with saline, ###p<0.001 and #p<0.05 in comparison to the group administered betulin at a dosage of 400 mg/kg, and &p<0.05 in comparison to the group administered betulin at a dosage of 100 mg/kg. Betu: betulin; Diaz: diazepam.

leads to significant increase in latency to seizure. Furthermore, we found that betulin treatment attenuates the increase in NO levels induced by PTZ, suggesting a modulation of NO signaling pathways. These findings contribute to our understanding of the mechanisms underlying the anticonvulsant effects of betulin and highlight the involvement of NO in this process.

Moreover, the modulation of NO levels by betulin in our study provides novel insights into its anticonvulsant effects. NO is a known signaling molecule involved in various physiological and pathological processes, including neuronal excitability and neuroinflammation (Yuste et al., 2015; Gambino et al., 2020). Neuroinflammation contribute significantly to the development and progression of seizures (Kleen and Holmes, 2008). Proinflammatory cytokines, such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α, have been identified as crucial factors in the pathogenesis of seizures, exerting an impact on the hyperexcitability of the brain (Soltani Khaboushan et al., 2022). Neuroinflammation triggers neuronal cell death through the accumulation of reactive oxygen and nitrogen species (RONS) (Roberts et al., 2010). The brain's susceptibility to oxidative stress, due to an imbalance between antioxidant defenses and RONS generation, leads to oxidative or nitrosative damage to neurons (Aranda-Rivera, Cruz-Gregorio et al. 2022).

NO's role in neuroinflammation involves its release from activated microglia, inducing glutamate release and promoting NMDA receptor activation, leading to neuronal death (Brown and Bal-Price, 2003; Liy et al., 2021). Chronic NMDA administration up-regulates proinflammatory markers, including IL-1β, TNF-α, and iNOS, suggesting a cross-talk between neuroinflammation and excitotoxicity involving NO release and iNOS up-regulation in the brain (Brown and Bal-Price, 2003). Furthermore, the transcriptional regulation of iNOS, a key player in neuroinflammatory processes, involves nuclear factor-κB (NF-κB) (Singh, Rai et al. 2020). This NF-κB-mediated iNOS expression triggers pathways related to RONS formation, leading to oxidative stress and brain damage (Oh et al. 2009; Shaked et al., 2012). In an experiment conducted by Ci et al., it was demonstrated that betulin possesses a significant inhibitory effect on the lipopolysaccharides (LPS)-induced expression of iNOS. Additionally, betulin was found to exert a protective effect on the production of ROS within the murine macrophage cell line (Ci, Zhou et al. 2017). Moreover, it has been observed that derivatives of betulin significantly attenuate the production of NO and IL-6, and suppress the expression of iNOS at the post-transcriptional level (Laavola, Haavikko et al. 2016). These findings underscore the potent anti-inflammatory properties of betulin derivatives, a conclusion that is consistent with the results of our investigation. The findings of our research underscore the multifaceted therapeutic potential of betulin. It was observed that betulin exerts both anti-inflammatory and antioxidant effects, as evidenced by the significant reduction in MDA levels and enhancement of the TAC. Furthermore, betulin was found to suppress the gene expression of nitric oxide synthase, particularly iNOS, thereby reducing NO levels.

In this context, a study conducted by Muceniece et al. demonstrated that the anticonvulsant activity of betulin could potentially be attributed to the activation of the $GABA_A$-receptor-mediated signaling pathway (Muceniece et al., 2008). Furthermore, it has been established that abnormal nNOS activity can trigger seizures induced by $GABA_A$ antagonism. Therefore, the blockade of nNOS emerges as a viable pharmacological strategy to manage the epileptogenic mechanisms underlying seizures induced by $GABA_A$ antagonism, and it can also enhance the anticonvulsant action of some conventional antiepileptic drugs (Rajasekaran et al., 2003).

In our investigation, administration of a sub-therapeutic dose of betulin (100 mg/kg) did not elicit an elevation in latency to seizure. However, concomitant application of L-NAME resulted in a significant augmentation of the latency to seizure. This observation suggests that NOS inhibition may potentiate the anticonvulsant properties of betulin. Contrary to our initial hypothesis, the addition of L-arg to a therapeutically effective dose of betulin (400 mg/kg) did not affect the latency to seizure. This unexpected outcome may be attributed to betulin's mechanism of action, which is postulated to involve the activation of GABAergic receptors (Muceniece et al., 2008). It appears that even an increase in NO levels, a reduction in TAC, and an escalation of oxidative stress within the PFC do not impede the anticonvulsant efficacy of high dosage of betulin. These findings imply that the nitrergic system may exert a more pronounced influence on the anticonvulsant effects of betulin at lower dosages than at higher ones.

Taken together, these findings suggest that the anticonvulsant effects of betulin may be partially due to the suppression of both iNOS and nNOS, leading to a reduction in NO production. This mechanism could contribute to the attenuation of neuroinflammation and oxidative stress, further emphasizing the therapeutic potential of betulin.

A notable limitation of this study lies in the evaluation of the molecular mechanisms underlying the role of NO primarily at the gene expression level. While the investigation provides valuable insights into how betulin may modulate NO-related gene expression in response to PTZ-induced seizures, it is essential to recognize that gene expression does not always directly correlate with protein levels or activity (Tartaglia et al., 2007; Edfors et al., 2016). To provide a more comprehensive understanding of the mechanisms involved, future research should incorporate additional analyses at the protein level. Techniques such as Western blotting, immunohistochemistry (IHC), or enzyme-linked immunosorbent assay (ELISA) could be employed to assess the actual protein expression and activity levels of NOS isoforms, providing a more direct link between the observed gene expression changes and their functional impact (Beesley, 1995).

Furthermore, the study's focus on male mice in investigating the role of NO in PTZ-induced seizures presents another limitation. Epilepsy exhibits sex-dependent differences in incidence, severity, and response to treatment (Scharfman and MacLusky, 2014; Catherine et al., 2020). Considering the growing recognition of sex-specific factors in neurological disorders, it would be valuable to extend the investigation to female mice.

This research lays the groundwork for further investigation, aiming to translate betulin's anticonvulsant effects into effective treatments for epilepsy, particularly in drug-resistant cases.

5. Conclusions

In conclusion, this research elucidates the complex relationship between NO modulation and betulin's anticonvulsant effects in a PTZ-induced seizure model. The results indicate that betulin's anticonvulsant activity is partially due to its ability to regulate NO levels, primarily through the suppression of iNOS and nNOS gene expressions in the prefrontal cortex. This leads to a decrease in NO production, potentially mitigating neuroinflammation and oxidative stress. However, further studies and more complex models are needed to fully understand betulin's therapeutic potential in epilepsy.

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CRediT authorship contribution statement

Fatemeh Eghbali: Methodology, Writing – original draft, Writing – review & editing. **Zahra Lorigooini:** Methodology. **Mohammad Rahimi-Madiseh:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Hossein Tahmasebi Dehkordi:** Methodology, Writing – original draft, Writing – review & editing. **Hossein Amini-Khoei:** Data curation, Formal analysis, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors have no conflicts of interest to declare regarding the study described in this article and the preparation of the report.

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Consent to Participate

Not applicable.

Consent to Publish

All authors reviewed and approved the manuscript.

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