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ORIGINAL PAPER

Effects of oleuropein on pentylenetetrazol-induced seizures in mice: involvement of opioidergic and nitrergic systems

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Abstract Oleuropein, a well-known olive polyphenol, has been shown to mediate neuroprotection in Alzheimer's disease and cerebral ischemia. We investigated the effects of oleuropein on pentylenetetrazole (PTZ)-induced seizures in male NMRI mice, with diazepam as the standard drug. We also examined the possible involvement of opioidergic/nitrergic pathways in the probable effects of oleuropein. Intraperitoneal (i.p.) administration of different doses of oleuropein (10, 20 and 30 mg/kg) significantly increased the seizure threshold 60 min prior to induction of seizure, in a dose-dependent manner. Administration of naltrexone (10 mg/kg, i.p.), an opioid receptor antagonist, completely reversed the anticonvulsant effects of oleuropein (10 mg/kg). On the other hand, the anticonvulsant effect of oleuropein (10 mg/kg) was blocked by a non-effective dose of nonspecific inhibitor of nitric oxide synthase (NOS), L-NAME (1 and 10 mg/kg, i.p) and a selective inhibitor of neuronal NOS, 7-nitroindazole (30 mg/kg, i.p.). However, the nitric oxide precursor, L-arginine (30 and 60 mg/kg, i.p.) potentiated the anticonvulsant activity of oleuropein (10 mg/kg). A selective inducible NOS inhibitor, aminoguanidine (100 mg/kg, i.p.) did not change the anticonvulsant activity of oleuropein. It seems that the opioidergic system and constitutive neuronal NOS may be involved in the anticonvulsant properties of oleuropein.

Keywords Oleuropein · Nitric oxide · Opioid · Pentylenetetrazole · Seizure · Mice

Introduction

Oleuropein is the major bioactive component of *Olea europaea*, which is widely known as the olive tree, and is present in high amounts in unprocessed olive fruit and leaves. Oleuropein have been shown to possess multiple neuroprotective effects such as brain hypoxia–reoxygenation [1, 2], audiogenic seizures related to a high-fat diet [3], hippocampal neuronal damage [4], cerebral ischemia [5, 6], brain damage after hypoxia–reoxygenation in diabetic rats [7], and aging [8] in experimental models. Despite these reported neuroprotective effects of oleuropein, the effectiveness of oleuropein in pentylenetetrazole (PTZ)-induced seizures remains obscure.

It has been shown that tolerance to the antinociceptive effect of morphine was inhibited by oleuropein [9]. It has also been found that oleuropein could prevent withdrawal signs in rats dependent on morphine [10]. It is well known that opioids have both anticonvulsant and proconvulsant effects on seizure threshold [11, 12]. On the other hand, our previous studies have shown that nitric oxide (NO) is linked to opioidergic modulation of seizure threshold [13, 14]. Furthermore, oleuropein has been reported to have both the ability to scavenge NO and increase the expression of nitric oxide synthase (NOS) enzymes in mouse peritoneal macrophages [15–18].Therefore, the aim of this study was to determine whether oleuropein may alter seizure threshold through opioidergic and nitrergic pathways on PTZ-induced seizures in mice (Scheme 1).

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Materials and methods

Animals

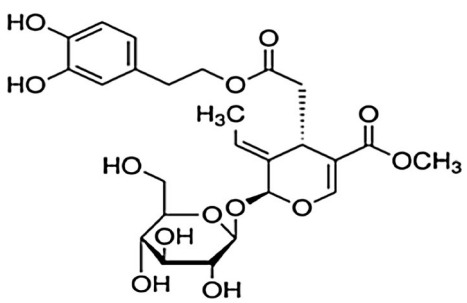
In this study, male NMRI mice weighing 23–30 g were used for acute administration [19]. The animals were housed in a temperature-controlled room (24 ± 1 °C) in standard polycarbonate cages with four or five mice in each cage on a 12-h light/dark cycle with free access to food and water. All behavioral experiments were performed between 9:00 am and 2:00 pm. All experiments and manipulations were carried out in accordance with the Institutional Animal Care and Use Committee (Department of Pharmacology, School of Medicine, Tehran University of Medical Sciences) guidelines. The animals were randomly assigned to different groups, plus each mouse was used only once and each treatment group consisted of 8–12 animals. Additionally, every effort was made to reduce animal suffering and to use only the number of animals necessary to produce reliable scientific data.

Chemicals

Oleuropein, PTZ, L-NAME [L-NG-nitro-L-arginine methyl ester hydrochloride], L-arginine, 7-nitroindazole (7-NI), aminoguanidine (AG) and naltrexone (NTX) were purchased from Sigma (St. Louis, MO, USA). All drugs except 7-NI were dissolved in sterile isotonic saline solution to a concentration at which the required doses were administered intraperitoneally (i.p.) in a volume of 10 ml/kg. PTZ was administered intravenously. 7-NI was suspended in 1% solution of Tween 80. The solutions were prepared immediately before each experiment. Appropriate vehicle controls were prepared for each experiment.

Seizure paradigms

A 30-gauge butterfly needle was inserted into the tail vein while the mice were restrained in a mouse restrainer. The needle was then secured to the tail with a narrow piece of adhesive tape, with the mouse moving freely. PTZ (0.5%) was infused at a constant rate of 1 ml/min [12, 13] using an infusion pump (Harvard, USA). Infusion was halted when forelimb clonus followed by full clonus of the body was observed. The minimal dose of PTZ (mg/kg mouse weight) needed to induce clonic seizures was used as an index of seizure threshold [13, 20].



Scheme. 1 Structure of oleuropein

Experiments

In experiment 1, to determine the optimum time needed to develop the effects of oleuropein acid on PTZ-induced seizure threshold, animals received an acute i.p. injection of saline or oleuropein (20 mg/kg) at 30, 60 and 90 min prior to determination of PTZ-induced clonic seizure threshold. This dose was chosen based on published data [9, 21].

In experiment 2, mice in separate groups received a single injection of different doses of oleuropein [0 (saline), 1, 5, 10, 20 and 30 mg/kg, i.p.] at 60 min before evaluation of the PTZ-induced seizure threshold. In this step, the potent anticonvulsant dose of oleuropein was assessed for further experiments.

In experiment 3, mice in separate groups received a single injection of an opioid receptor antagonist, NTX [0 (saline), 10 mg/kg, i.p.] alone and at 30 min before oleuropein (10 mg/kg, i.p.). The PTZ-induced seizure threshold was assessed 60 min after the injection of oleuropein.

In experiments 4 and 5, animals in separate groups received an injection of nonspecific NO synthase [NOS] inhibitor, L-NAME [0 (saline), 1 and 10 mg/kg, i.p.] as well as the precursor of NO, L-arginine [0 (saline), 30 and 60 mg/kg, i.p.] alone and at 15 min before the effective dose of oleuropein (10 mg/kg, i.p.). In addition, 7-NI as a selective neuronal NOS inhibitor at a dose of 30 mg/kg, and AG as a selective inducible NOS inhibitor at a dose of 100 mg/kg were administered 15 min before the effective dose of oleuropein (10 mg/kg, i.p.) or saline in experiment 6. PTZ-induced seizure threshold was assessed 60 min after the injection of oleuropein acid.

Diazepam (0.05 mg/kg, i.p.) was injected 30 min before seizure threshold measurement, as a positive control. The doses of L-NAME, L-arginine, NTX [13, 22], 7-NI and AG [23, 35] were chosen based on previously published studies.

Measurement of NO metabolites

NO end product (nitrite) was measured using the Griess reaction [23, 24]. Mice were killed by cervical dislocation, and the blood was drawn from the carotid arteries and the jugular veins at cervical dislocation. Blood was centrifuged to separate plasma from red blood cells, and the supernatants were stored at −20 °C for 1–3 h until assay.

The samples were mixed with the same volume of Griess reagent to form a purple azo dye in a reaction pathway. The Griess reagent (1.25% HCl containing 5 g/l sulfanilamide with 0.25 g/l N-naphtyl ethylenediamine) was added at a rate of 0.1 ml/min. The absorbance of the dye product was measured at 540 nm with regard to a standard nitrite curve generated using $NaNO_2$ by ELISA reader. The results were expressed as nM/ml.

Statistical analysis

Data are presented as mean ± SEM of PTZ minimal dose (mg/kg) and were analyzed using the SPSS statistical software package (ver. 15). One- or two-way analyses of variance (ANOVA) followed by post hoc Tukey's tests was used to analyze the data where appropriate. Tests of homogeneity of variance were used to ensure normal distribution of the data. A P value of <0.05 was defined as statistically significant.

Results

Effect of time in oleuropein treatment on seizure threshold

Figure 1 shows the time course of the effect of oleuropein on seizure threshold. As shown, i.p. administration of oleuropein (20 mg/kg) did not affect the PTZ-induced seizure threshold at 30 and 90 min, whereas it significantly reduced the seizure threshold at 60 min after administration [$F\ (3,\ 23) = 11.911$, $P < 0.001$, $P < 0.05$, respectively].

Effect of different doses of oleuropein on seizure threshold

Figure 2 illustrates the effect of acute i.p. administration of oleuropein (1, 5, 10, 20 and 30 mg/kg) on seizure threshold. Seizure threshold was determined 60 min after administration of different doses of oleuropein. The low dose of oleuropein (1 mg/kg) did not alter seizure threshold. However, ANOVA analysis and subsequent post hoc analysis showed a significant increase in seizure threshold at ≥5 mg/kg [$F\ (5, 47) = 13.851$; $P < 0.001$; Fig. 2].

Effect of NTX on effective doses of oleuropein

Figure 3 illustrates the effect of NTX (10 mg/kg, i.p.) alone or in combination with oleuropein (10 mg/kg, i.p.) on the clonic seizure threshold induced by PTZ. Comparison of the effect of oleuropein with the effect of saline show no significant difference in seizure threshold [$F\ (1, 15) = 2.635$; $P > 0.05$, compared with the saline-treated group; Fig. 3].

Acute administration of NTX (10 mg/kg, i.p.) 15 min before oleuropein (10 mg/kg, i.p.) significantly altered the anticonvulsant effect of oleuropein [$F\ (1, 17) = 11.234$; $P < 0.01$, compared with the corresponding oleuropein-treated group; Fig. 3].

Effect of L-NAME on the anticonvulsant effect of oleuropein

Figure 4 illustrates the effect of different doses of L-NAME (1 and 10 mg/kg, i.p.) alone or in combination with oleuropein (10 mg/kg, i.p.) on the clonic seizure threshold induced by PTZ. Comparison of the effect of L-NAME with the effect of saline showed no significant difference in seizure threshold [$F\ (2, 23) = 1.125$; $P > 0.05$, compared with the saline-treated group; Fig. 4].

Acute administration of different doses of L-NAME (1 and 10 mg/kg, i.p.) 15 min before oleuropein (10 mg/kg, i.p.) significantly altered the anticonvulsant effect of oleuropein [$F\ (5, 47) = 10.041$; $P < 0.001$, compared with the corresponding oleuropein acid-treated group; Fig. 4].

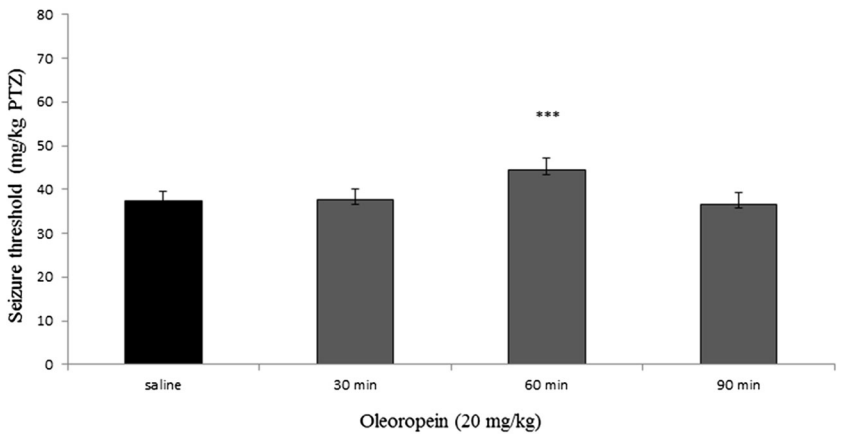


Fig. 1 Effect of oleuropein (20 mg/kg, i.p) on PTZ-induced clonic seizure threshold in mice. Oleuropein was administered at 30, 60 and 90 min before PTZ and its effects were compared with those of the control group administered PTZ at the same time. Data are expressed as mean ± SEM of seizure threshold in at least 8 mice. ***$P < 0.001$ compared with corresponding saline group

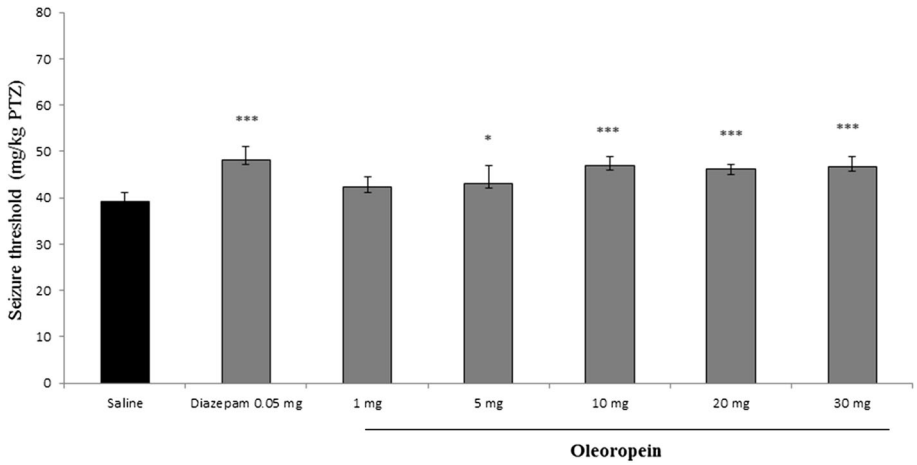


Fig. 2 The effect of different doses of oleuropein (1, 5, 10, 20 and 30 mg/kg, i.p.) administration on the PTZ-induced seizure threshold in mice. Oleuropein was administered 60 min prior to PTZ. Data are expressed as mean ± SEM of seizure threshold in at least 8 mice. $^{*}P < 0.05$, $^{***}P < 0.001$ compared with saline-treated group

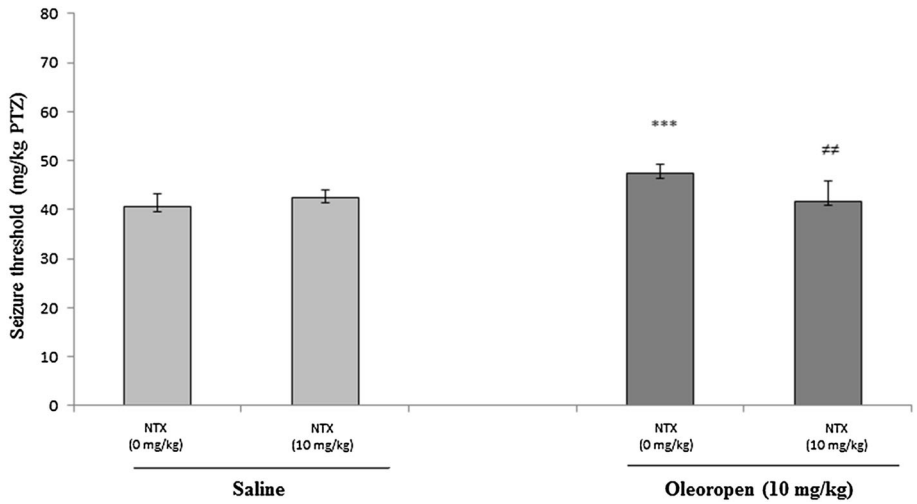


Fig. 3 Effect of naltrexone on the anticonvulsant effect of oleuropein. Naltrexone (10 mg/kg, i.p.) was administered 15 min before oleuropein (10 mg/kg, i.p.). Data are expressed as mean ± SEM of seizure threshold in at least 8 mice. $^{\neq\neq}P < 0.01$ compared with oleuropein-treated group; $^{***}P < 0.001$ compared with saline-treated group

Effect of L-arginine on the anticonvulsant effect of oleuropein

Figure 5 shows the effect of different doses of L-arginine (30 and 60 mg/kg, i.p.) alone or in combination with oleuropein (10 mg/kg, i.p.) on the clonic seizure threshold induced by PTZ. Comparison of the effect of L-arginine with the effect of saline showed no significant difference in seizure threshold [F (2, 23) = 2.635; $P > 0.05$, compared with the saline-treated group; Fig. 5].

Acute administration of different doses of L-arginine (30 and 60 mg/kg, i.p.) 15 min before oleuropein (10 mg/kg, i.p.) did not change the anticonvulsant effect of oleuropein [F (5, 47) = 11.839; $P < 0.001$, compared with the saline-treated group; Fig. 5].

Effect of 7-NI and AG on the anticonvulsant effect of oleuropein

Figure 6 shows the effect of 7-NI (selective neuronal NOS inhibitor) and AG (selective inducible NOS inhibitor) alone or in combination with oleuropein (10 mg/kg, i.p.) on the clonic seizure threshold induced by PTZ. Comparison of the effect of 7-NI (30 mg/kg, i.p.) and AG (100 mg/kg, i.p.) with the effect of saline showed no significant difference in seizure threshold ($P > 0.05$ compared with the oleuropein-treated group). Furthermore, administration of AG before oleuropein did not affect the anticonvulsant property of oleuropein; however, the anticonvulsant effect of oleuropein was completely prevented by an injection of 7-NI 15 min before oleuropein [F (5, 47) = 9.501; $P < 0.001$].

NO metabolite (NO*x*) levels in plasma

Table 1 shows a comparison between plasma NO*x* levels in the different experimental groups. Plasma NO*x* levels were significantly elevated in the oleuropein (10 mg/kg, i.p.)-, and L-arginine (60 mg/kg, i.p.)-treated groups, and also in the mice that were administered L-arginine (60 mg/kg, i.p.) 15 min before oleuropein (10 mg/kg, i.p.) compared to the saline-treated group (44.21 ± 2.70, 43.02 ± 3.26,

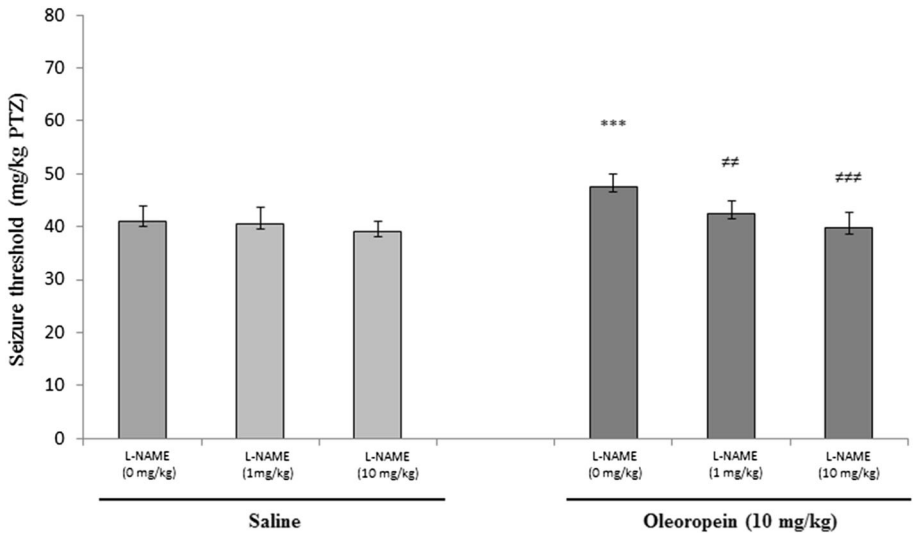


Fig. 4 Effect of L-NAME on the anticonvulsant effect of oleuropein. L-NAME (1 and 10 mg/kg, i.p.) administered 15 min before oleuropein (10 mg/kg, i.p.). Data are expressed as mean ± SEM of seizure threshold in at least 8 mice. $^{\neq\neq}P < 0.01$; $^{\neq\neq\neq}P < 0.001$ compared with oleuropein-treated group; $^{***}P < 0.001$ compared with saline-treated group

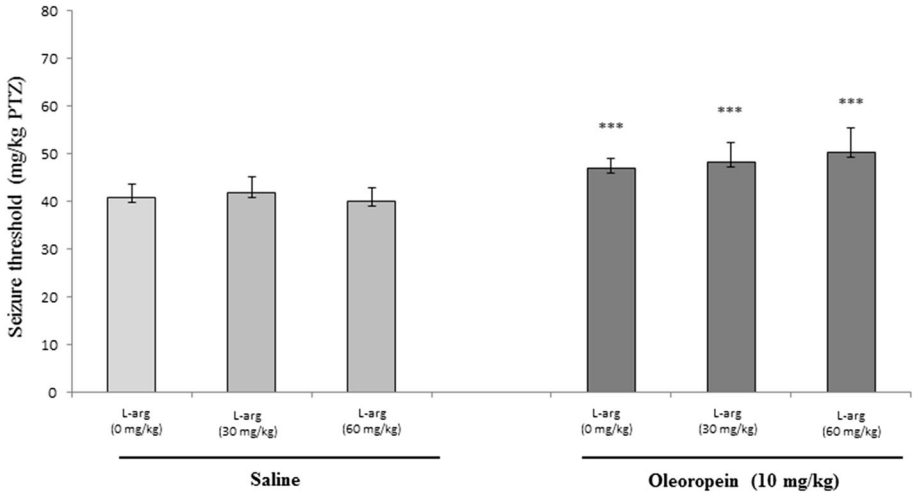


Fig. 5 Effect of L-arginine on the anticonvulsant effect of oleuropein. L-Arginine (30 and 60 mg/kg, i.p.) was administered 15 min before oleuropein (10 mg/kg, i.p.). Data are expressed as mean ± SEM of seizure threshold in at least 8 mice. $^{***}P < 0.001$ compared with saline-treated group

45.11 ± 2.96, and 37.75 ± 2.27 nmol/ml, respectively). However, nitrite levels were not significantly different in the mice that received L-NAME (10 mg/kg, ip) 15 min before oleuropein (10 mg/kg, i.p.) compared with the saline-treated group (37.33 ± 2.27, and 37.75 ± 2.27 nm/ml, respectively).

Discussion

In the current study, we showed for the first time that oleuropein could modulate the anticonvulsant effect in a PTZ-induced seizure model in mice. We demonstrated that NTX (opioid receptor antagonist) blocked the effect of oleuropein. In addition, the anticonvulsant effect of oleuropein was completely reversed by acute pretreatment with L-NAME (a nonselective NOS inhibitor) and 7-NI (a selective inhibitor of neuronal NOS). However, L-arginine (a precursor of NOS) potentiated the anticonvulsant effect of oleuropein on seizure threshold. The anticonvulsant activity of oleuropein did not change after treatment with AG as a selective inhibitor of inducible NOS. Based on these data, we speculate that the opioidergic system and neuronal nitrergic pathway could be involved in the anticonvulsant properties of oleuropein.

Oleuropein, a natural polyphenolic compound belonging to the secoiridoids group, possesses diverse pharmacological and healing properties. A growing body of evidence supports the protective characteristics of oleuropein in the central nervous system against audiogenic seizures related to a high-fat diet [3], Alzheimer's disease [25, 26], hippocampal neuronal damage [4], and cerebral ischemia [5, 6, 27]. In our study, oleuropein at doses of 10, 20 and 30 mg/kg showed anticonvulsant activities. The mechanisms suggested for this reaction of oleuropein were reduction of amyloid β-protein (Aβ) degradation [3, 25], calcium antagonistic activity [28], suppression of the

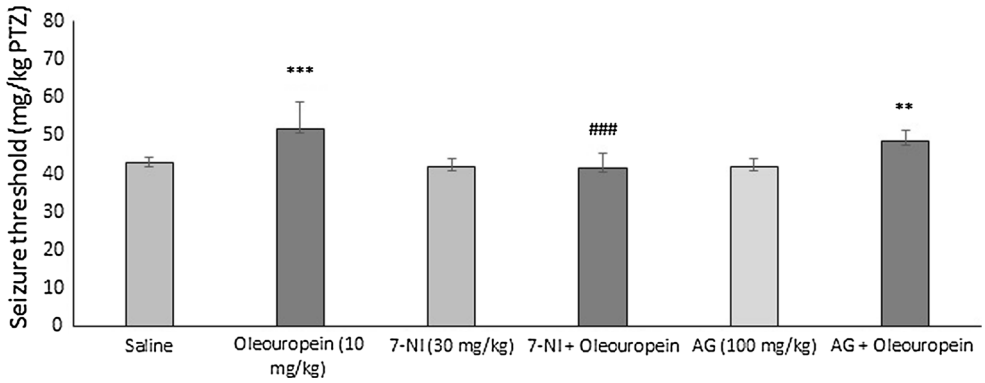


Fig. 6 Effect of 7-nitroindazole (7-NI) and aminoguanidine (AG) on the anticonvulsant effect of oleuropein. 7-NI (30 mg/kg, i.p.) and AG (100 mg/kg, i.p.) were administered 15 min before oleuropein (10 mg/kg, i.p.). Data are expressed as mean ± SEM of seizure threshold in at least 8 mice. $^{###}P < 0.001$ compared with oleuropein-treated group; $^{**}P < 0.01$, $^{***}P < 0.001$ compared with saline-treated group

Table 1 Comparison between plasma NO*x* levels in mice in the different experimental groups

Groups	NO_x concentration (nmol/ml)	Significance
Saline	37.75 ± 2.27	
Oleuropein (10 mg/kg)	44.21 ± 2.70	$P < 0.01$ vs saline
L-NAME (10 mg/kg)	35.31 ± 2.76	$P > 0.05$ vs saline
L-Arginine (60 mg/kg)	43.02 ± 3.26	$P < 0.05$ vs saline
Oleuropein (10 mg/kg) + L-NAME (10 mg/kg)	37.33 ± 2.27	$P > 0.05$ vs saline
Oleuropein (10 mg/kg) + L-aiginine (60 mg/kg)	45.11 ± 2.96	$P < 0.01$ vs saline

inflammatory response [29, 30], and clearing various endogenous and exogenous free radicals and oxidants [18, 31].

It has been reported that oleuropein could potentiate the antinociceptive property of morphine in a subeffective dose and suppress low-dose morphine hyperalgesia in rats through Ca^{2+} channel blocking activity [10]. In addition, oleuropein prevents the development of morphine antinociceptive tolerance through inhibition of morphine-induced L-type calcium channel overexpression [9]. In the current study, NTX suppressed the anticonvulsant activity of oleuropein. These data suggest the modulation of opioidergic receptors in the effects of oleuropein in PTZ-induced seizure in mice.

NO is a known modulator of seizure susceptibility with diverse anti- and proconvulsant effects in different models of seizure [13]. Furthermore, previous reports have shown that three different isoforms of NOS are found to be modulators of both anti- and proconvulsant effects in different seizure models. For example, Sardo et al. reported that the anti-epileptic effect of levetiracetam was mediated via neuronal NOS [34]. Although in our recent study, the selective inducible NOS inhibitor reversed the anticonvulsant effect of vasopressin on seizure threshold [35]. In another study, both the pro- and anticonvulsive effect of D-penicillamine was inhibited by 7-NI, a selective neuronal inhibitor of NOS [23]. Our evidence suggests that the anticonvulsant activities of oleuropein may be mediated through an NO-dependent mechanism on the basis that L-NAME and 7-NI blocked the aforementioned phase of oleuropein. As shown in previous studies, oleuropein enhances NO production by mouse macrophages [17, 26], and causes an increase in NOS expression in these cells [15, 32]. Furthermore, the anti-hypertensive effects of oleuropein might be mediated by improving the release of NO [33].

In conclusion, the current findings underline for the first time that oleuropein shows anticonvulsant activity in PTZ-induced seizures. This phenomenon could be blocked by opioid receptor antagonists and neuronal NOS inhibitors, suggesting the involvement of opioidergic/nitrergic pathways in the convulsive impacts of oleuropein in the PTZ model of clonic seizures in mice.

Compliance with ethical standards

Conflict of interest The authors confirm that there is no conflict of interest.

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