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ARTICLE

The anticonvulsant effects of *Ducrosia anethifolia* (Boiss) essential oil are produced by its main component alpha-pinene in rats

Os efeitos anticonvulsivantes do óleo essencial de *Ducrosia anethifolia* (Boiss) são realizados pelo seu principal componente alfa-pineno em ratos

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ABSTRACT

Ducrosia anethifolia has been recommended as a remedy for neurological disorders. However, the anticonvulsant effects of *D. anethifolia* essential oil (DAEO) and its major constituent α-pinene have not yet been clarified. **Methods:** A rat model of pentylenetetrazole (PTZ)-induced convulsions was used. Oxidant and antioxidant parameters were assayed in the temporal lobe. **Results:** The data showed that DAEO (50, 100 and 200 mg/kg, i.p.) and α-pinene (0.2 and 0.4 mg/kg i.p.) delayed the initiation time, and reduced the duration of myoclonic and tonic-clonic seizures following PTZ injection. The PTZ produced oxidative stress so that malondialdehyde and hydrogen peroxide levels were increased and catalase and peroxidase activity decreased. Pretreatment with DAEO and α-pinene significantly inhibited the above-mentioned enzymatic changes in PTZ-treated animals. **Conclusion:** The results suggest that α-pinene, at least in part, was responsible for the induction of the anticonvulsant and antioxidant effects of DAEO in rats.

Keywords: Pentylenetetrazole; seizures; oxidative stress..

RESUMO

A *Ducrosia anethifolia* tem sido recomendada como remédio para os distúrbios neurológicos. No entanto, os efeitos anticonvulsivantes do óleo essencial de *Ducrosia anethifolia* (DAEO) e do seu principal constituinte alfa-pineno (α-pineno) ainda não foram clarificados. **Métodos:** Foi utilizado um modelo de rato de convulsões induzidas por pentilenotetrazol (PTZ). Os parâmetros oxidante e antioxidante foram ensaiados no lobo temporal do cérebro. **Resultados:** Os dados mostraram que DAEO (50, 100 e 200 mg / kg, i.p.) e α-pineno (0,2 e 0,4 mg / kg i.p.) retardaram o tempo de iniciação e reduziram a duração das crises mioclônicas e tônico-clônicas após a injeção de PTZ. O PTZ produziu estresse oxidativo, de modo que os níveis de malondialdeído (MDA) e de peróxido de hidrogênio aumentaram e a atividade da catalase e da peroxidase diminuiu. O pré-tratamento com DAEO e α-pineno inibiu significativamente as alterações enzimáticas mencionadas em animais tratados com PTZ. **Conclusão:** O resultado sugere que α-pineno, pelo menos em parte, é responsável pela indução dos efeitos anticonvulsivantes e antioxidantes da DAEO em ratos.

Palavras-chave: Ducrosia anethifolia; α-pinene; Pentilenotetrazol; Crise; Estresse oxidativo.

Epilepsy is one of the oldest conditions known to man and is the third most common neurological disorder after stroke and Alzheimer's disease. Approximately 1% of the world's population suffers from epilepsy[1]. Anti-epileptic drugs are usually the first choice of treatment for epilepsy but approximately one-third of people with epilepsy do not respond to the drugs. Anti-epileptic drugs do not cure epilepsy, but can prevent seizures from occurring. Recently, it has been proposed that seizures and status epilepticus may be associated with oxidative stress[2]. Oxidative stress, due to the increase in the activity of glutamatergic transmitters, plays a crucial role in the induction of neuronal cell death[3]. Since the brain utilizes the greatest amount of oxygen compared with other organs, it is particularly at risk of oxidative stress[4].

Experimental models of epilepsy have been developed to find the basic mechanisms of epileptic seizures and new therapeutic approaches. The chemical kindling

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induced by the pentylenetetrazole (PTZ) is one of the most-widely used models for the induction of convulsions in animals.

Medicinal plants have recently become a major target in the search for new drugs and have led to compounds to treat epilepsy accompanied by oxidative stress[5,6]. *Ducrosia anethifolia* Boiss, known in Persian as Moshgak, Roshgak, and Moshkbu, belongs to the Apiaceae family. It is one of the three species of Iranian *Ducrosia* growing wild in southeastern Iran, in the mountainous regions of the Kerman province[7]. In Iranian traditional Medicine, the whole herb—especially its aerial parts—has been used as an analgesic for headache, backache, as well as for the treatment of colic, and colds. It is also used to relax the body and mind, allowing a restful sleep[8]. Furthermore, antianxiolytic effects of *D. anethifolia* essential oil (DAEO) have been reported[9]. The antioxidant, antimicrobial, antimycobacterial, antifungal, and central nervous system depressant effects of this plant and other species of *Ducrosia* have been reported in pharmacological and biological studies[10]. Phytochemical studies of DAEO revealed that aliphatic aldehydes and other monoterpene hydrocarbons such as limonene, citronellal, terpinolene, myrcene, α-pinene, pulegone, p-cymene and coumarins such as pangelin are the main components of *D. anethifolia* aerial parts[11]. High performance liquid chromatography (HPLC) analysis of DAEO indicated the presence of terpenoids such as α-pinene as one of the major components. Terpenes constitute the major portion of the essential oils and, somehow, are responsible for the medicinal plant's pharmacological activities such as antinociceptive, anti-inflammatory and anticonvulsant effects[12].

It has been reported that α-pinene has anticonvulsant and antioxidant properties[13]. However, there is no scientific information to validate the anticonvulsant activity of this plant in experimental animals. Therefore, the present study was designed to determine the possible effects of DAEO, and its major component α-pinene, on PTZ-induced seizure and brain oxidative stress in male rats.

METHODS

Animals

Adult male Wistar rats weighting 200-250g were prepared from the Animal House of Shahid Bahonar University of Kerman. The animals were housed in a room with photoperiod control (a 12-hour light/dark cycle) and temperature (22 ± 2°C). Food and water was available *ad libitum*. All experimental procedures were approved by the Animal Research Ethics Committee of the Kerman Neuroscience Research Center, Kerman, Iran (EC/95).

Drugs

Pentylenetetrazole, α-pinene and diazepam were purchased from Sigma-Aldrich Co. The drugs were dissolved in a saline solution (0.9%) and injected intraperitoneally (i.p.) in a volume of 1 ml/kg of the rat's body weight.

Plant material

Fresh aerial parts (leaves and flowers) of *D. anethifolia* were collected, in July, from the Lalehzar mountainous area in Kerman province, Iran, at an altitude of 2,800 m. The voucher specimens were deposited at the herbarium of Shahid Bahonar University of Kerman (Code number: 1371). The material was dried at room temperature and used for distillation. The essential oil was isolated by hydrodistillation of the fresh aerial parts for 4 hours, and then dried over anhydrous sodium sulfate 14 and stored in a refrigerator (4°C).

Acute toxicity

Seven rats were treated with the DAEO (500 mg/kg, i.p.) and the mortality and morbidity were determined.

PTZ-induced seizures

Pentylenetetrazole (80 mg/kg, i.p.) was injected to induce convulsions in rats. Diazepam (2 mg/kg, i.p.) and DAEO (25, 50, 100 and 200 mg/kg, i.p.) and α-pinene (0.2 and 0.4 mg/kg, i.p.) were administered 30 minutes before receiving PTZ. The seizure parameters were precisely monitored for 40 minutes after each PTZ injection in all groups. The following parameters were measured using a stopwatch in seconds, and behaviors were recorded with a CD camera.

The resultant seizures were classified according to the modified Racine scale[14] as follows:

Stage 0: no response.

Stage 1: ear and facial twitching.

Stage 2: myoclonic jerks without rearing.

Stage 3: myoclonic jerks, rearing.

Stage 4: turning over onto side position, tonic-clonic seizures.

Stage 5: turning over onto back position, generalized tonic-clonic seizures.

1) Latency: the time between PTZ injection and the onset of seizures[15].

2) Duration: the time interval from the onset to termination of seizures or death of the animal.

3) Percent of death: the number of rats that died after PTZ injection among the rats of a particular group.

4) Protection percentage: the number of rats that responded to the test[16]. P%=1-(nt/Nt) (nc/NC) ×100.

Biochemical measurements

After behavioral assessment, the animals were euthanized under deep anesthesia, and the temporal lobes of the brains were dissected and stored at –80°C until the day of assay.

Brain lipid peroxidation

Lipid peroxidation products such as malondialdehyde (MDA) are considered to be reliable indicators of oxidative damage[17]. Temporal lobe tissue (0.5 g) was homogenized in 10 mg of 0.1% trichloroacetic acid; the homogenate was centrifuged at 15,000 rpm for 15 minutes to 1.0 mg aliquot of the supernatant; and 4.0 mg of 0.5% thiobarbituric acid in 20% trichloroacetic acid was added. The mixture was heated at 95°C for 30 minutes and then cooled in an ice bath. After centrifugation (10,000 rpm for 10 minutes), the absorbance of the supernatant was recorded at 532 nm (Biochrom WPA Biowave II UV/Visible Spectrophotometer). The thiobarbituric acid reactive substances content was calculated according to its extinction coefficient of $155 mM^{-1}cm^{-1}$ and expressed in units (U). One 'U' is defined as μmol of MDA formed $min^{-1}mg^{-1}$ protein.

Hydrogen peroxide

Hydrogen peroxide (H_2O_2) was determined by the method described by Velikova et al., (2000). Temporal lobe tissue (0.5 g) was finely ground with trichloroacetic acid (5 ml of 0.1 % w/v) and centrifuged at 10,000 × *g* for 15 minutes. Phosphate buffer (0.5 ml, pH 7.0) and 1 ml potassium iodide were added to the 0.5 ml supernatant. Its absorbance was recorded at 390 nm after overtaxing using a UV visible spectrophotometer.

Total soluble proteins

Total proteins were estimated using the Bradford method and bovine serum albumin was used as the standard.

Antioxidant enzymes activities

Temporal lobe tissue (0.5 g) was finely ground under chilled conditions in 3 ml of phosphate buffer (50 mM with pH 7.5) for the extraction of antioxidant enzymes. Centrifugation of the mixture was performed at 10,000 × *g* for 10 minutes at 4°C. The supernatant was recentrifuged at 15,000 × *g* for 10 minutes and the resultant extract stored at -20°C for determination of the activity of antioxidant enzymes.

Evaluation of catalase activity

The activity of catalase (CAT) was estimated by monitoring the decrease in absorbance of H_2O_2 within 30 seconds at 240 nm. The assay solution contained 50 mM potassium phosphate buffer (pH 7.0) and 15 mM H_2O_2 and 100 μl enzyme extract[18].

Evaluation of peroxidase activity

Peroxidase (POD) activity was assayed according to the method of Plewa et al.[19], based on the amount of tetraguaiacol absorbed after formation, by oxidation, of guaiacol catalyzed by this enzyme in 3 minutes at a wavelength of 470 nm using an extinction coefficient of tetraguaiacol, ε = 26.6 $mM^{-1}cm^{-1}$.

HPLC analysis

The obtained essential oil was analyzed using HPLC (Agilent Technologies, 1200 Infinity series, USA) equipped with a 1260 Infinity Quaternary Pump and a 1260 Infinity Variable Wavelength Detector. An Agilent 1260 Infinity Manual Injector fitted with a 20 μL sample loop was used to introduce the samples. The analytes were separated on a Restek Ultra C18 (250 mm × 4.6 mm, 5μm) column (USA). Chromatograms were processed by an Agilent HPLC Chem Station (Rev. B.04.03).

Statistical analysis

The data are expressed as mean ± SEM. Comparison between groups was made by analysis of variance followed by the Tukey test. Differences between experimental groups of each point with $p < 0.05$ were considered statistically significant.

RESULTS

Acute toxicity

The essential oil of *D. anethifolia* has shown no mortality up to a dose of 500 mg/kg. However, we used doses of 25, 50, 100 and 200 mg/kg in this study.

Anticonvulsant activity assessment

Effect of DAEO on PTZ-induced seizures

The essential oil showed dose-dependent effects against PTZ-induced seizures. It could significantly reduce the number of convulsing animals. Pretreatment with DAEO (50, 100 and 200 mg/kg) and α-pinene (0.2 and 0.4 mg/kg) significantly reduced mortality rate and attenuated PTZ-induced seizures (Table).

Effect of DAEO and α-pinene on the onset of seizure

The DAEO (50, 100 and 200 mg/kg, i.p.) significantly delayed the onset of PTZ-induced seizures. However, diazepam and α-pinene had no significant effects on the onset of seizure (Figure 1).

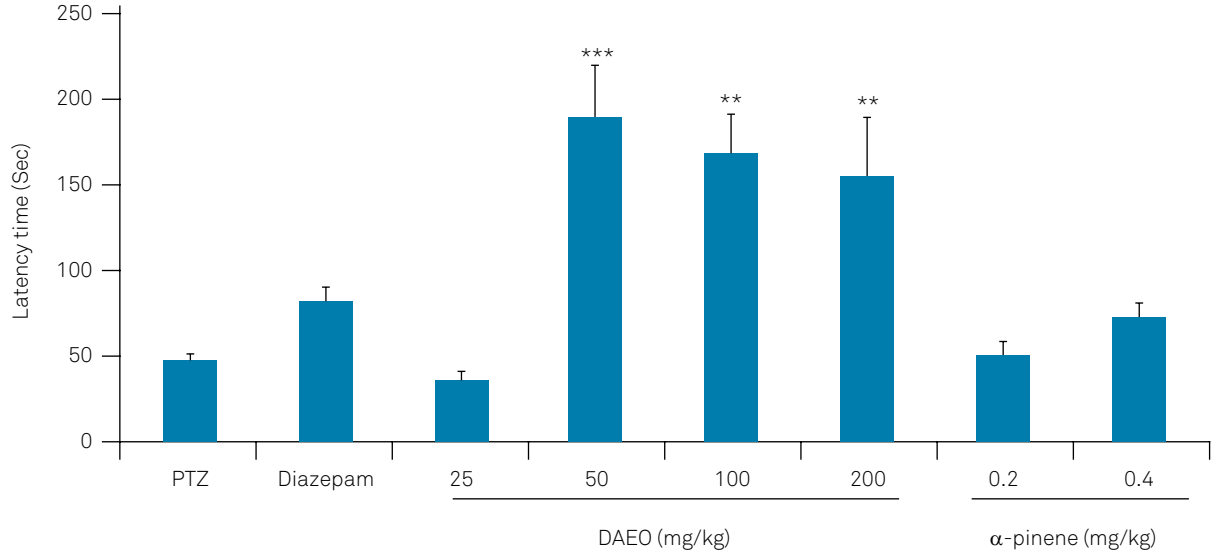
Effect of DAEO and α-pinene on the duration of seizure

The essential oil at doses of 50, 100 and 200 mg/kg, α-pinene (0.2 and 0.4 mg/kg) and diazepam could significantly alter the duration of seizures in PTZ-treated rats. However, 25 mg/kg of DAEO had no effect on the duration of seizures (Figure 2).

Table. The effect of *Ducrosia anethifolia* essential oil and α-pinene on pentylenetetrazole (PTZ) induced seizures in rats.

Treatment and doses (mg/kg, i.p)	Duration of seizure (sec.)			% Mortality	% Protection
	Myoclonic	Tonic	Tonic-clonic		
PTZ	34 ± 03	31 ± 42	165 ± 86	100	14
PTZ + Diazepam 2 mg/kg	2 ± 14***	4 ± 85***	4 ± 14***	0	100
PTZ + *D. anethifolia* 25 mg/kg	20 ± 01	40 ± 01	162 ± 71	71	29
PTZ + *D. anethifolia* 50 mg/kg	10 ± 42**	13 ± 57*	13 ± 42***	0	100
PTZ + *D. anethifolia* 100 mg/kg	13 ± 71**	18 ± 14	35 ± 57***	14	86
PTZ + *D. anethifolia* 200 mg/kg	17 ± 42*	19 ± 14	27 ± 14***	14	86
PTZ + α-pinene 0.2 mg/kg	16 ± 71*	10 ± 57**	31 ± 57***	42	58
PTZ + α-pinene 0.4 mg/kg	13 ± 43**	8 ± 42**	21 ± 71***	28	72

Data are presented as duration of myoclonic, tonic and tonic-clonic seizures and represent percentage of the mortality and protection criteria (n = 7). ***p < 0.001, **p < 0.01, *p < 0.05, compared with PTZ-treated control rats. PTZ: pentylenetetrazole



Histograms represent mean ± SEM for seven animals. ***p < 0.001, **p < 0.01, versus PTZ group by analysis of variance with Tukey's post hoc test.
Figure 1. The effect of *Ducrosia anethifolia* essential oil (DAEO) and α-pinene on the onset of seizure of pentylenetetrazole (PTZ)-induced convulsion in rats.

Biochemical measurements

MDA levels

The PTZ injection significantly increased brain temporal lobe MDA levels, which were significantly attenuated by DAEO (50, 100, 200 mg/kg) and α-pinene (0.2 and 0.4 mg/kg) (Figure 3).

H_2O_2 levels

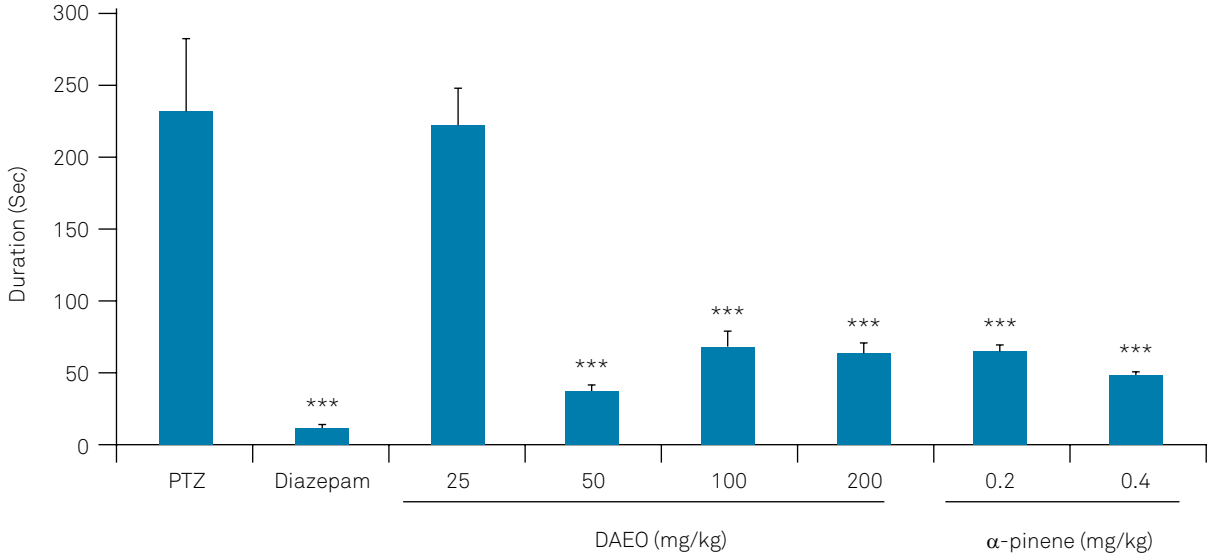
The PTZ-treated rats showed a significant increase in H_2O_2 levels in the temporal lobe. Alternatively, DAEO, α-pinene and diazepam significantly decreased PTZ-induced H_2O_2 production (Figure 4).

The effect of DAEO and α-pinene on brain CAT and POD activities in PTZ-treated animals

The brain CAT and POD activities were significantly decreased following PTZ administration. However, DAEO (50,100 and 200 mg/kg), α-pinene (0.2 and 0.4 mg/kg) and diazepam could prevent the effect of PTZ on CAT and POD activities (Figure 5 and 6).

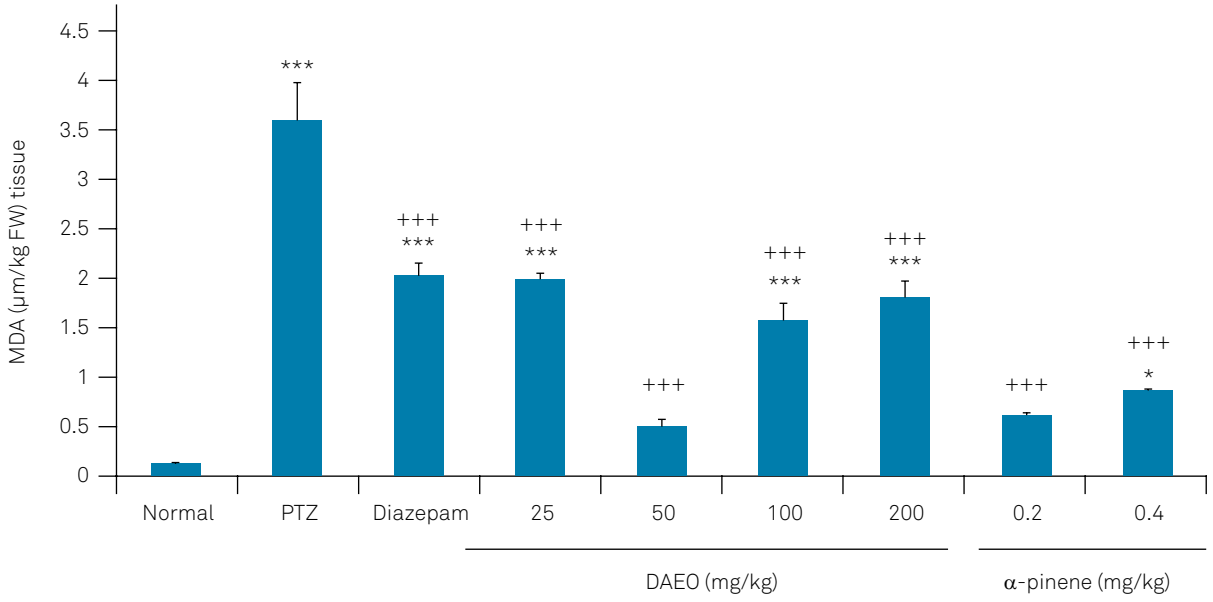
HPLC analysis

According to the obtained HPLC spectrum of essential oil of *D. anethifolia*, there was a major peak following retention times (min): 6.950 (Figure 7). The peak for the reference standard, α-pinene, appeared at the retention time (min) of 6.866.



Histograms represent mean ± SEM (n=7). ****p < 0.001 versus PTZ-treated group. The data were analyzed by one-way analysis of variance with Tukey's post hoc test.

Figure 2. The effect of *Ducrosia anethifolia* essential oil (DAEO) and α-pinene on the duration of PTZ-induced seizure in Rats.



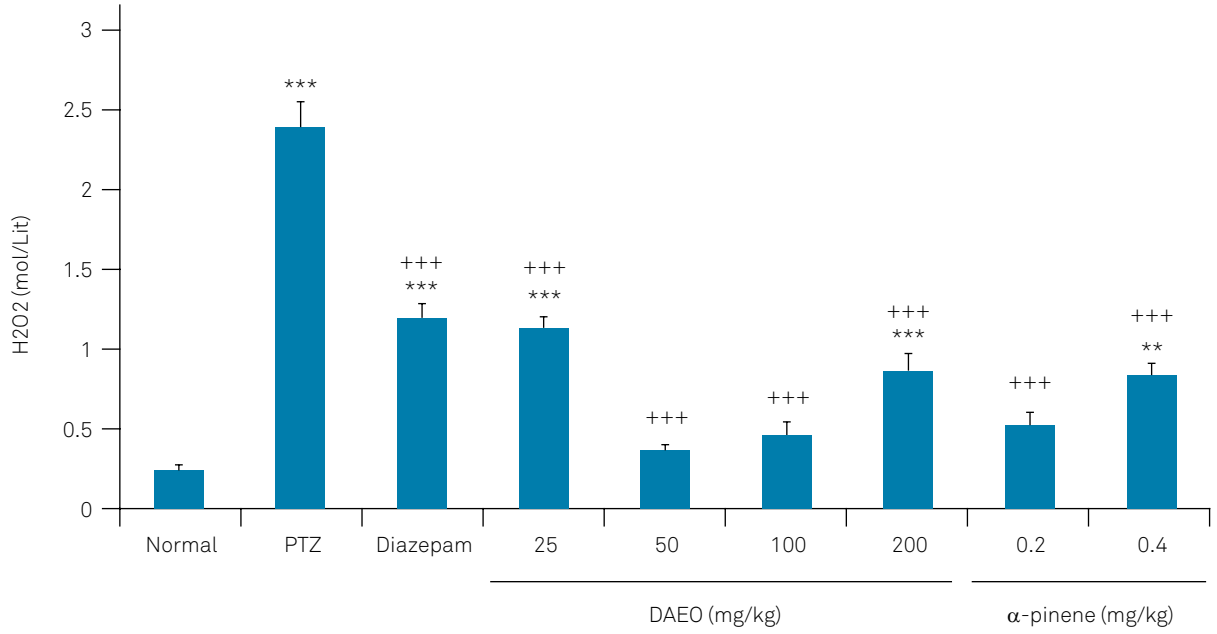
Data represent means ± SEM (n=7), ***p < 0.001 and *p < 0.05 compared with nontreated normal rats. +++p < 0.001 versus PTZ-injected group.

Figure 3. The effect of *Ducrosia anethifolia* essential oil (DAEO) and α-pinene on the temporal lobe MDA levels in the PTZ seizure models.

DISCUSSION

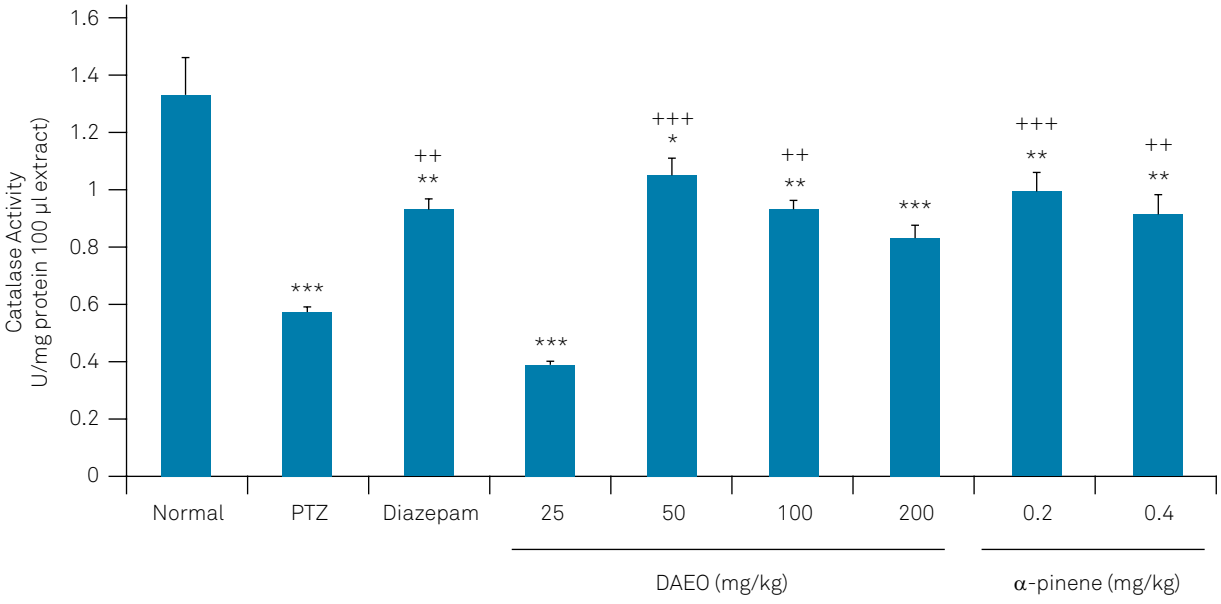
In the present work, the effects of DAEO and α-pinene were studied. *Ducrosia anethifolia* essential oil and α-pinene were initially evaluated in a behavioral study that gave a good indication of the reduction of seizures. Additionally, the results showed that DAEO and α-pinene were able to significantly decrease the oxidative stress factors after seizures induced by PTZ.

The PTZ method is a valid model of convulsion for the study of generalized myoclonic (absence) seizures[20,21,22]. It has been demonstrated that oxidative stress resulting from free radicals plays a critical role in the genesis of epilepsy and in post-seizure neuronal death. The brain is particularly susceptible to oxidative stress damage[4,23,24]. Traditionally, medicinal plants with antioxidant properties have been candidates for preventing oxidative damage and epilepsy[25]. The phytochemical and HPLC analysis by Hajhashemi et al.[9] showed that DAEO had a wide spectrum of bioactive compounds, and terpenoids were its major components[9]. The antinociceptive, anticonvulsant and anti-inflammatory properties of monoterpenes, such as α-pinene, carvacrol, γ-terpineol, citronellol



Data represent means ± SEM (n=7), ***p < 0.001 and **p < 0.01 compared with the control nontreated groups. +++p < 0.001 compared with PTZ-treated animals.

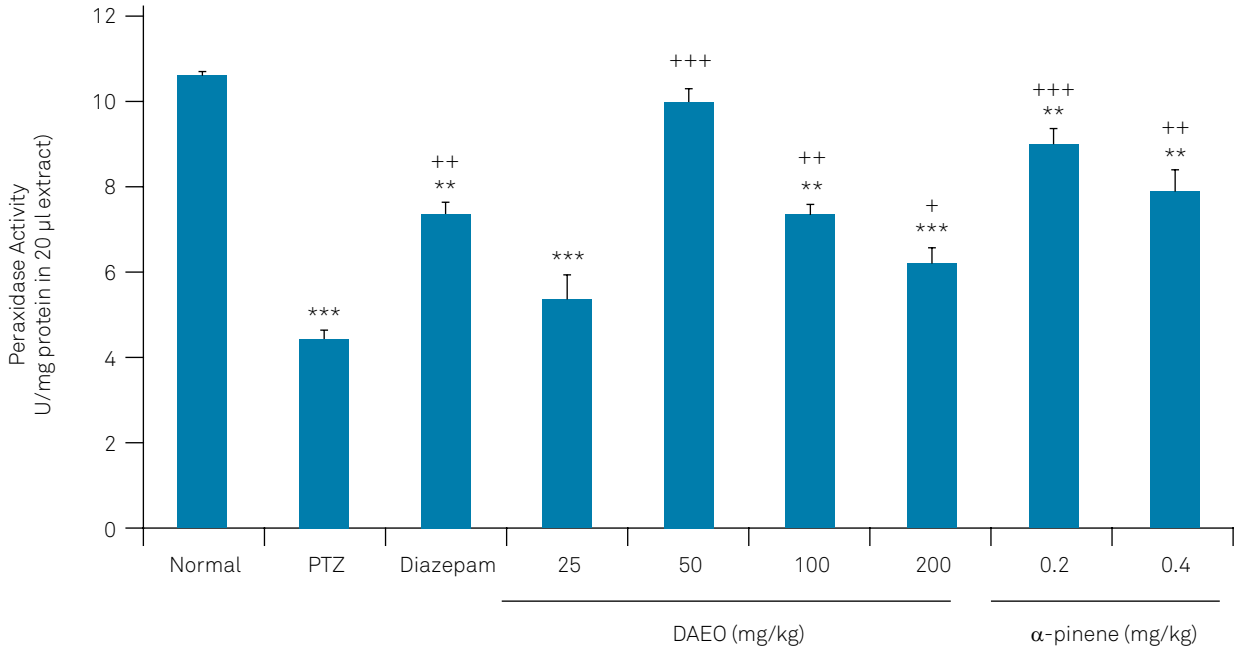
Figure 4. The effect of *Ducrosia anethifolia* essential oil (DAEO) and α-pinene on the temporal lobe H_2O_2 levels in the rat PTZ seizure models.



Data represent means ± SEM (n=7), ****p < 0.001, **p < 0.01 and *p < 0.05 compared with the control non-treated groups. +++p < 0.001 and ++p < 0.01 compared with PTZ-treated animals.

Figure 5. The effect of *Ducrosia anethifolia essential* oil (DAEO) and α-pinene on catalase activity in the temporal lobe of the brain in the PTZ seizure model.

and linalool have been reported[26,27]. Pentylenetetrazole induces convulsion by inhibiting GABA receptors–chloride channel complexes. It appears that the inhibitory effect of DAEO against PTZ-induced seizure may occur through the rise of the convulsion threshold in the brain via the stimulation of GABA receptors[28]. The α-pinenes, as major components of DAEO, have a promoting effect on $GABA_A$ receptors and increase the postsynaptic GABA-dependent chloride flows, as well as being a potent inhibitor of acetylcholinesterase[13]. The major inhibitory neurotransmitter in the brain is GABA and the inhibition of its neurotransmission has been thought to be a critical factor in epilepsy[29]. The standard antiepileptic drugs, phenobarbital and diazepam, can induce their antiepileptic effects by enhancing GABA neurotransmission. Glutamate and glutamatergic receptors are located



Data represent means ± SEM (n=7), ****p < 0.001, **p < 0.01 compared with the control untreated groups. +++p < 0.001 and ++p < 0.01 compared with PTZ-treated animals.

Figure 6. The effect of *Ducrosia anethifolia* essential oil (DAEO) and α-pinene on peroxidase activity in the temporal lobe in the PTZ seizure model.

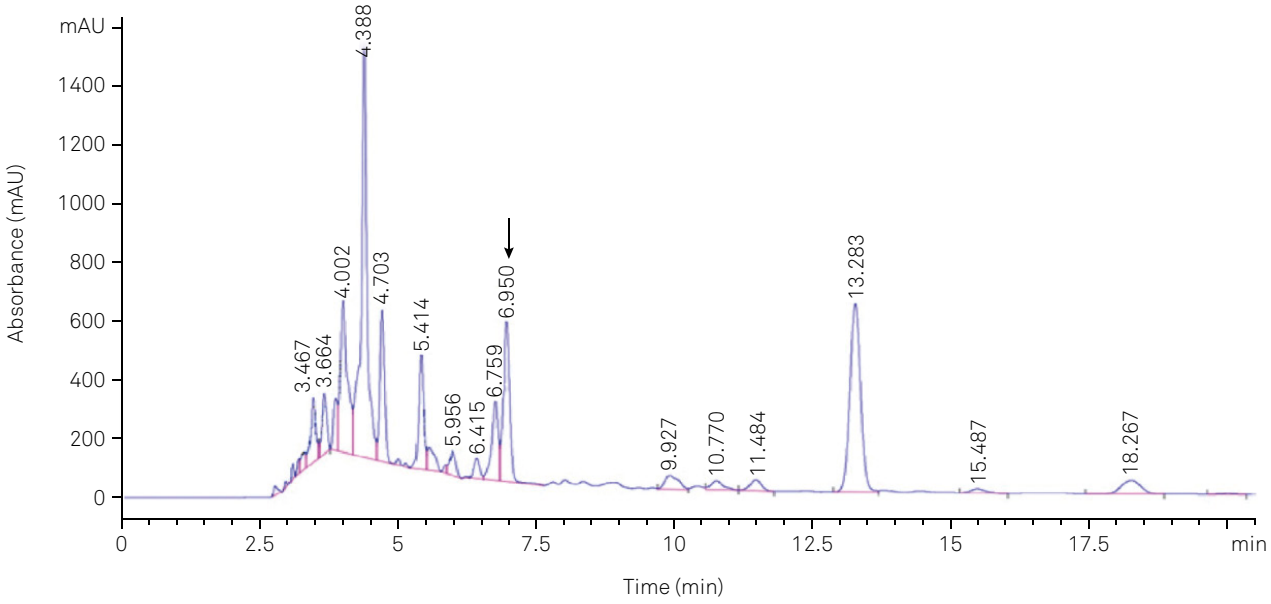


Figure 7. HPLC chromatogram of *Ducrosia anethifolia* essential oil (DAEO).

in both central and peripheral nervous systems and may be responsible for most of the excitatory neurotransmission.

In addition to GABA dysregulation, it has been indicated that excitatory amino acids are also involved in the initiation and propagation of seizures[30,31]. Citronellal, citronellol, myrcene and β-pinene, the DAEO monoterpenes, have NMDA receptor antagonist activities and can protect neurons against overstimulation[30,32]. Activation of NMDA receptors generally increases intracellular calcium influx, which raises neuronal excitation and excitability mainly via stimulation of cAMP-dependent signaling molecules including adenylyl cyclases and protein kinase A[33]. Especially, it has been reported that down-regulation of the cAMP-response element-binding protein is correlated with the suppression of epileptic seizures[34]. It has been reported that linalool, a DAEO constituent compound, exerts a considerable anticonvulsant activity in a rat model of PTZ-kindling via modulation of glutamatergic currents[35]. In addition,

linalool inhibits adenylate cyclase in chick retinas[36]. Thus, DAEO anticonvulsant capacity, at least in part, is mediated by modulation of intracellular second messengers such as calcium and glutamate. However, additional studies are still required to clarify this important issue in more details.

In the present study, PTZ-induced seizures could increase the levels of oxidative stress indicators such as MDA and H_2O_2, and decrease the activities of antioxidant enzymes, CAT and POD[37,38]. It has been demonstrated that the use of free radical scavengers in the treatment of epilepsy provides an important perspective that will be the driving force for future drug design of novel antiepileptics[39]. Pretreatment with DAEO and α-pinene could prevent the seizures and thus decrease oxidative stress. The data showed a dose-dependent effect of DAEO against seizure-induced oxidative stress in experimental models of seizures.

Potential antioxidant therapy that includes either natural antioxidants or agents is capable of augmenting the functions of these enzymes[40]. Earlier reports have shown that the natural drugs like DAEO have antioxidant properties because of the presence of α-pinene, citronellal, γ-terpinene, myrcene and limonene[41].

Taken together, the data suggest that DAEO and α-pinene have antiepileptic activities. This effect may be due to their antioxidant properties and possible activation of $GABA_A$ receptors. Our experiment contributes to our knowledge of the pharmacology of *D. anethifolia* (Boiss).

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