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Paeoniflorin Diminishes Maximal Electroshock - and PTZ - induced Convulsions in Mice

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1. Introduction

Paeonia Radix is one of the most commonly used herbal drug in traditional Chinese medicine[1] and folkloric medicine of different countries since ancient times[2]. A number of chemical and pharmacological studies have been conducted on the roots of *Paeonia* species. Monoterpen glycosides are the active constituents, among them; paeoniflorin is the main compound in genus *Paeonia*[3]. In traditional medicine aqueous decoctions of *Paeonia* have been used against several types of seizures[1,4,5].

Epilepsy is a common chronic neurological disorder characterized by recurrent seizures, resulting from excessive electrical activity of the brain. Medical treatment of epilepsy mainly consists of preventing seizure activity by anti-epileptic drugs. Anti-epileptic drugs prevent seizures in about two third of the patients[6,7]. Most of the anti-epileptic drugs are not individually effective in inhibiting all types of seizures and they mostly

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can suppress only one type of seizure and even sometimes they worsen the other epileptic disorder types. For instance, fenitoin, a first line drug for partial and tonic-clonic epilepsies, aggravates absence type of epilepsy. Some of the epileptic patients are resistant to currently used drugs. Therefore, research for new drugs with different mechanisms of action and broader spectrum of activity is still required.

Screening plant-originated molecules is a promising approach to develop new antiepileptic agents. Various compounds derived from *Paeonia* were shown to have antiseizure effects in several studies[8,9]. Although *Paeonia* species have been used to treat all types of epilepsies for centuries in traditional medicine, antiseizure effects of paeoniflorin in generalized tonic-clonic and/or absence type of epilespy have not been evaluated yet. Sugaya *et al*[10] extracted several compounds from *Paeonia* species and found that they alter PTZ-induced EEG power spectrum changes in anesthezied rats and they reported that paeoniflorin was one of the active compound among the other molecules tested in this study[10]. Although, the exact mechanism of antiseizure action of paeoniflorin is not clear, previous studies demonstrated that it exerts inhibitory effect on L-type Ca^{2+} channels[11], iberiotoxin-sensitive large conductance calcium-activated potassium channels[12] and voltage-gated sodium channels[13,14].

In this study, we aimed to evaluate the efficacy of paeoniflorin in generalized seizures in mice. In order to test the antiseizure activity of paeoniflorin, we employed MES and PTZ which are both standard procedures for testing the effectiveness of antiepileptic agents in generalized tonic-clonic and absence type of epilepsies, respectively[15,16].

2. Material and Methods

2.1. Plant material

Paeonia mascula subsp. *arietina* was collected from Sikirin Tepe, Bitlis, Turkey, in June, 2004. A voucher specimen (ADD-11999) has been deposited at the Herbarium of the Department of Biology, Faculty of Sciences, Hacettepe University, Beytepe, Ankara, Turkey.

2.2. Reagents

All chemicals were analytical reagent grade. Deionized water was prepared using a Barnstead Nanopure Diamond Analytical (USA) ultrapure water system.

2.3. Extraction and isolation of the compound

The air-dried powdered roots of Paeonia mascula subsp. arietina (480g) were extracted with MeOH under reflux (40°C). The solvent was removed by rotary evaporation yielding 30.191 g methanolic extract. The methanolic extract was subjected to vacuum liquid chromatography using reversed-phase material (Sepralyt 40 mm, 100 g), as eluents, employing H_2O (400 mL), H_2O- MeOH (75:25, 100 mL, 50:50 mL, 25:75 mL) and MeOH (400 mL); to give 27 fractions (100 ml/fractions), which were combined to nine main groups (I-IX) based on their TLC profiles: Fr. II (1.103 g) was subjected to normal-phase silica-gel (100 g) CC with CH_2Cl_2-MeOH-H_2O mixtures with increasing polarity (80:20:1®80:20:2) to give compound paeoniflorin (477 mg).

Paeoniflorin is an amorphous white powder; $[a]_D$: -12.9^0(c = 1.0,MeOH); The ^{1}H-, ^{13}C-NMR spectra and physical constants were similar to the previous report[3,17].

2.4. Animals

All experimental procedures involving animals were conducted in accordance with the guidelines for animal experiments of Hacettepe University Ethical Committee (Approval number: 2008/16-2) together with the recommendations from the Declarations of Helsinki. Male mice, 2-4 months old, weighing 25-40 g were used in the experiments. Animals were maintained in a room where temperature (24±2°C) and relative humidity (55±15%) were kept within constant limits with a light-dark cycle of 12 h/12 h, (lights were on at 07:00). Water and food were available *ad libitum*. Groups of 12 to 18 mice were used for each dose of the drugs and during the experiments mice were housed in individual cages. Experiments were carried out between 12:00 and 18:00 hrs.

2.5. Drugs

The following drugs were used; phenytoin (Sigma, USA) 15 mg/kg, PTZ (Sigma) 80mg/kg and paeoniflorin (obtained from *Paeonia* species

from Turkey) at 100, 250 and 500 mg/kg. The drugs were dissolved in saline and were administered intraperitoneally in a volume of 10 ml/kg of body weight. Control mice received saline and standard drug (positive control) group received phenytoin alone.

2.6. Statistical analysis

All data are expressed as the mean±SEM. Data were analyzed with Student's unpaired *t*-test and accepted as significant when $P<0.05$.

3. Experimental Procedure for Antiepileptic Activity

3.1. Maximal electroshock

Convulsions were induced with rectangular current pulses delivered by a Grass S44 stimulator (Grass, USA) and a constant current unit (Grass CCU1A) using corneal electrodes. Electrodes were soaked with saline containing 1% lidocaine was applied to the eyes in order to ensure sufficient electrical conduction and to avoid pain[18]. MES was applied at current intensities between 32.5-42 mA with 3 ms duration and 60 Hz for 1 sec. Maximal seizure was defined by the tonic extension of the hindlimbs to an angle close to 180° to the plane of the body axis.

A maximal electroshock seizure threshold test was also performed. For the assessment of threshold for convulsions, four groups of mice consisting of at least 12 animals were used. Groups were tested with various electrical stimuli in different current intensities. The shocks were aimed to yield 10-30%, 30-50%, 50-70% and 70-90% of animals with fully developed seizures. Following this, a current intensity-effect curve was constructed according to a log-probit method by Litchfield and Wilcoxon[19]. Using this curve, a median current strength (CS_{50} in mA) and its 95% confidence limits with SEM were calculated[20]. The CS_{50} value obtained from the control mice was used to evaluate the efficacy of paeoniflorin on MES-induced convulsions at different doses and time intervals following drug administration. MES was applied 40, 90, and 120 minutes after drug administration. In the evaluation of anticonvulsive effect, following parameters were compared: 1) Number of animals having convulsions. 2) Duration of hindlimb extension. 3) Duration of post-convulsive recovery period, including recovery from righting position and from stupor state.

3.2. PTZ test

Convulsions were induced with 80 mg/kg pentylenetetrazolium (PTZ) given intraperitoneally. Maximal seizure was defined by the tonic extension of the hindlimbs to an angle close to 180° to the plane of the body axis. Paeoniflorin was given at 100 and 250 mg/kg doses 90 minutes prior to PTZ injection. The time period before the initiation of the first generalized convulsion and the duration of the convulsion were accepted as a measure of anticonvulsive effect.

3.3. Rotarod test

Animals treated with paeoniflorin were also tested with a rotarod device 4.5 cm in diameter with a rotation speed of 12 r.p.m. in order to determine the possible effects of paeoniflorin on motor performance.

4. Results

4.1. Effects of paeoniflorin on MES-induced seizures

In order to assess the effect of paeoniflorin on the seizure threshold for MES, we first determined the CS_{50} value. Control mice had CS_{50} value of 35.3± 1.0 mA (n=12 animals for different current intensities). After 250 mg/kg paeoniflorin treatment, the seizure threshold increased to 39.4±1.4 mA (Table I, n=12 animals, $p<0.05$).

Immediately after the electroshock administration in the control animals, a tonic flexion of all limbs (with a slight tremor lasting approximately 2 s) was observed (n=18, Table II). Following this phase, tonic extension of the hindlimbs occurred and lasted for 15±0.2 s. After the cessation of seizure activity, animals displayed a period of stupor for 38±2 s and then they recovered. Paeoniflorin at 100 mg/kg was ineffective at preventing convulsions. However, the number of animals displaying convulsions after 250 and 500 mg/kg paeoniflorin administration was significantly lower after 90 min of injection. Convulsions were observed only in 25 % of mice, 90 min after 250 mg/kg of paeoniflorin injection. This anticonvulsant effect persisted after 120 minutes. Greater inhibitory effect was observed at 500 mg/kg. Seventeen percent of the mice displayed convulsions 120

TABLE I

The effect of paeoniflorin on electroshock threshold in mice

Treatment	(mg/kg)	CS_{50}	N	S.E.M.
Control	**(Saline)**	35.3 (33.3-37.4)	12	1.0
Paeoniflorin	250	39.4 (36.1-41.2)*	12	1.4

Results are presented as median current intensities (CS50 values with 95% confidence limits) necessary to produce tonic hindlimb extension in 50% of animals tested in the electroshock-induced seizure threshold test. Paeoniflorin was administered intraperitoneally 90 min before the electroshock. Data was statistically evaluated by unpaired Student's t-test. * P<0.05 vs. control group.

TABLE II

Effects of Paeoniflorin on the Electroshock-induced Convulsions in Mice

Treatment (mg/kg) Convulsive	**Time after injection (min)**	**N of mice having convulsions**	**Duration of hindleg extension (s)**	**Post-recovery period (s)**
Control Saline	40	9/18	15.0 ± 0.2	38.6 ± 2.7
	90	9/18	15.7 ± 0.3	41.0 ± 3.5
	120	9/18	14.1 ± 0.5	45.7 ± 3.7
Paeoniflorin 100	40	6/12	14.0 ± 0.5	41.2 ± 2.7
	90	5/12	14.4 ± 0.5	44.4 ± 8.4
	120	6/12	14.5 ± 0.5	49.3 ± 8.7
Paeoniflorin 250	40	6/12	13.7 ± 0.6	40.6 ± 3.6
	90	3/12	12.3 ± 1.2	50.3 ± 12.8
	120	3/12	15.0 ± 1.5	48.7 ± 9.6
Paeoniflorin 500	40	6/12	14.3 ± 0.3	43.0 ± 4.7
	90	3/12	14.0 ± 0.9	54.0 ± 7.3
	120	2/12	15.2 ± 0.7	72.0 ± 8.9
Phenytoin 15	40	0/12	0	0
	90	1/12	13.0	43.2
	120	0/12	0	0

Table presents results of control, paeoniflorin and phenytoin groups in MES test. Paeoniflorin was given at increasing doses to the groups. Times of intraperitoneal injections for paeoniflorin were 40, 90 and 120 minutes before MES. All values presented as mean±SEM.

TABLE III

Effects of Paeoniflorin on the PTZ-induced Convulsions in Mice

Treatment (mg/kg)		Time after injection (min)	N of mice having convulsions	Latency for the 1st generalized convulsion (s)	Duration of convulsion (s)
Control	Saline	90	8/8	245.5 ± 50.3	11.1 ± 1.1
Paeoniflorin	100	90	8/8	346.0 ± 45*	12.0 ± 1.1*
Paeoniflorin	250	90	6/8	684.8 ± 105.8*	16.3 ± 0.8*

Table presents results of control and paeoniflorin groups in PTZ test. Paeoniflorin was given at two different doses to different groups. Time of intraperitoneal injection for paeoniflorin was, 90 minutes before PTZ injection. All values presented as mean±SEM. * $P<0.05$ vs. control group.

minutes after paeoniflorin injection. The duration of hindlimb extension in paeoniflorin-treated animals was not different from the controls at all doses tested. The recovery period was prolonged in all animals treated with paeoniflorin. This effect was significant at 250 and 500 mg/kg.

Paeoniflorin administration caused a mild sedation and decreased locomotor activity. This sedative effect was detectable at 100 mg/kg and it increased in a dose-dependent fashion, but these animals still responded to the external stimuli. Paeoniflorin-induced sedative effect was not evaluated quantitatively. However, all of the animals injected with 500 mg/kg paeoniflorin maintained to stay on the rotarod longer than 1 min at 12 r.p.m showing that motor control was not impaired.

Phenytoin was tested as a standard drug (positive control) to compare the antiseizure activity of paeoniflorin. As it is well documented[20-22], phenytoin (15 mg/kg) prevented the convulsions induced by MES in 11 animals out of 12 (n=12, Table II) and these animals did not display stupor or behavioral alterations after electroshock administration.

4.2. Effects of paeoniflorin on PTZ-induced seizures

Following PTZ administration all animals in control (saline) and 100 mg/kg paeoniflorin groups had generalized tonic-clonic seizures (Table III). On the other hand, only 6 out of 8 mice had convulsions after PTZ injection in 250 mg/kg paeoniflorin group. However, the latencies for the initiation of these seizures significantly increased in paeoniflorin groups

in a dose dependent manner ($p<0.05$). Latencies were 245 ± 5 s, 346 ± 45 s, and 684 ± 105 s in the control group, 100 mg/kg paeoniflorin group and 250 mg/kg paeoniflorin group, respectively.

Duration of seizures also showed a similar dose-dependent prolongation. In the control group, duration was 11.1±1 s and after 100 mg/kg paeoniflorin administration duration was increased to 12±1 s and it was 16.3±0.8 s in the 250 mg/kg paeoniflorin group. These prolongation of seizure durations following 100 and 250 mg/kg paeoniflorin administrations were statistically significant when compared to the control values ($P<0.05$ for both 100 and 250 mg/kg paeoniflorin groups).

4.3. Rotarod test and other effects of paeoniflorin

In order to assess the possible toxic effects of paeoniflorin at the maximum dose, we observed the animals for 15 days and all animals survived without any noticeable signs of toxicity or complications. Animals did not display any abnormality in their gait and posture within this period. Additionally, we assesed the possible effects of paeoniflorin on the motor coordination and/or fatigue resistance on mice by using Rotarod test. Animals from both paeoniflorin and control groups performed similarly in Rotarod test showing paeoniflorin did not alter motor performance of the mice.

5. Discussion

The outcome of this study indicates that paeoniflorin, the active constituent of *Paeonia* plant, displays an antiseizure activity in both tonic-clonic and absence type of generalized seizure tests in mice. Paeoniflorin effectively reduced the number of animals having convulsions subsequent to the electroshock administration and these animals displayed significantly exceeding seizure thresholds for MES. This antiseizure activity of paeoniflorin was observed at 250 mg/kg and higher doses for MES whereas it was apparent at 100 and 250 mg/kg doses in PTZ test. Paeoniflorin-induced antiseizure activity appears to be dose-dependent. Only two out of twelve mice displayed convulsion at the maximum dose (500mg/kg) of paeoniflorin in MES experiments at 120 minutes. Although paeoniflorin effectively increased the seizure threshold for MES, it did

not alter the duration of the hindlimb extension in animals having convulsions. When the antiseizure activity of paeoniflorin is compared with that of phenytoin, paeoniflorin exerted antiseizure activity with a lower potency. Phenytoin and paeoniflorin seems to have different antiseizure activity spectrums[23].

In order to evaluate the activity of paeoniflorin as an absence type of epilepsy drug we employed a commonly used screening test for the drugs effective against absence type of epilepsy[24]. In this study, paeoniflorin did not alter the number of animals having convulsions after PTZ injection. However, paeoniflorin significantly prolonged latency period for the appearance of the first convulsion as well as the seizure duration in the PTZ tests. These findings indicate that paeoniflorin may also have some activity against absence type of epilepsies.

Contrary to phenytoin[25], paeoniflorin seems to be effective on both absence and tonic-clonic types of generalized seizures. These findings may imply that paeoniflorin utilizes a different mechanism of action than phenytoin. Furthermore, it may be proposed that combination of phenytoin with paeoniflorin may help to reduce phenytoin dose and consequently, its side effects as well. Since there is scarce information about the mechanism of paeoniflorin, similar plant based pharmacological studies may provide additional information about its mode of action. A recent ethnopharmacological study demonstrates that brain monoamines such as serotonin can behave as an inhibitory molecule in seizure activity[26]. Interaction of paeoniflorin with such inhibitory neurotransmission during antiseizure activity needs clarification. According to our findings, anticonvulsant activity of paeoniflorin became prominent 90 min after the injection and this effect persisted over 120 min. These findings suggest that paeoniflorin is a slow-onset drug in terms of its antiseizure activity and most likely this is a consequence of its pharmacokinetic profile. Moreover, further research on oral bioavailability and evaluation of possible synergic interactions with standard antiepileptic drugs are required for the assessment of paeoniflorin in this context to conclude the value of paeoniflorin as a candidate molecule in the treatment of epilepsy. Nevertheless, this study strongly implies that ethnopharmacological use of paeoniflorin has a realization in the treatment of epilepsy in folkloric medicine.

The major limitation of this study is the finding that the effective doses of paeoniflorin on seizures were higher than 250mg/kg. In another

study focusing on medicinal plants, effective doses were found to be as 200 and 400 mg/kg and our results show similar potency in this aspect[26]. These high doses seem to be irrelevant in clinical settings (human use)[27]. However, it should be taken into account that this acute dose may be much lower in its chronical use. Additionally, in our study, this molecule was tested in mice and pharmacological effective doses of paeoniflorin may be different in humans because of interspecies differences. In support of this view, anecdotal evidence from herbal medicine also implies that lower doses seem to be effective in patients with epilepsy.

Paeoniflorin induced sedation and inhibited locomotor activity at doses greater than 250 mg/kg. These effects do not seem to be related to a neuromuscular blocking action, since animals maintained their gait, and posture, and their motor performance on the rotarod test was within the control limits indicating that paeoniflorin does not cause incapacitance of the animals at these doses. In addition, paeoniflorin-treated animals were responsive to external stimuli. Therefore, paeoniflorin-induced inhibition of locomotor activity seems to be related to its actions on the central nervous system. Prolonged post-convulsive recovery period may also be explained with peoniflorin's inhibitory effect in the central nervous system.

This is the first study demonstrating the antiseizure activity of paeoniflorin against generalized seizures induced either by MES or PTZ in the experimental animals. Although the mechanism of action of antiseizure activity of paeoniflorin is not elucidated in this study, it seems to be different from that of phenytoin. Since the effectiveness of paeoniflorin in PTZ-induced absence type seizure model is also demonstrated in this study, this property of the glycoside may broaden its antiseizure spectrum. Although extracts of peony have been cited from western medicine since 17th century, only recently the responsible molecules could be found[28]. This molecule can be a candidate for a new anti-seizure drug or as an add-on therapy with other antiepileptic drugs. Further pharmacological studies and molecular modifications on paeoniflorin are essential for the assessment of this compound.

Research focusing on plant originated chemicals based on ethnopharmacological relevance is a fruitful area and current research on this subject continues to yield new molecules[29]. Evaluation of anticonvulsive properties of other monoterpene glycosides, tannins[30] and

Paeonimetabolin-I[31,32] which exert anti-convulsant action from paeony species may stimulate a research field for the appraisal of new anti-seizure compounds and further modifications of these molecules may provide new options for the treatment of seizures.

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Summary

Paeonia species have been used to treat epilepsia in traditional medicine, however the effects of paeoniflorin, the main constituent of *Paeonia* species have not been studied scientifically. We studied the effects of paeoniflorin with generalized tonic-clonic convulsion model induced by Maximal Electroshock (MES) and pentylenetetrazolium (PTZ) in mice. In MES test paeoniflorin was given intraperitoneally at doses of 100, 250 and 500 mg/kg. The current strength which yielded convulsions in 50 % of the animals was calculated by MES threshold test. The effects of paeoniflorin on convulsions were evaluated firstly via MES test. Paeoniflorin exerted anticonvulsive effect at 250 and 500 mg/kg and decreased the number of animals having convulsion. Anticonvulsive effect of paeoniflorin was observed 90 min after injection. In PTZ test we observed that 100 and 250 mg/kg paeoniflorin prolongs both the time period required for generalized convulsions and the duration in a dose dependent fashion. Based on these findings, we conclude that paeoniflorin inhibited generalized tonic-clonic convulsions induced by MES, and partially attenuated seizures in absence seizure model. Paeoniflorin and other monoterpenic glycosides of *Paeonia* species may be considered as a candidate for the development of a new anticonvulsive drug.

Keywords: Paeony, *Paeonia*, paeoniflorin, maximal electroshock, PTZ, antiseizure.

Özet

Paeoniflorin Farede Maksimal Elektroşok ve PTZ Nöbetlerini Azaltır

Paeonia türleri halk tıbbında epilepsinin tedavisi amacıyla kullanılan türlerdir, ancak *Paeonia* türlerinin ana etken maddesi olan paeoniflorinin etkileri bilimsel olarak incelenmemiştir. Bu çalışmada paeoniflorinin Maksimal Elektroşok (MES) ve pentilentetrazol (PTZ) ile oluşurulan jeneralize tonik-klonik konvülsiyon modelindeki etkileri araştırılmıştır. MES testinde paeoniflorin 100, 250 ve 500 mg/kg dozlarında intraperitoneal yolla verilmiştir. MES eşik testi ile hayvanların yüzde ellisinde konvülsiyona neden olan akım ölçülmüştür. Paeoniflorinin konvülsiyonlar üzerine etkileri once MES testi ile değerlendirilmiştir. Paeoniflorin 250 ve 500 mg/kg dozlarında antikonvülsif etki göstermiş ve konvülsiyona giren hayvan sayısını azaltmıştır. Paeoniflorinin antikonvülsif etkileri enjeksiyondan 90 dakika sonra gözlenmiştir. PTZ testinde paeoniflorinin 100 ve 250 mg/kg dozlarında jeneralize konvülsiyonlar için gereken süreyi ve konvülsiyon süresini doza bağımlı olarak uzatmıştır. Bu bulgular ışığında, paeoniflorinin MES'le indüklenen jeneralize tonik-klonik konvülsiyonları inhibe ettiğini değerlendirdik. Paeoniflorin ve paeonia türlerinde bulunan diğer monoterpenik glikozitler yeni antikonvülsif ilaçların geliştirilmesi için aday olarak değerlendirilebilir.

Anahtar Kelimeler: Şakayık, *Paeonia*, paeoniflorin, maksimal elektroşok, PTZ, antikonvülsif.

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