

# Research Paper: Anticonvulsant Effect of *Alcea aucheri* on Pentylentetrazole and Maximal Electroshock Seizures in Mice



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## ABSTRACT

**Introduction:** This study was designed to investigate the possible anticonvulsant effect of acute administration of an aqueous extract of flowers of *Alcea aucheri* (EFA) in two in vivo seizure models.

**Methods:** Seizures were induced in male adult Swiss mice by administration of Pentylentetrazole (PTZ) or Maximal Electroshock (MES). Mice were randomly subjected to receive saline, EFA (8.75-175 mg.kg<sup>-1</sup>), or diazepam intraperitoneally (i.p.) 15 or 30 min before PTZ injection. In another experiment, mice were treated (i.p.) with saline, EFA (8.75-350 mg.kg<sup>-1</sup>), or phenytoin 15 or 30 min before the MES test. Diazepam and phenytoin were used as reference drugs.

**Results:** EFA (175 mg.kg<sup>-1</sup>) significantly increased the PTZ-induced seizure threshold compared with the saline control group 15 min after its administration. In the MES test, the extract (35 mg.kg<sup>-1</sup>) increased the latency to onset of tonic Hind Limb Extension (HLE) (seizure activity) compared with the saline group 15 min after treatment. Also, 30 min after treatment, EFA (35, 70, and 175 mg.kg<sup>-1</sup>) increased the latency to onset of the seizure, decreased the duration of the seizure (70 mg.kg<sup>-1</sup>), and decreased seizure occurrence (350 mg.kg<sup>-1</sup>) compared with those of the saline group. At both time points, the extract at all doses significantly reduced the mortality rate compared with the saline group.

**Conclusion:** These findings provide evidence of a possible anticonvulsant effect of *A. aucheri* in PTZ and MES seizure models in mice.

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## Highlights

- Intraperitoneal administration of *Alcea aucheri* flower extract increased threshold for PTZ seizure.
- *Alcea* extract increased latency to tonic HLE, and decreased HLE duration in MES seizure.
- *Alcea* extract also protected mice against seizure, and decreased mortality rate in MES seizure.
- *Alcea aucheri* possibly have anticonvulsant effects in PTZ and MES-induced seizure models in mice.

## Plain Language Summary

Epilepsy is the third most common neurological disorder after stroke and Alzheimer disease, which affects around 50 million people worldwide. It is a chronic non-communicable disease of the brain characterized by seizures. Seizures are recurrent brief episodes of involuntary movements that may involve a part of the body or the entire body. Plants of the genus *Alcea* (*Althaea*) have been traditionally used for neurological conditions. However, their anticonvulsant effects remain to be investigated; neither clinical nor experimental assessments are present to indicate the anticonvulsant effect for the *Alcea* spp. Therefore, this study aims to investigate the anticonvulsant effect of aqueous extract of flowers of *A. aucheri* (EFA) on seizure induced by Pentylentetrazole (PTZ) and Maximal Electroshock (MES) in mice. According to the results, EFA increased the PTZ-induced seizure threshold and, the latency to onset of the MES-induced seizure; 15 min after treatment. Moreover, in MES test, EFA increased the latency to onset of the seizure, decreased the duration of the seizure, and decreased seizure occurrence, and reduced the mortality rate, compared with related saline group, 30 min after treatment.

### 1. Introduction

Epilepsy is the third most common neurological disorder after stroke and Alzheimer disease (Hirtz, Thurman, Gwinn-Hardy, 2007), which affects around 50 million people worldwide. It is a chronic non-communicable disease of the brain characterized by recurrent seizures. Seizures are brief episodes of involuntary movements that may involve a part of the body (partial seizure) or the entire body (generalized seizure). The episodes are sometimes accompanied by loss of consciousness and the control of bowel or bladder function (Fiest et al., 2017; Vezzani, French, Bartfai, 2011; WHO, 2001a).

Pharmacotherapy is the mainstay of treatment for epileptic patients. However, current available anticonvulsant drugs can efficiently control epileptic seizures in about 50% of the patients; 25% of the cases may show improvement, whereas the rest do not benefit significantly (Schmidt & Loscher, 2005). Also, antiseizure drugs are associated with specific toxicities, such as nystagmus, diplopia, ataxia, idiosyncratic blood dyscrasia, gingival hyperplasia, hirsutism, and teratogenic effects (Katzung, Masters, & Trevor, 2018). Accordingly, there is a major unmet clinical need traditionally for new antiepileptic

drugs with improved anticonvulsant efficacy and safety profile. Medicinal plants have long been used for neurological disorders and can be a good source of new therapeutic agents.

The medicinal plants of the genus *Alcea* have been used traditionally as food (the root of the plant) and also for the treatment of dermatologic, respiratory, gastrointestinal, and urinary disorders (the flowers of the plant). *Alcea* spp. also has been used as a sedative agent (Zargari, 1991; Blumenthal, Goldberg, & Brinckmann, 2000). Some of these traditional knowledge has been supported by some research (Deters, Zippel, Hellenbrand, Pappai, Possemeyer, Hensel, 2010; El Ghaoui, Ghanem, & Chedid, 2008; Sutovska et al., 2011; Mombeini, Gholami-Pourbadie, & Kamalinejad, 2017).

According to the following knowledge we aimed at conducting this research: a. *Alcea* spp. have been traditionally used for neurological conditions (Zargari, 1991); b. An acute administration of *A. aucheri* (Boiss) Alef. extract has central depressant effects in rats; c. Recent phytochemical analysis has shown the presence of flavonoids in the flowers of *A. aucheri* (Mombeini et al., 2017); and d. The flavonoids have a selective affinity for central benzodiazepine receptors (Medina et al.,

1997). To our knowledge, there is no information about the effect of *A. aucheri* extract on animal models of epilepsy. Therefore, this study was designed to investigate the possible anticonvulsant effect of the aqueous extract of flowers of *A. aucheri* (EFA) on Pentylentetrazole (PTZ)- and Maximal Electroshock (MES)-induced seizures in mice.

## 2. Methods

### 2.1. Animals

Male adult Swiss mice weighting 24-30 g (Razi Institute, Tehran, Iran) were housed in standard conditions, including controlled temperature ( $23\pm 1^\circ\text{C}$ ), 12 h dark/12 h light cycle, and with access to food and water *ad libitum*. Naive mice were used for the experiments and each mouse was used only once. All procedures were conducted in accordance with the Shahid Beheshti University of Medical Sciences Guidelines for the Care and Use of Laboratory Animals and were approved by the local Medical Research Ethics Committee. Each tested animal was immediately euthanized after seizure tests.

### 2.2. Plant materials

The fresh whole herb of *Alcea aucheri* was collected from Khuzestan province, in March 2018 and identified by the Department of Pharmacognosy, School of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran. A voucher specimen (SBMU-8021) was kept in the herbarium of the School of Pharmacy for future reference.

### 2.3. Preparation of aqueous extract

The fresh and healthy flowers were separated instantly, and then washed twice and dried in the shade at room temperature for 3 days. The dried flowers (100 g) were ground using a grinder for 30 s. Then, the powdered *A. aucheri* was macerated using 1000 mL of boiling distilled water and allowed to infuse for 2 h at room temperature. The extract was filtered, concentrated over the water bath and then under vacuum, and stored at  $4^\circ\text{C}$  in the refrigerator until use. The extract yield of 7.2% (w/w) was obtained.

### 2.4. Drugs

Pentylentetrazole was purchased from Sigma (UK). Diazepam hydrochloride (10 mg/2 mL; Daru Pakhsh Pharmaceutical Co.; Tehran, Iran) and phenytoin sodium (250 mg/5 mL; Caspian Tamin Pharmaceutical Co., Rasht, Iran) were used as the positive control drugs. PTZ

was dissolved in physiological saline as a 1% solution. Diazepam and phenytoin were diluted with saline. Moreover, different concentrations of EFA were prepared by serial dilution from a stock solution of 70 mg/mL of the extract dissolved in saline. EFA at the doses of 8.75-350 mg.kg<sup>-1</sup> of body weight of mice was used for behavioral tests. These doses were chosen according to our previous study, where we showed that a single dose of EFA has acute sedative and anxiolytic effects in experimental models (Mombeini et al., 2017). Therefore, we selected a limited range of effective sedative doses (17.5, 35, & 175 mg.kg<sup>-1</sup>) and tested them in a pilot study in PTZ- and MES-induced seizures. In addition, the time intervals of 15 min and 30 min between EFA injection and seizure inductions were found as the optimum time intervals for the evaluation of the acute anticonvulsant effect of the extract, according to findings of a pilot study (unpublished observations). Then, we gradually expanded the dosage range according to the results.

All drugs were administered in a volume of 10 ml/kg body weight of the mouse. PTZ was administered intravenously, whereas other drugs were administered intraperitoneally (i.p.). All drugs and the PTZ solution were made freshly on the day of the experiment before administration.

### 2.5. Behavioral assays

#### 2.5.1. Seizure threshold determination

The threshold for PTZ-induced seizures was measured by an infusion of PTZ into the tail vein of freely moving mice at a constant rate of 0.6 ml/min via a 30-gauge needle, connected by a polyethylene tube to a Hamilton microsyringe. The correct placement of the microsyringe was verified by the appearance of blood in the infusion tube. During the infusion, the animal was held gently using the tip of the tail. As previously described (Amini-Khoei et al., 2015; Haj-Mirzaian et al., 2019), the animal was monitored throughout the infusion and the time latency from the start of PTZ infusion to the onset of seizures was recorded. The infusion was halted when the general clonus was observed. The general clonus was characterized by a forelimb clonus followed by whole-body clonus. The minimal dose of PTZ (mg.kg<sup>-1</sup>) to induce general clonus was recorded and considered as an index of seizure threshold. For each animal, the convulsive threshold dose (mg.kg<sup>-1</sup> of body weight) required to elicit general clonus was calculated from the time of infusion (latency), infusion rate, the concentration of PTZ, and body weight.

**Table 1.** Experimental design of PTZ seizure test

EFA / Controls treatment (i.p.)		Observation of Behavior and Recording			
		Time	Seizure	Time	Stopping
		A	Induction	B	PTZ infusion
		(Min)		(Sec)	
PTZ tests	8.75, 17.5, 35, 70, 175 mg/kg EFA; saline; 3 mg/kg Dz	15	i.v. PTZ (1%)	Infusion duration	Onset of seizure
	8.75, 17.5, 35, 70, 175 mg/kg EFA; saline; 3 mg/kg Dz	30	i.v. PTZ (1%)	Infusion duration	Onset of seizure

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Time A column: Time between EFA/controls and PTZ administration; Time B column: Intervals from the start of infusion of PTZ solution to appearance of general clonus. EFA: Extract of flower of *Alcea aucheri*, Dz: Diazepam

**2.5.2. Maximal electroshock-induced seizure (MES)**

Tonic convulsions were produced using an alternating current (fixed current intensity: 35 mA, pulse duration: 0.2 s, frequency: 50 Hz, and pulse width: 0.5 ms) delivered via ear clip electrodes by a generator (Borj San-at, Tehran, Iran) (Ahmadiani, Mandgary, & Sayyah, 2003; Shirazizand, Ahmad-Molaei, & Motamedi, 2013). Electrodes were moistened by saline before attaching to the animal’s ears to improve electrical contact. The criterion for the occurrence of seizure activity was the tonic hind limb extension (HLE) (i.e. the hind limbs of animals outstretched 180° to the plane of the body axis) (Holmes & Zhao, 2008). At the time of electroshock induction, the animals were observed for 10 s for the seizure activity (HLE). Seizure variables, including latency to the onset of the seizure, duration of HLE, percent of protection against seizure, and mortality rate after electroshock convulsions were recorded for each mouse. Animals failing to show tonic hind limb extension were scored as protected and expressed in percentage.

**2.6. Experimental design**

**Table 2.** Experimental design of MES seizure test

EFA / Controls Treatment (i.p.)		Observation of Behavior And Recording			
		Time	Seizure	Time	Time
		A	Induction	B	C
		(min)		(sec)	(sec)
MES tests	8.75, 17.5, 35, 70, 175 mg/kg EFA; saline; 25 mg/kg PHN	15	MES (35 mA, 0.2 s, 50 Hz)	Latency to seizure	Seizure (HLE) duration
	8.75, 17.5, 35, 70, 175, 350 mg/kg EFA; saline; 25 mg/kg PHN	30	MES (35 mA, 0.2 s, 50 Hz)	Latency to seizure	Seizure (HLE) duration

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Time A column: Time between EFA/controls administration and the electroshock induction; Time B column: Time intervals of the electroshock induction and appearance of seizure. Time C column: Duration of seizure, i.e. hind limb extension, HLE. EFA: Extract of flower of *Alcea aucheri*; PHN: Phenytoin

Six to twelve mice were used in each treatment group. Mice were allowed at least 2 h for adaptation to the new environment (behavioral laboratory) before seizure assays. The treatments were randomized, and the observer was blind to the grouping. In the PTZ test, PTZ was administered intravenously 15 or 30 min after i.p. injection of EFA (8.75-175 mg.kg-1), diazepam (3 mg.kg-1) (Vlainic & Pericic, 2009), or saline (i.e. PTZ-15, PTZ-30; respectively).

In the MES test, electroshock was induced 15 or 30 min after i.p. injection of *A. aucheri* extract (8.75-350 mg.kg-1), phenytoin (25 mg.kg-1) (Reddy, Dubey, Handu, 2018), or saline (i.e. MES-15, MES-30, respectively). All experiments were conducted between 10:00 am and 4:00 pm. Tables 1 and 2 present the experimental designs in details.

**2.7. Statistical analysis**

Data on threshold for PTZ seizure, latency to the onset of HLE, and duration of HLE (MES test) were analyzed using one-way analysis of variance ANOVA followed by Student-Newman-Keuls post-hoc test. The Chi-squared

test followed by the Fisher's exact post-hoc test were used where appropriate to assess differences in incidence parameters (mortality or protection).  $P < 0.05$  was considered statistically significant. GraphPad Prism software (version 7 GraphPad Software, Inc., USA) was used for all statistical analysis and graph presentation.

### 3. Results

#### 3.1. The effect of EFA on PTZ seizure threshold

The ANOVA results indicated a significant effect of the extract on the PTZ-induced seizure threshold in the PTZ-15 group ( $F_{6,6} = 39.55$ ,  $P < 0.0001$ ) (Figure 1). Newman-Keuls t post-hoc comparison revealed a significant difference in the PTZ threshold between the extract-treated (175 mg.kg<sup>-1</sup>), or diazepam-treated, and saline control groups. Also, the ANOVA results showed a significant effect on the PTZ-induced seizure threshold in the PTZ-30 test following treatment [ $F_{6,6} = 51.33$ ,  $P < 0.0001$ ] (Figure 1). The post-hoc comparison showed a significant difference between the diazepam and saline groups.

#### 3.2. The effect of EFA on maximal electroshock-induced seizure

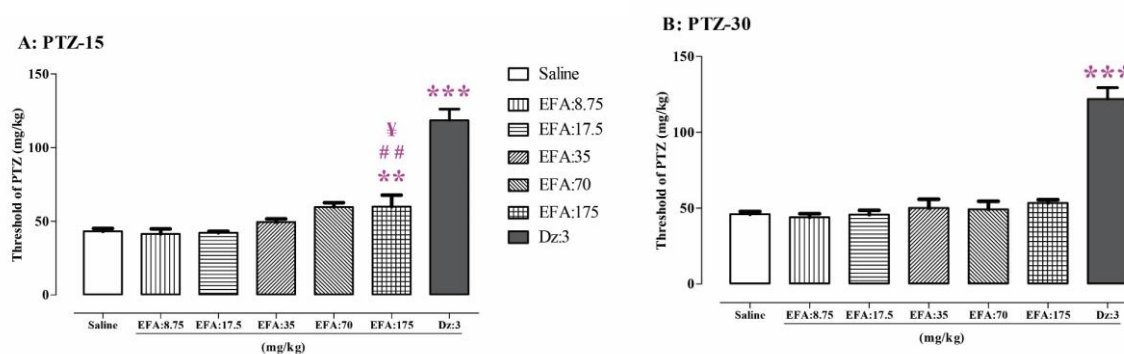
According to the ANOVA results, there was a significant difference in the latency to the onset of HLE in the MES-15 test [ $F_{5,5} = 3.17$ ,  $P < 0.01$ ] (Figure 2). The post-hoc test showed a significant difference in the latency to onset of HLE between the extract (35 mg.kg<sup>-1</sup>) and saline control groups.

A similar difference was observed in the latency to onset of HLE in the MES-30 test [ $F_{6,6} = 4.27$ ,  $P = 0.0021$ ] (Figure 2). The post-hoc comparison showed significant differences in the latency to onset of HLE between the extract (35, 70, and 175 mg.kg<sup>-1</sup>) and saline groups. Besides, the ANOVA results showed a significant difference in the duration of HLE after treatment [ $F_{6,6} = 4.25$ ,  $P = 0.01$ ]. The post-hoc comparison revealed a significant difference in the duration of HLE between the extract (70 mg.kg<sup>-1</sup>) and saline groups (Figure 2).

Also, the Chi-squared test indicated a significant difference in the percentage of protection against MES-induced seizure between the extract (350 mg.kg<sup>-1</sup>) or phenytoin and saline groups ( $P < 0.05$  and  $P < 0.0001$ ; respectively) (Table 3). Besides, the Chi-squared test indicated a significant difference in the mortality rate of animals after treatment. At both time points, EFA at all doses significantly reduced the mortality rate of the convulsed mice compared with the saline group ( $P < 0.0001$ ) (Table 3).

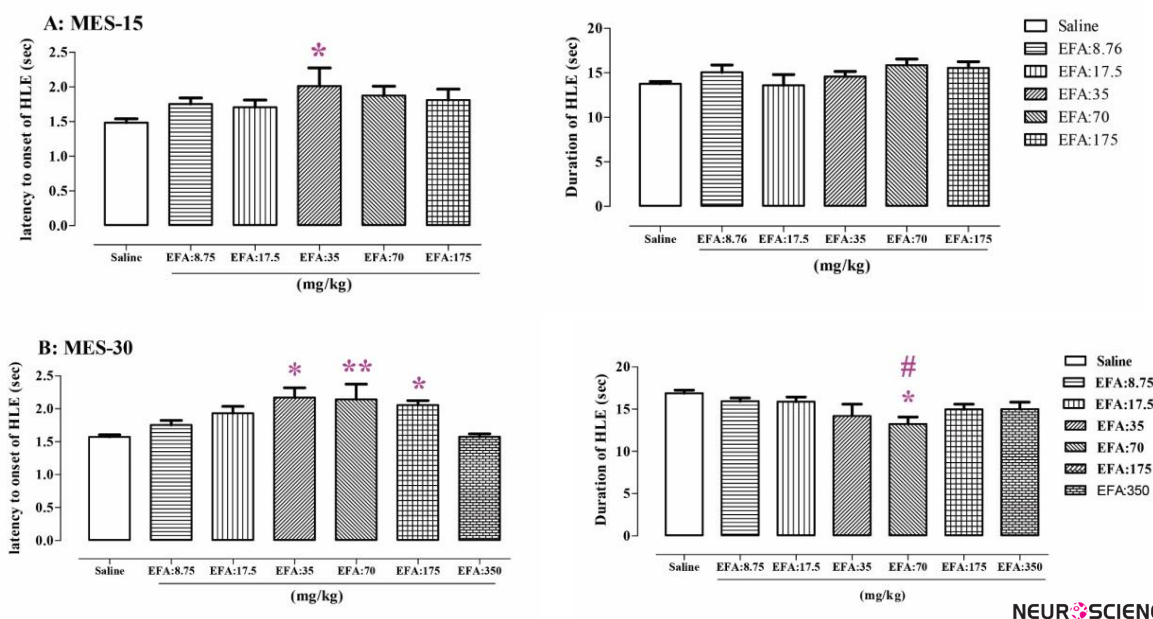
### 4. Discussion

The findings of the present study showed that the aqueous extract of flowers of *A. aucheri* had an anticonvulsant activity in two in vivo models of convulsion in mice. This is the first report on the anticonvulsant activity of *A. aucheri*. In the present study, we used PTZ and MES seizure models because these tests have been used as gatekeepers in antiepileptic drug discovery for over seven decades (Loscher, 2011). Also, both can reasonably predict human efficacious doses and exposures in



**Figure 1.** Effect of the aqueous extract of flowers of *A. aucheri* (EFA) on the pentylenetetrazole-induced seizure threshold in mice. Drugs were administered intraperitoneally 15 or 30 min before seizure threshold determination.  $N = 6-12$ . Data are expressed as Mean  $\pm$  SEM and were analyzed by the one-way ANOVA followed by the Student-Newman-Keuls post hoc test; \*\*  $P < 0.01$  and \*\*\*  $P < 0.0001$  compared with the saline control group; ¥  $P < 0.05$ ; ##  $P < 0.01$  compared with the EFA group (8.75 and 17.5 mg.kg<sup>-1</sup>, respectively); Dz: Diazepam; PTZ: Pentylenetetrazole.





**Figure 2.** Effect of the aqueous extract of flowers of *A. aucheri* (EFA) on maximal electroshock-induced seizure test in mice.

Drugs were administered intraperitoneally 15 min (upper panel, N=6-12) or 30 min (lower panel, N = 10-12) prior to electroshock induction. Data are expressed as Mean±SEM and were analyzed by one-way ANOVA followed by the Student-Newman-Keuls post hoc test; \*P<0.05 compared with saline group; \*\*P<0.01 compared with saline group; #P<0.05 compared with EFA at 8.75 mg.kg-1;

EFA: Aqueous extract of flower of *A. aucheri*; HLE: Hind limb extension

**Table 3.** Effect of the aqueous extract of flower of *A. aucheri* (EFA) on protection against maximal electroshock (MES)-induced seizure.

Control/Treatment (mg/kg)	MES-15 a		MES-30 b	
	Seizure protection	Mortality	Seizure protection	Mortality
Saline	0	38.46	0	41.66
EFA: 8.75	0	0*	8.33	8.33*
EFA: 17.5	0	0*	30	0*
EFA: 35	0	0*	30	0*
EFA: 70	0	0*	10	0*
EFA:175	0	0*	0	0*
EFA: 350	-	-	60 ¥	0*
PHN: 25	100*	0*	100*	0*

Saline, EFA or phenytoin (PHN) were administered intraperitoneally 15 or 30 min before seizure induction (i.e. MES-15, MES-30). The Chi-squared test was used for statistical analysis. aN=6-12; bN=10-12. ¥p < 0.05, \*p < 0.0001, compared with saline group. PHN :phenytoin.

epilepsy (Yuen, & Troconiz; 2015). The PTZ test represents a valid model for human-generalized myoclonic seizure and, absences seizure; whereas the MES test is thought to be a predictive model for generalized tonic-clonic seizures.

Our findings showed that EFA increased the PTZ seizure threshold in the PTZ-15 test (Figure 1). In the MES test, EFA increased the latency to onset of seizure at both time points, decreased seizure duration, and protected mice against seizure in the MES-30 test (Figure 2). Furthermore, EFA at all doses reduced the mortality rate of mice after electroshock convulsion (Table 3). According to these findings, it can be concluded that *A. aucheri* has anticonvulsant effects on PTZ- and maximal electroshock-induced seizure models. The present findings raise some questions about the mechanism of the anticonvulsant effect of EFA or possible ingredient responsible for these effects. Using the employed experimental tests, it is not possible to elucidate the mechanism of action, through which EFA exerts its anticonvulsant effects. Our preliminary phytochemical analysis showed the presence of phenolic compounds, polysaccharides, and flavonoids in the extract (Mombeini et al., 2017). It is not clear which compound(s) is responsible for the observed anticonvulsant effects of EFA; however, based on the literature, all ingredients in the EFA may be involved. Phenolic acids (phenolic compounds) are considered as one of the compounds responsible for the anticonvulsant effects of EFA. The identified phenolic acids in the flowers of *Alcea* spp. are caffeic (a derivative of rosmarinic acid), salicylic, vanillic, ferulic, and p-coumaric acids (Dudek, Matlawska & Szkudlarek, 2006). Caffeic acid and rosmarinic acid have anticonvulsant effect on seizures induced by PTZ using the kindling model in mice. Rosmarinic acid increased latency to seizure and decreased the incidence of seizures, and both of rosmarinic acid and caffeic acid reduced DNA damage index. (Coelho et al., 2015). Furthermore, Grigoletto et al. showed that rosmarinic acid dose-dependently increased the latency to myoclonic jerks and generalized seizures in the PTZ model and increased the latency to myoclonic jerks induced by pilocarpine in mice (Grigoletto et al., 2016).

Polysaccharides available in EFA may be also contributed to the observed effects. Herbal- or fungal-derived polysaccharides can protect murine against neuronal damage due to excessive glutamatergic activity (Ho, Yu, Yik, So, Yuen, & Chang, 2009; Baggio et al., 2010). Anti-epileptic effects *Ganoderma lucidum* (GL) spore has been shown in in vivo and in vitro studies. This effect is mediated through inhibition of the calcium accumula-

tion in epileptic rat hippocampal neurons in cell culture, by GL polysaccharides, and subsequently stimulation of Calcium/calmodulin-dependent protein kinase II (CaMK II  $\alpha$ ) expression. CaMK II  $\alpha$  inactivation is associated with many experimental models of epilepsy (Wang et al., 2014). Recently, Nonato et al. showed that polysaccharide of *Genipa americana* leaves increased the latency for PTZ-induced seizures, and the latency to death in mice. Both effects were reversed by flumazenil (Nonato et al., 2018).

Finally, the flavonoids in *A. aucheri* may be responsible for the observed effects. Flavonoids with anticonvulsant activity have been found in some medicinal plants used in traditional medicine, such as *Anisomeles malabarica* (Choudhary, Bijjem, & Kalia, 2011). This effect has been attributed to the affinity of flavonoids for the central benzodiazepine receptors (Medina et al., 1997; Hanrahan, Chebib, & Johnston, 2011). Furthermore, flavonoids (and polyphenols) can increase cerebral blood flow and reduce neuronal oxidative metabolism, by which they can protect the brain against oxidative stress injury in epilepsy (Fraga, Croft, Kennedy, 2019).

In summary, our findings demonstrated that *A. aucheri* has anticonvulsant effects against pentylenetetrazole and maximal electroshock models of seizure in mice. The active ingredient(s) and the pharmacological mechanism(s) that might account for the anticonvulsant effect of *A. aucheri* flowers have yet to be determined.

## Ethical Considerations

### Compliance with ethical guidelines

All procedures were conducted in accordance with the Shahid Beheshti University of Medical Sciences Guidelines for the care and use of Laboratory Animals and were approved by the local Medical Research Ethics Committee.

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### Authors' contributions

Conceptualization and investigation: Tajmah Mombeini; Plant identification and extract preparation: Mahammad Kamalinejad; Conducted the experiments: Ram-

tin Ejtemaei, Babak Asadpour Behzadi; Data analysis: Tajmah Mombeini; Writing-review & editing: Tajmah Mombeini, Freidoun Tahmasbi; Supervision: Tajmah Mombeini, Ahmad Reza Dehpour; and All authors have read and approved the manuscript before submission.

### Conflict of interest

The authors declared no competing interest.

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