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ARTICLE

Anticonvulsant Activity of Essential Oil From Leaves of *Zhumeria majdae* (Rech.) in Mice: The Role of $GABA_A$ Neurotransmission and the Nitric Oxide Pathway

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The essential oil from the leaves of *Zhumeria majdae* Rech. (ZMEO) has been shown to have several beneficial effects in the clinic. In this work we examined the anticonvulsant activities of ZMEO in an experimental mouse model of seizure and aimed to identify any possible underlying mechanisms. ZMEO (5, 10, 20, and 40 mg/kg intraperitoneally (i.p.)) or diazepam, as the reference anticonvulsant drug (25, 50 and 100 µg/kg i.p.), were administered 60 minutes prior to pentylenetetrazol (PTZ) injection (intravenously (i.v.) or i.p.) and changes in threshold, latency, and frequency of clonic seizure were examined. The PTZ i.p.-induced model of seizure was also applied for examining the protective effects of ZMEO pretreatment against PTZ-induced mortality. In some studies, the anticonvulsant effect of the combination of diazepam and ZMEO was also studied. The protective effects of ZMEO against hindlimb tonic extensions (HLTEs) were also examined by maximal electroshock (MES) seizure testing. The γ-aminobutyric acid (GABA)ergic mechanism and nitric oxide (NO) pathway involvement in anticonvulsant activity of ZMEO were assessed by pretreating animals with flumazenil, N^{ω}-nitro-L-arginine methyl ester (L-NAME), aminoguanidine, and L-arginine in a PTZ-induced model of seizure. Administration of 20 mg/kg ZMEO significantly increased chronic seizure threshold and latency while reducing frequency of convulsions and mortality in the PTZ-induced model. In the doses studied, ZMEO could not protect mice from HLTE and mortality induced by MES. Pretreatment with L-arginine and diazepam potentiated the anticonvulsant effects of ZMEO, whereas pretreatment with L-NAME, aminoguanidine, and flumazenil reversed anticonvulsant activity. The anticonvulsant activity of ZMEO may be mediated in part through a GABAergic mechanism and the NO signaling pathway.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

☑ The essential oil derived from leaves of *Zhumeria majdae* Rech. (ZMEO) has recently been shown to have several beneficial effects in clinical practice. However, little is known about ZMEO's potential anticonvulsant activity and its underlying pharmacologic mechanism of action.

WHAT QUESTION DID THIS STUDY ADDRESS?

☑ Does ZMEO demonstrate significant anticonvulsant activity in experimental models of seizure? Is this anticonvulsant activity linked to the activation of γ-aminobutyric acid type A ($GABA_A$) receptor or nitric oxide (NO) pathway?

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

☑ With linalool as its most abundant component, ZMEO demonstrated a meaningful anticonvulsant activity in both intravenous and intraperitoneal pentylenetetrazol-induced mouse models of seizure. ZMEO, however, was not effective in the maximal electroshock model. These effects were partially mediated through activation of $GABA_A$ receptor and NO synthesis.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

☑ Our data present ZMEO as a novel antiepileptic agent, especially for application in the treatment of myoclonic/generalized absence epilepsy, which is usually refractory to treatment. Furthermore, understanding the underlying mechanism of ZMEO's anticonvulsant activity will help physicians in its rational application as a complementary component in combinational therapies to improve treatment of resistant epilepsies.

Affecting about 50–70 million people worldwide, epilepsy is responsible for 0.75% of the global burden of disease.[1-3] The number of newly diagnosed cases of epilepsy worldwide is estimated to be around 2.4 million per year, with annual incidence of 50 in 100,000 and prevalence of 700 in 100,000, making it one of the most frequent chronic

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neurologic disorders.[2] Approximately, 80% of epileptic patients reside in low- and middle-income countries (including Asia, sub-Saharan Africa, and Latin America), where, due to poverty and/or unavailability of antiepileptic drugs (AEDs), three quarters of them lack proper therapy.[2,4] Unfortunately, the number of newly diagnosed cases of epilepsy in these low-income zones is about twofold higher than in high-income countries.[2] In addition, about one third of epilepsy cases are nonresponsive or only partially responsive to currently existing AEDs. Similar numbers of patients also experience severe or unacceptable adverse effects in response to AED therapy.[5,6] Therefore, the challenge remains to identify new sources with greater affordability, higher clinical efficacy, and a safer toxicity profile. In recent few decades, much attention has been focused on plant extracts and essential oils as indigenous sources for development of new drugs. In this context, a diverse group of plant extracts and essential oils, including extracts from *Berberis vulgaris* (L.), *Passiflora incarnate* (L.), and *Pseudospondias microcarpa* (A. Rich) Engl., have shown promising anticonvulsant effects in different models of seizure.[7-9]

There is a trend for identifying other new plant sources endemic in low- and middle-income countries for much easier accessibility and affordability. *Zhumeria majdae* Rech. (Labiatae) is an endemic fragrant shrub found in southern regions of Iran, especially the Hormozgan province, and typically grows on bare, rocky slopes. The folk name of this plant is "*Mohrkhosh*." It is routinely utilized in Iranian traditional medicine as a carminative for infants and also for treatment of stomach aches, headaches, and dysmenorrhea in adults.[10] Several recent reports have demonstrated antiplasmodial,[11] anti-inflammatory, and antioxidant activities of *Z. majdae* essential oil (ZMEO) and its extracts, both *in vivo* and *in vitro*.[12-15] Now, it is clear that the antioxidant activity of *Z. majdae* extract is largely due to its polyphenolic components.[12,15] Recently, Mandegary et al. reported the anticonvulsant activity of essential oil and methanolic extract of *Z. majdae* in the maximal electroshock (MES) model of tonic seizure and showed that administration of *Z. majdae* could delay clonus and tonic seizures after intraperitoneal (i.p.) administration of pentylenetetrazol (PTZ).[16] Here, in parallel to a previous report, we attempted to further confirm the anticonvulsant activity of ZMEO and identify the probable underlying mechanisms, using different models of seizure.

MATERIALS AND METHODS

Collection of plant material and preparation of the essential oil

Fully matured leaves of *Z. majdae* were collected between March and April 2015 from the Genow-protected zone, located in Hormozgan province in southern of Iran. After identification and confirmation of leaves by Asadpour, a voucher code 1091-AUPF was sent to the herbarium at the School of Pharmacy, Tehran University of Medical Sciences, and the Islamic Azad University, Tehran, Iran. Hydrodistillation of the collected samples was then performed in a Clevenger-type apparatus for 3 hours and the oil collected was dried (using anhydrous sodium sulfate) and then stored in a glass bottle at –18°C until further biochemical analysis or application in pharmacologic tests.

Gas chromatography–mass spectroscopic analysis of *Z. majdae* essential oil

Gas chromatography–mass spectroscopy (GC-MS) was performed utilizing an HP-6890 gas chromatograph (GC) coupled with an HP-5973 Mass Selective Detector (MSD) (Agilent Technologies, Santa Clara, CA) functioning in 70-eV mode. Identity of each component of essential oil was determined by comparing its mass spectra with those deposited in the mass spectral libraries, including Wiley NBS75K.L and NIST/EPA/NIH (2002 version; National Institute of Standards and Technology, Gaithersburg, MD), using different search engines.[17,18]

Animals

The study was carried out on adult male NMRI mice, 20–25 g in weight. The animals were housed with five or six animal per cage under regular 12-hour light/dark cycles and allowed to access food and water *ad libitum*, except for short periods when the pharmacologic tests were being carried out. Experiments were performed between 10.00 AM and 1:00 PM each day under regular room lighting and at ambient temperature. All animal experiments were complied with the ARRIVE guidelines and carried out in accordance with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 8023, revised 1978). Mice were euthanized by cervical dislocation after completion of the tests. The whole study and the procedures included were approved by the ethics committee of the Tehran Islamic Azad University of Medical Sciences on August 1, 2013.

Experimental procedures

Intravenous PTZ-induced seizures model. Clonic seizure induced by PTZ is a standard experimental model of seizure, equivalent to clonic petit mal seizures in humans with both face and construct validity. The i.v. PTZ-induced model of seizure was used for studying the acute effect of ZMEO pretreatment on clonic seizure threshold (CST).[19] Within the studied range of ZMEO pretreatment times (30, 60, and 120 minutes prior to PTZ administration), the best timepoint was the one with the largest increase in threshold of seizure, after administration of an effective concentration of ZMEO (20 mg/kg; **Figure 1g**). Based on the results, the best anticonvulsant activity was achieved when ZMEO was administered 60 minutes prior to PTZ infusion. Consequently, a 60-minute time interval was included between administration of ZMEO and infusion of PTZ in all experiments with measurement of clonic seizure threshold as their end point. Mice were classified into eight groups (eight mice per group) and treated with either ZMEO (5, 10, 20, or 40 ml/kg i.p.), diazepam (25, 50, or 100 µg/kg i.p.), or vehicle (sweet almond oil; 10 mg/kg i.p.) 60 minutes prior to i.v. infusion of PTZ (0.5% PTZ solution, 0.5 ml/min). Infusion was halted when full clonus of the body was seen. This usually occurs soon after initiation of forelimb clonus (**Figure 1a**).[20] At this point, the total amount of PTZ delivered per kilogram of body weight in each mouse was calculated according to the following formula and referred as clonic seizure threshold:

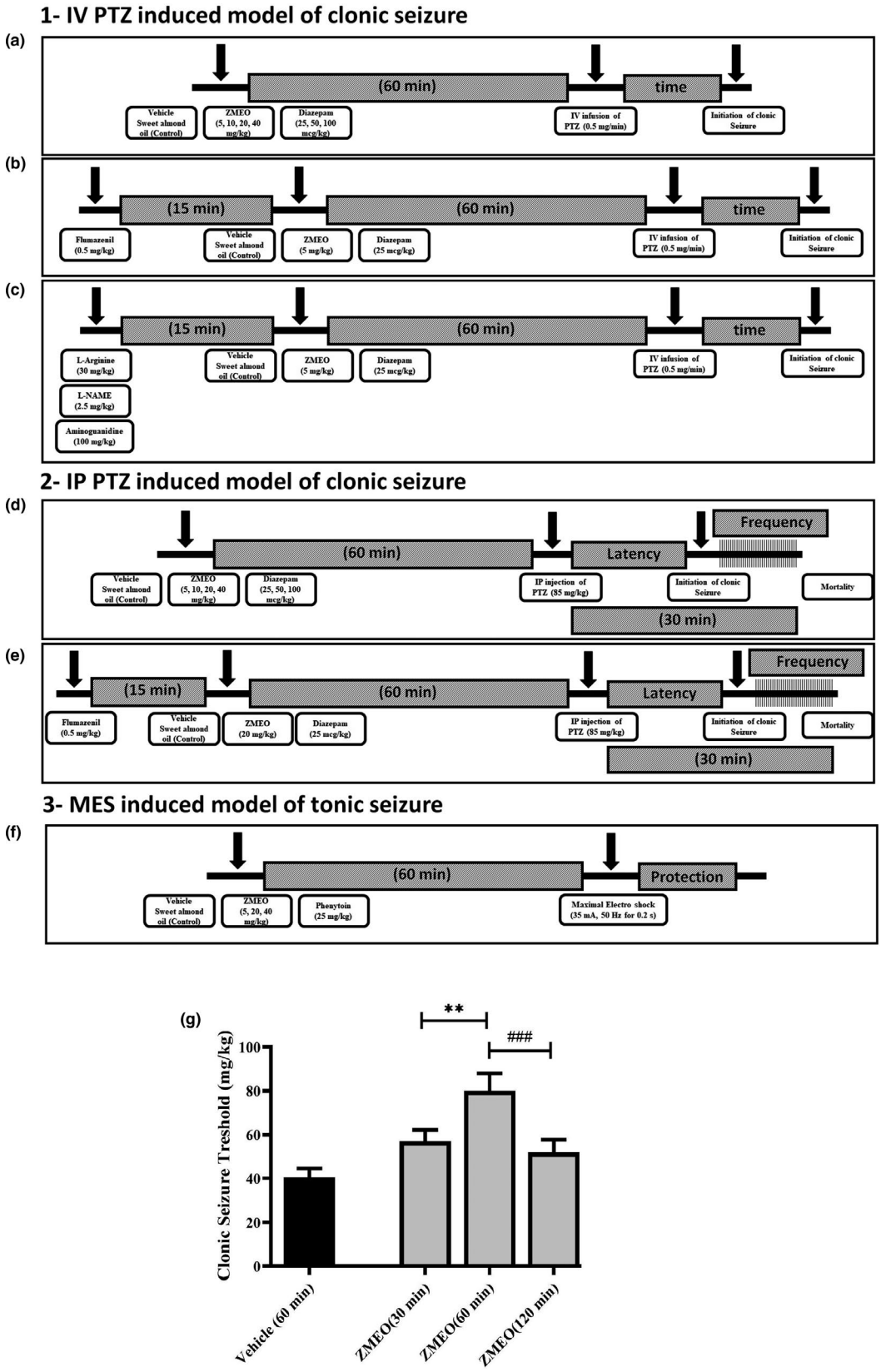


Figure 1 A schematic representation of the study design. Animal models of seizure utilized in present study for investigating anticonvulsant activity of ZMEO, as well as the sequences of drug administration have been depicted in (**a-f**) and effect of pretreatment time on anticonvulsant activity of 20 mg/kg ZMEO has been demonstrated in (**g**). ZMEO, *Zhumeria majdae* (Rech.).

$$\text{PTZ (mg/kg)} = \frac{\text{Infusion time (min)} \times \text{Rate of infusion (mL/min)} \times \text{milligrams PTZ/mL} \times 1{,}000\,\text{g}}{60\,\text{s} \times \text{weight of animals (g)}}$$

For examination of possible enrollment of γ-aminobutyric acid type A ($GABA_A$) receptors in protection induced by ZMEO in i.v. PTZ-induced model of seizure, flumazenil (0.5 mg/kg i.p.), a selective antagonist at the benzodiazepine binding site on the $GABA_A$ receptor, or vehicle was administered 15 minutes prior to injection of ZMEO (5 mg/kg i.p.) and diazepam (25 µg/kg i.p.). In cases where effect of flumazenil on combination of diazepam and ZMEO was studied, diazepam and ZMEO were administered 5 and 15 minutes after injection of flumazenil, respectively. After 60 minutes, mice were infused at a constant rate with PTZ (0.5 ml/min, 0.5%). Alterations in CST were recorded for each group separately and compared (**Figure 1b**).

The possible role of the nitric oxide (NO) pathway involvement in the anticonvulsant activity of ZMEO was examined by pretreating mice with subeffective doses of L-arginine (30 mg/kg i.p.), a precursor for nitric oxide synthesis; N^{ω}-nitro-L-arginine methyl ester (L-NAME) (2.5 mg/kg i.p.), a nonselective inhibitor of nitric oxide synthase (NOS); and aminoguanidine (100 mg/kg i.p.), a selective inhibitor of inducible NOS (iNOS). This was done 15 minutes prior to administration of ZMEO (10 and 20 mg/kg i.p.). After 60 minutes, mice were infused with PTZ. Alterations in CST were then reported in response to pretreatment with modulators (**Figure 1c**). Doses of pharmacologic modulators were chosen according to pilot experiments and similar studies in the literature.21,22

Intraperitoneal PTZ-induced seizures. Short-term i.p. administration of PTZ (85 mg/kg) was done to evaluate the frequency and latency of generalized clonic seizure and incidence of death. Mice were classified into eight groups (eight mice per group) and received ZMEO (5, 10, 20, or 40 ml/kg i.p.), diazepam (25, 50, or 100 µg/kg i.p.), or vehicle (sweet almond oil; 10 mg/kg i.p.) 60 minutes prior to IP infusion of PTZ, respectively (**Figure 1d**). The duration of monitoring after PTZ administration was limited to 30 minutes, and a latency of 1,800 seconds was recorded for experiments in which no generalized seizure occurred.[23]

For examination of possible enrollment of $GABA_A$ receptor in protection induced by ZMEO in the i.p. PTZ-induced seizure model, flumazenil (0.5 mg/kg) or vehicle was administered 15 minutes before injection of ZMEO (20 mg/kg) and diazepam (25 µg/kg). In cases where the effect of flumazenil on the combination of diazepam and ZMEO was studied, diazepam and ZMEO were administered 5 and 15 minutes after injection of flumazenil, respectively. After 60 minutes, mice were intraperitoneally injected with PTZ (85 mg/kg). Alterations in latency and frequency of clonic convulsions were then recorded for each group separately and compared (**Figure 1e**).

Maximal electroshock seizure test. The seizures were induced by MES in male NMRI mice with the help of an electroconvulsiometer by passing current of 35 mA at 50 Hz for 0.2 second using ear clip electrodes. Mice were classified into five groups, each consisting of 10 mice. ZMEO (5, 20, and 40 mg/kg), phenytoin (25 mg/kg), or vehicle (sweet almond oil; 10 mg/kg i.p.) were given 1 hour prior to seizure induction. Drops of saline were instilled in both ears to ensure current transmission. The ability to reduce mortality and incidence rate of tonic hindlimb extension (THLE) were used as end points for evaluating the protective effects of ZMEO against the MES-induced seizure model.[24,25]

Statistical analysis

Throughout the experiments, sample sizes of eight to ten were used. Clonic seizure frequency, latency, and threshold obtained from the i.p. and i.v. PTZ-induced models of seizure are expressed as mean ± SEM. One-way analysis of variance (ANOVA) with Newman-Keuls *post hoc* test was used for comparing clonic seizure thresholds and frequencies between groups. The Kruskal-Wallis nonparametric test was used to compare clonic seizure latencies among groups, as data did not demonstrate a normal distribution. Two-way ANOVA with Bonferroni's *post hoc* test was applied for analyzing experiments in which the possible roles of the GABAergic system and NO pathway in anticonvulsant activity of ZMEO were studied. In the i.p. model of PTZ-induced seizure, the Kaplan-Meier test was applied for evaluating survival relative to time. Differences in survival were then analyzed using the log-rank test. Statistical analysis was performed using GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA). $P < 0.05$ was considered statistically significant for all experiments.

RESULTS

Chemical composition of ZMEO

The yield of ZMEO extraction based on native plant material was 9.3%. The main terpenoids identified in ZMEO were linalool (61.4%) followed by camphor (27.5%). Other major components found include cis-linaloloxide (1.11%), limonene (0.98%), and geraniol (0.9%) As shown in **Figure 2** and **Table 1**, 16 major compounds formed ~ 98.8% of the total essential oil. With regard to the predominance of linalool and camphor in the formulation, the biologic effects observed may in large part be attributed to the pharmacologic activity of these two terpenes.

Effect of ZMEO on i.p. and i.v. PTZ-induced seizure models

Administration of ZMEO in the i.v. PTZ-induced seizure model resulted in a dose-dependent increase in clonic seizure threshold, with statistical significance beginning at 10 mg/kg ($P < 0.05$), suggesting protective effects of ZMEO against petit mal clonic seizure ($F_{(3, 28)} = 79.87$, $P < 0.0001$; **Figure 3b**). Notably, 40 mg/kg ZMEO demonstrated a submaximal effect compared with 20 mg/kg ZMEO. In addition, one-way ANOVA demonstrated that ZMEO could significantly reduce frequency of clonic convulsions at all doses examined ($P < 0.0001$) in the i.p. PTZ-induced seizure model ($F_{(3, 36)} = 40.28$, $P < 0.0001$; **Figure 3a**). Kruskal-Wallis analysis consistently revealed a statistically significant increase in latency of clonic convulsions only at 20 mg/kg ZMEO in the i.p. PTZ-induced seizure model ($P < 0.05$; **Figure 3a**). Diazepam as the reference anticonvulsant agent increased the clonic seizure threshold, with

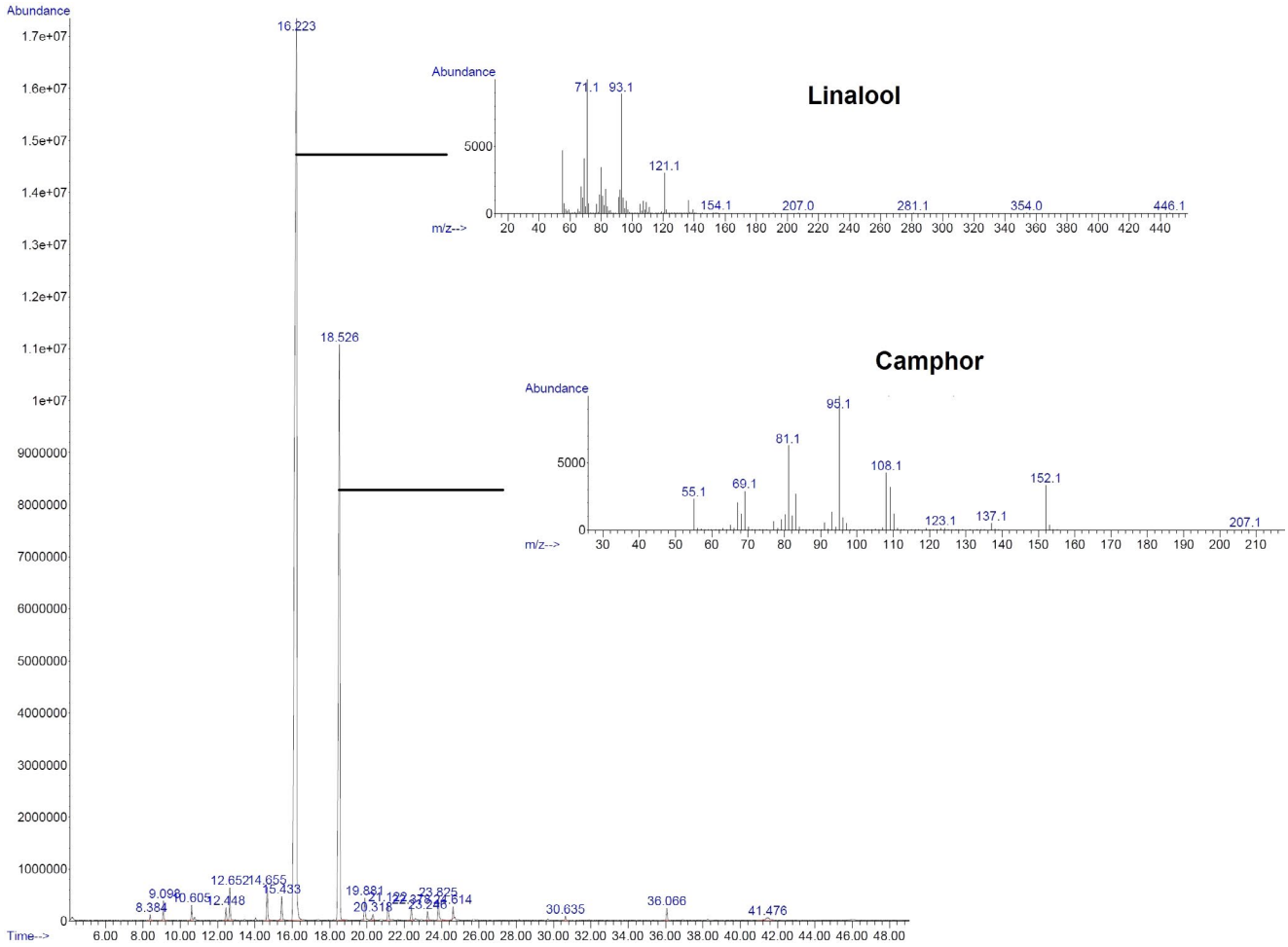


Figure 2 Experimental chromatogram of ZMEO obtained by GC-MS. The mass analysis of Linalool and Camphor has also been provided in scheme. ZMEO, *Zhumeria majdae* (Rech.).

statistical significance beginning at 50 µg/kg in the i.v. PTZ-induced seizure model ($F_{(3, 28)}$ = 19.34, $P < 0.0001$; **Figure 3d**). Also, pretreatment with diazepam meaningfully delayed onset of clonic convulsions ($P < 0.001$ for 50 and 100 µg/kg; **Figure 3c**) and reduced the frequency of clonic convulsions ($F_{(3, 36)}$ = 116.1, $P < 0.0001$; **Figure 3c**) at all concentrations in the i.p. PTZ-induced seizure model.

As shown in **Figure 4a**, administration of ZMEO dose-dependently (with statistical significance beginning at 20 mg/kg; $P = 0.002$, $\chi^2(df = 3) = 14.71$) improved animal survival after induction of convulsions by i.p. administration of PTZ. When compared with the vehicle group, mortality in mice pretreated with diazepam (25 mg/kg i.p.) was not significantly different from that in the control group ($P = 0.079$, $\chi^2(df = 1) = 3.08$). In contrast, concurrent administration of 5 mg/kg ZMEO with 25 µg/kg diazepam prevented death by i.p. administration of PTZ in all mice ($P = 0.0003$, $\chi^2(df = 1) = 13.39$; **Figure 5b**).

Effect of ZMEO on maximal electroshock-induced seizure model

Administration of electrical shock produced THLEs in almost all mice in the vehicle group (**Table 2**). Administration of ZMEO at the doses studied also did not produce any protective effects against THLEs. In contrast, administration of 25 mg/kg phenytoin completely protected mice against THLE and MES-induced death.

$GABA_A$ receptor is partially involved in protective anticonvulsive effects of ZMEO

As depicted in **Figure 5a,b**, administration of 20 mg/kg ZMEO or 25 µg/kg diazepam significantly increased latency while decreasing frequency of clonic convulsions in the i.p. PTZ-induced seizure model. Furthermore, a synergistic increase in latency and a decrease in number of clonic convulsions were observed when ZMEO and diazepam were administered together in the model. Interestingly, pretreatment with flumazenil (0.5 mg/kg i.p.), which when given alone did not demonstrate any significant effect on latency or frequency of clonic convulsions, completely reversed the anticonvulsant effects of ZMEO, diazepam, and their combination by decreasing latency and increasing frequency of clonic seizures in the i.p. PTZ-induced seizure model up to levels observed in vehicle-administered mice (frequency: $F_{(1, 56)}$ = 74.42, $P < 0.0001$; latency: $F_{(1,56)}$ = 1,356,

Table 1 The 16 most abundant components of ZMEO in order of their retention time and their abundancy and structure

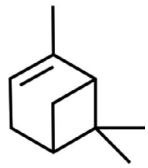
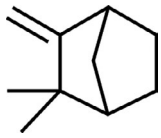
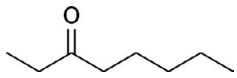
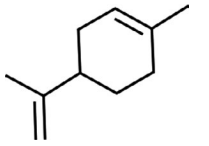
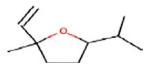
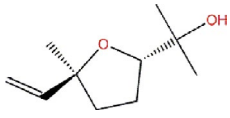
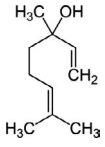
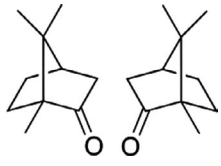
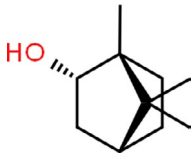
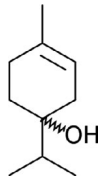
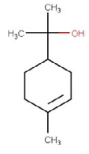
Compounds	Retention time (minutes)	Amount in ZMEO (%)	Structure
α-Pinene	8.38	0.16	
Camphene	9.09	0.56	
3-Octanone	10.6	0.43	
Limonene	12.65	0.98	
cis-Linaloloxide	14.65	1.11	
trans-Linaloloxide	15.43	0.81	
Linalool	16.22	63.4	
Camphor	18.52	27.48	
Borneol	19.88	0.83	
Terpinene-4-ol	21.22	0.31	
p-Menth-1-en-8-ol	23.24	0.63	

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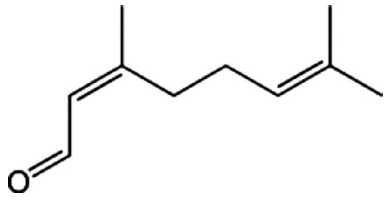
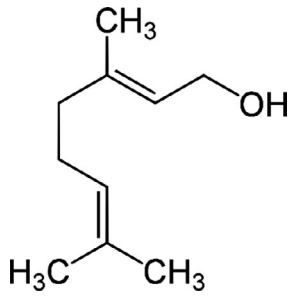
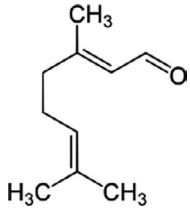
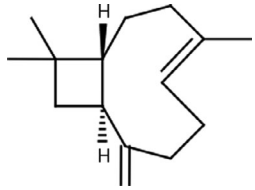
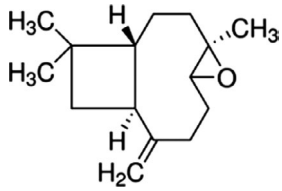
Table 1 (Continued)

Compounds	Retention time (minutes)	Amount in ZMEO (%)	Structure
Z-citral	23.82	0.32	
Geraniol	24.61	0.9	
Geranial	30.63	0.28	
trans-Caryophyllene	36.06	0.14	
Caryophyllene oxide	41.47	0.4	

ZMEO, *Zhumeria majdae* (Rech.).

$P < 0.0001$). As shown in **Figure 5c**, administration of ZMEO (5 mg/kg i.p.) and diazepam (25 µg/kg i.p.) did not significantly increase threshold of clonic convulsions in the i.v. PTZ-induced seizure model. However, concurrent administration of the two therapies resulted in a significant increase in threshold of clonic seizure. This finding suggests a synergistic effect for concurrent administration of these agents, which may occur through a common pathway in the i.v. PTZ-induced seizure model. Interestingly, pretreatment with flumazenil (0.5 mg/kg i.p.), which on its own did not demonstrate any significant effect on threshold, completely reversed the anticonvulsant effect of a combination up to the levels observed in vehicle-administered mice ($F_{(1, 28)} = 30.82$, $P < 0.0001$). Furthermore, the protective effects of ZMEO and combination of ZMEO and diazepam against PTZ-induced mortality was completely reversed by pretreating mice with flumazenil (**Figure 4b**). These results strongly suggest that $GABA_A$ receptors are at least partially involved in the anticonvulsant activity observed with ZMEO.

NO pathway is partially involved in protective anticonvulsive effects of ZMEO

The lower row in **Figure 5** demonstrates the modulatory effects of L-arginine, L-NAME, and aminoguanidine effects on the anticonvulsant activity of ZMEO in the i.v. PTZ-induced seizure model. Short-term treatment with ZMEO (10 and 20 mg/kg i.p.) significantly increased threshold of clonic convulsions. L-Arginine (30 mg/kg i.p.) pretreatment did not have any significant effect on threshold of convulsions compared with vehicle-administered mice. However, pretreatment with L-arginine significantly increased ZMEO's

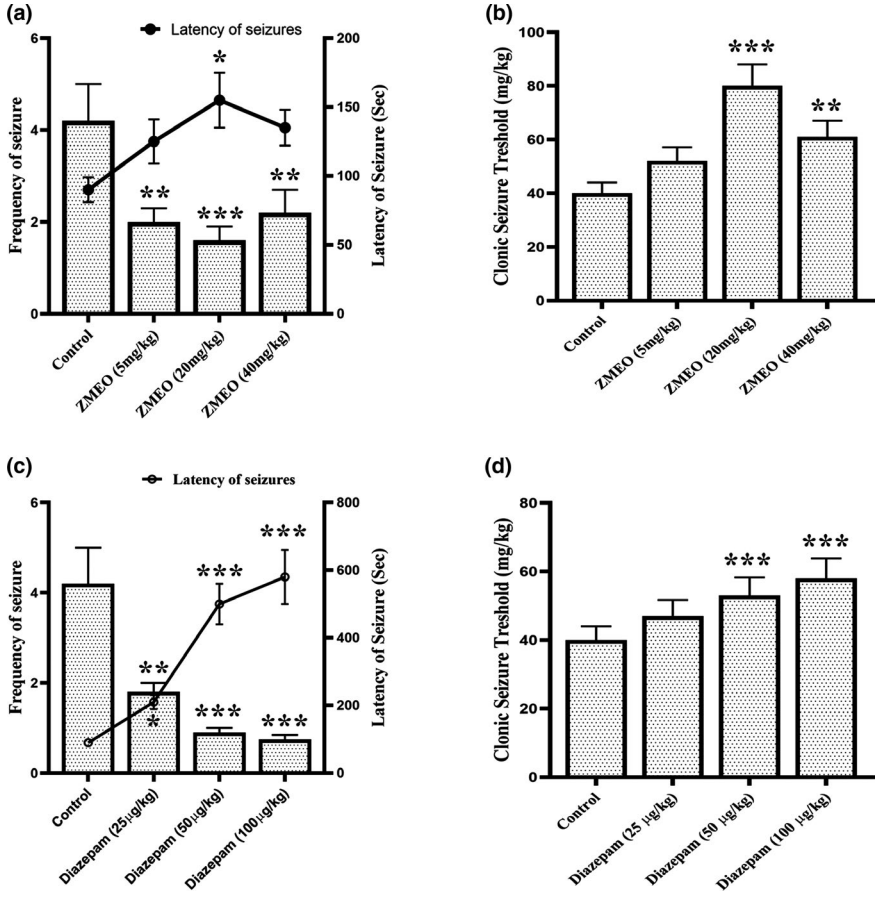


Figure 3 Anticonvulsant activity of ZMEO (5–40 mg/kg) and diazepam (25–100 µg/kg) on (**a,c**) frequency, (**a,c**) latency of convulsions in i.p. PTZ-induced seizure model, and (**b,d**) threshold of convulsions in i.v. PTZ-induced seizure model. Data represent mean ± SEM and each group comprised of eight mice. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 vs vehicle group (control). Analyses were performed using one-way ANOVA and Newman-Keuls *post hoc* test. ANOVA, analysis of variance; i.p., intraperitoneal; i.v., intravenous; ZMEO, *Zhumeria majdae* (Rech.).

protective effects by increasing the threshold of clonic seizures ($F_{(1, 42)}$ = 21.66, P < 0.0001; **Figure 5d**). Pretreatment of mice with either L-NAME (2.5 mg/kg i.p.) or aminoguanidine (100 mg/kg i.p.) significantly reduced anticonvulsant activities of ZMEO, as is evident from a decline in threshold of clonic convulsions in the i.v. PTZ-induced seizure model (L-NAME ($F_{(1, 42)}$ = 134.6, P < 0.0001); aminoguanidine ($F_{1,24}$ = 115, P < 0.0001); **Figure 5e,f**).

DISCUSSION

Based on results obtained from GC-MS analysis, linalool and camphor were the main components of ZMEO. Linalool is a tertiary alcohol belonging to acyclic monoterpenoid, which is also a common floral scent found in nature. Several studies have reported on the potential inhibitory effect of linalool on neural excitability in a variety of experimental mouse models of seizure. For instance, administration of linalool was found to delay onset of seizure in the NMDA model or protect against picrotoxin, quinolinic acid, pentylenetetrazol, and transcorneal electroshock-induced models of seizure.26 Proposed pharmacologic mechanisms for these anticonvulsant effects include dose-dependent antagonism of glutamate NMDA receptors, agonistic effect on $GABA_A$ receptors and inhibition of intracellular accumulation of cyclic adenosine monophosphate (cAMP),[27] modulation of nicotinic receptor–ion channel kinetics, alteration in calcium channel blocking, and an inhibitory effect on acetylcholine secretion.[28] Based on findings by De Sousa *et al.*,[25] single enantiomers of linalool demonstrate different potencies when compared with their racemic formulations. It has been shown that the (S)/(+) enantiomer is less effective compared with the (R)/(–) enantiomer and racemic formulation. The anticonvulsant activity of two latter forms of linalool has been shown to be comparable with activities seen with diazepam and phenytoin.[29]

The capability of different components of ZMEO in opening the $GABA_A$ receptor has been demonstrated in several reports utilizing the patch-clamp technique. Based on work by Kessler *et al.*, compared with GABA application alone, application of linalool (forming 63.4% of ZMEO) can increase mean current amplitude by two-fold.[30,31] In addition, although camphor (forming 27.4% of ZMEO) can mildly potentiate $GABA_A$ receptor–mediated currents, borneol (forming 0.9% of ZMEO) can effectively enhance it in patch-clamp studies.[32] As about 93% of

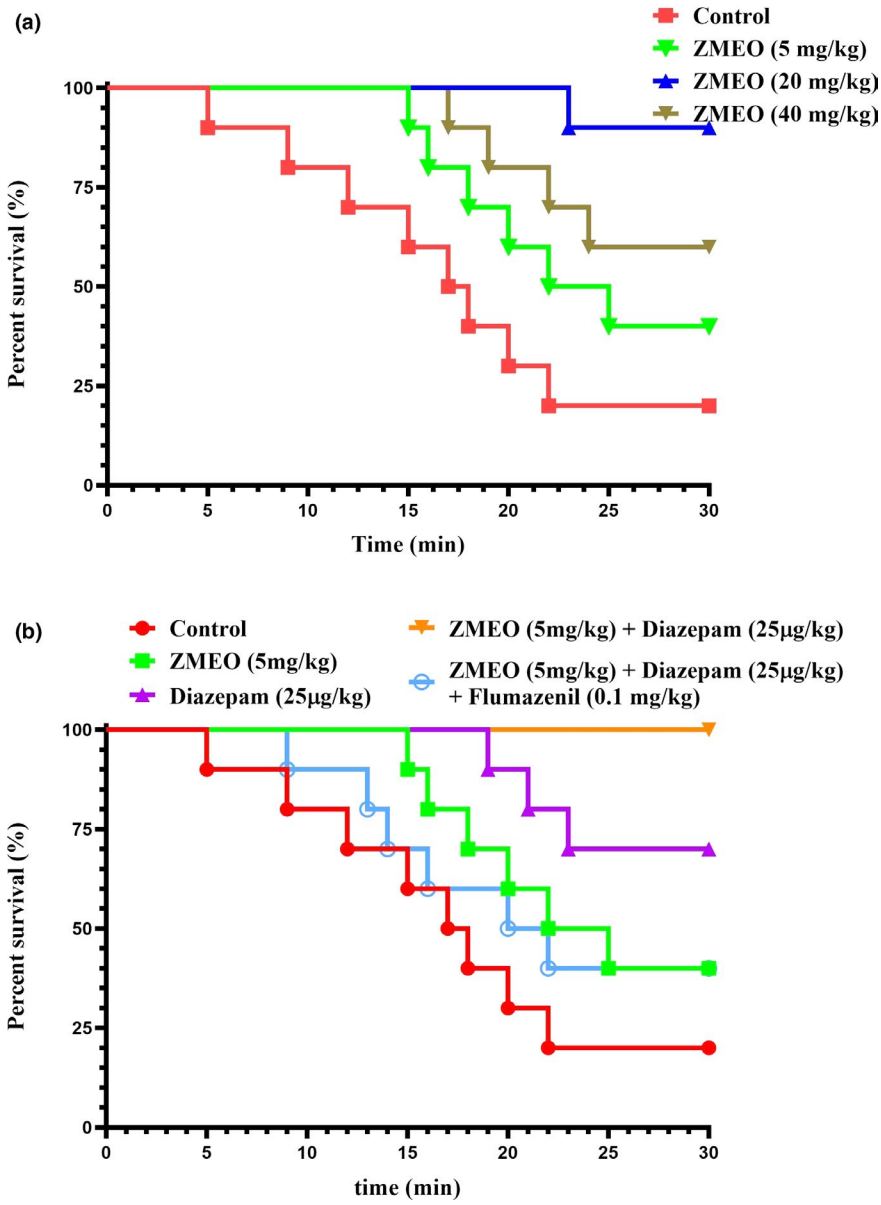


Figure 4 Kaplan-Meier analysis for estimation of overall survival of animals receiving (**a**) ZMEO (5, 20, and 40 mg/kg) and (**b**) diazepam (25 µg/kg), diazepam + ZMEO, and flumazenil (0.5 mg/kg) pretreated diazepam + ZMEO, in a i.p. PTZ-induced seizure model over a 30-minute observation period (n = 10). ZMEO, *Zhumeria majdae* (Rech.).

ZMEO's components are capable of potentiating $GABA_A$ receptor current in patch-clamp–based studies, it can be reliably predicted that overall EO formulation can also modulate $GABA_A$ receptors. Other components with a lower ZMEO formulation, including α-pinene, geranial, geraniol, caryophyllene, and limonene, are also capable of enhancing $GABA_A$ receptor current.[31] In parallel, in experiments other than epilepsy, modulatory effects of ZMEO components on voltage-dependent Na^+ channels have also been observed. For instance, the antinociceptive effect of linalool was shown to originate from a decrease in voltage-dependent Na^+ current in neurons located in the dorsal root ganglia, or terpinen-4-ol, which was shown to be capable of decreasing Na^+ current in electrophysiology studies.[33]

Currently, MES- and PTZ-induced models of seizure are the most widely utilized tests for preclinical examination of anticonvulsant activity in new investigational AEDs. In this context, MES is capable of identifying compounds with preventive effects against spread of seizure, whereas PTZ mostly identifies agents with the capacity to increase seizure threshold.[34,35]

With a few exceptions, an appropriately designed i.p. PTZ-induced seizure model was shown to be consistent for assessment of human myoclonic jerks and spike-wave seizures. With this test, the suppressive effects of an investigational AED on clonic seizure induced by a proconvulsive i.p. dose of PTZ can be monitored. Thus, the i.p. PTZ-induced model of seizure is capable of only evaluating the ability of a compound to block a specific end point in quantal mode. That is, it can only inform us as to whether or not

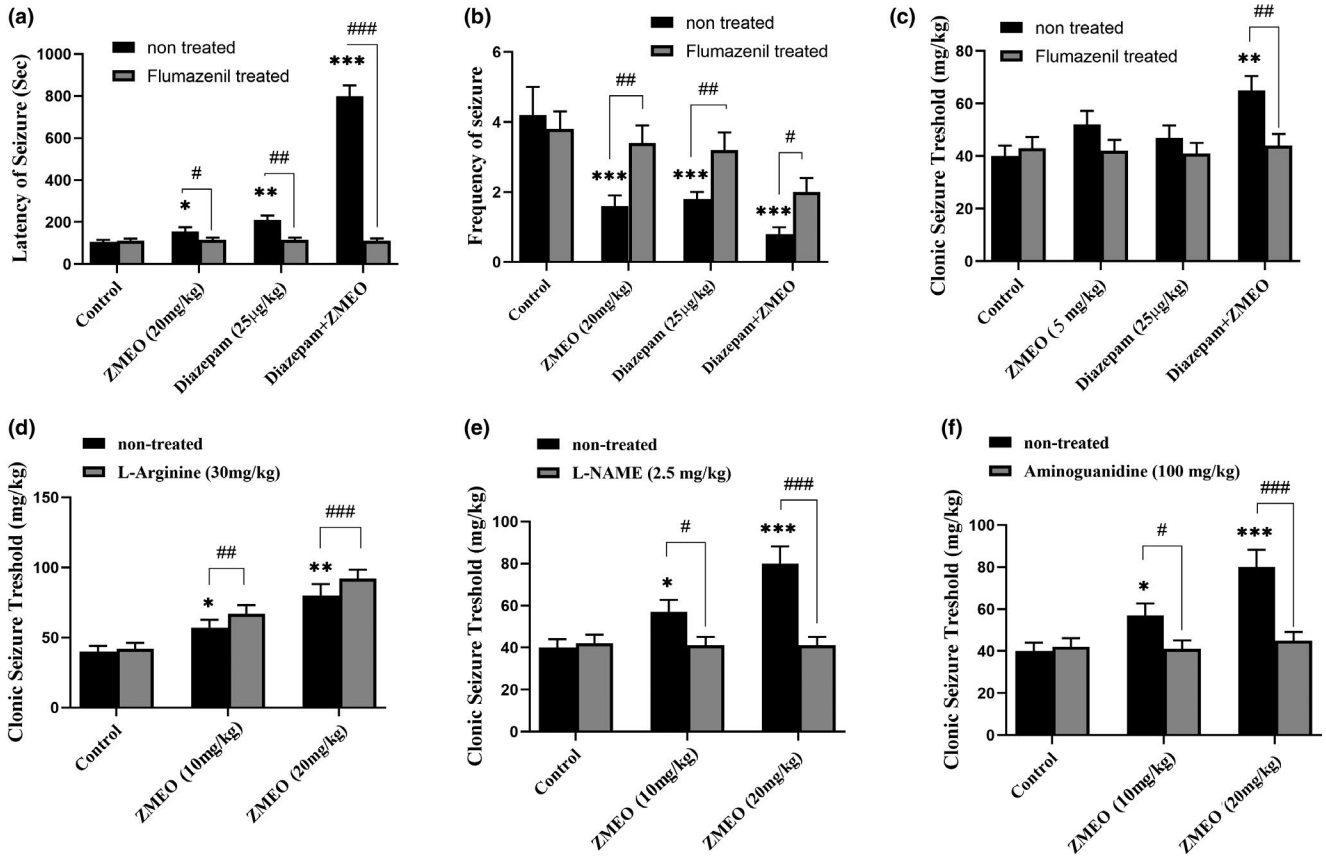


Figure 5 Effects of flumazenil pretreatment on (**a**) latency and (**b**) frequency of ZMEO (5 or 20 mg/kg) and diazepam (25 µg/kg) receiving mice in i.p. PTZ-induced seizure model, and (**c**) threshold of ZMEO (5 or 20 mg/kg), diazepam (25 µg/kg), and ZMEO + diazepam (5 mg/kg and 25 µg/kg) receiving mice in i.v. PTZ-induced seizure model. Each group consisted of eight mice and data are presented as mean ± SEM. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 vs vehicle group (control) (one-way ANOVA and Newman-Keuls *post hoc* testin used for frequency and threshold, and Kruskal-Wallis nonparametric test used for latency). #*P* < 0.05, ##*P* < 0.01, and ###*P* < 0.001 vs flumazenil-treated group (two-way ANOVA followed by Bonferroni's test). (**d**) Effects of pretreating mice with L-Arginine (30 mg/kg), (**e**) L-NAME (2.5 mg/kg), and (**f**) aminoguanidine (100 mg/kg) on the threshold of ZMEO (10 and 20 mg/kg) in i.v. PTZ-induced seizure model. L-Arginine, L-NAME, aminoguanidine, or vehicle (control) were administered 15 minutes before injection of ZMEO and 75 minutes before induction of seizure by i.v. infusion of PTZ. Each group consisted of eight mice and data represent mean ± SEM. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 vs vehicle group (control) (one-way ANOVA and Newman-Keuls *post hoc* testing). Significance of differences between groups were analyzed using two-way ANOVA and Bonferroni's *post hoc* test: #*P* < 0.05, ##*P* < 0.01, and ###*P* < 0.001. ANOVA, analysis of variance; i.p., intraperitoneal; i.v., intravenous; L-NAME, N^{ω}-nitro-L-arginine methyl ester; PTZ, pentylenetetrazol; ZMEO, *Zhumeria majdae* (Rech.).

a specific compound can prevent a seizure end point.[36,37] Although measurement of alterations in parameters, including latency to onset, severity, and duration of clonus seizure response, have been applied recently for quantifying responses in the i.p. model, high inter-animal variability in latency of seizure onset resulted in a significant decrease in precision of the results.[38] Finally, evaluation of an investigational AED's ability to improve survival may be effectively evaluated with this test.[39]

In contrast, the i.v. PTZ-induced model of seizure is widely applied to provide data other than those provided by the i.p. PTZ-induced seizure model. The i.v. model offers a highly sensitive parametric approach for measuring seizure threshold.[40] That is, instead of only identifying an agent with a comprehensive preventive effect on seizure occurrence, all agents capable of delaying its appearance can be identified. Thus, a quantifiable end point can be achieved by performing this test. Regarding the diversity of end points associated with each model, in the present study both i.p. and i.v. PTZ-induced models of seizure were used for acquiring a comprehensive data set for anticonvulsant activity of ZMEO. The i.p. model was applied mainly for evaluating improvements in survival rate, latency to onset, and severity and duration of clonus seizure response in ZMEO-pretreated mice and i.v. PTZ-induced mice for measuring seizure susceptibility in each individual animal.

Like other EOs, absorbed ZMEO in blood after IP administration will freely pass through the blood-brain barrier (BBB) to reach its site of action in the central nervous system (CNS). This happens mainly owing to its very small size (< 400 Da) and high lipid solubility in all components found in EOs.[41,42] The dose-response curve obtained in the i.v. PTZ-induced seizure model in the present study did not display the classic sigmoidal dose-response pattern. Instead, as depicted in **Figure 3a,b**, ZMEO treatment produced a

Table 2 Protective effects of ZMEO and phenytoin on MES-induced seizures in mice

Group	Dose (mg/kg)	Incidence of tonic extensions (%)	Death (%)
Control		100	33.3
ZMEO (mg/kg)	5	100	33.3
	20	100	50
	40	100	33.3
Phenytoin (mg/kg)	25	0	0

Data represent the percentage of mice (n = 10) producing THLE and mortality.
MES, maximal electroshock; ZMEO, *Zhumeria majdae* Rech.

biphasic dose-response curve, raising the seizure threshold up to a maximum of about twofold that of control at its maximum effective dose (20 mg/kg), while beginning to progressively lose its anticonvulsant activity and demonstrate reduced anticonvulsant activity as the dose increased from 20 to 40 mg/kg. A similar pattern was also observed for latency of seizure onset upon administration of increasing doses of ZMEO. Similarly, studies have shown that primidone, phenytoin, and carbamazepine also had U-shaped, nonlinear responses in PTZ seizure–induced models. Similar to ZMEO, anticonvulsant effects were reported at relatively low doses, while the effects were significantly decreased or completely vanished at higher doses.[38] This pattern of response, at least for phenytoin and carbamazepine, has been ascribed to their proconvulsive behavior at high concentrations, both in rodents and humans.[43-45]

Indeed, the proconvulsive activity observed with carbamazepine, phenytoin, and herein ZMEO may be attributed to the hyperexcitability of neuronal networks mediated by intensive activation of $GABA_A$ receptors at high doses of these agents. It has been shown that an intensive short-term $GABA_A$ receptor activation can switch primary hyperpolarizing responses (i.e., anticonvulsant effects) of GABA to depolarizing or excitatory responses in normal brain. During an intensive activation of $GABA_A$ receptor (herein administration of high doses of ZMEO), even in the absence of structure-based change of the chloride transporters, increased Cl^- influx will overcome its extrusion and result in accumulation of chloride and an increase in $[Cl^-]_i$, which in turn can induce a depolarizing shift in reversal potential of the $GABA_A$ receptor in postsynaptic cells. However, as the affinity and subtypes of $GABA_A$ receptors are regionally different in the CNS, similar doses of ZMEO may demonstrate protective effects in some sections. Thus, the overall effect may be only a reduction in ZMEO protective effects but not a complete reversal of the effect.[46]

In addition, in a very similar study, evaluating the effects of opioids on seizure threshold, Lauretti *et al.* demonstrated that morphine, fentanyl, and pethidine are capable of demonstrating such biphasic responses in four models of seizure, namely PTZ, kainic acid, *N*-methyl-D,L-aspartate (NMDLA), and bicuculline. Regardless of the individual pattern of each opiate in the different models studied (whether anti- or proconvulsive responses), coadministration of naloxone could reverse the opiate effect in the low dose range, strongly suggestive of the enrollment of μ-opioid receptors in the observed effects. In contrast, coadministration of naloxone in the high dose range demonstrated complex responses, with pethidine being completely insensitive in all studied models and fentanyl and morphine in the bicuculline-induced seizure model. The main outcome of this study on mechanistic insight into these biphasic responses is that other non-opiate receptors may also be involved.[47] Accordingly, Homayoun *et al.* demonstrated that anticonvulsive effects of morphine could be suppressed with administration of clonidine, an α_2-adrenoceptor agonist, or potentiated by yohimbine, an antagonist for this receptor.[48] In the case of ZMEO, consisting of 16 components, exploring the mechanism of this biphasic response may become somehow more complex. However, as stated earlier, one possible mechanism may be the activation or involvement of receptors other than $GABA_A$.

Flumazenil is a selective antagonist for the benzodiazepine site on $GABA_A$ receptors, which is mainly applied in the clinic for reversing benzodiazepine overdose.49-51 Flumazenil has been widely applied as a powerful tool for exploring benzodiazepine/$GABA_A$ receptor interaction in animals and humans, with the purpose of searching endogenous or exogenous ligands for this receptor.52 Based on our results, involvement of $GABA_A$ receptor was confirmed in three ways: (1) As depicted in **Figure 5c**, coadministration of diazepam and ZMEO, both in subeffective concentrations, induced protective effects in the PTZ model. Therefore, two components must have had an additive or synergistic effect together, which could have antagonized the proconvulsive effects of PTZ. Because, at the molecular level, a generally accepted mechanism of PTZ is noncompetitive antagonism of the $GABA_A$ receptor complex, a combination of the two drugs must have reversed the antagonizing effect of PTZ on $GABA_A$ receptor. As diazepam efficacy in this context was not high enough at the concentrations administered, the extra stimulatory effect on $GABA_A$ receptor must have been compensated by ZMEO.53 Furthermore, because ZMEO at high doses could increase the threshold of PTZ-induced seizure, it must have at least partially reversed the antagonizing activity of PTZ on $GABA_A$. (2) Administration of flumazenil completely antagonized both components effects. As the only known mechanism of flumazenil is ligation to $GABA_A$ receptor, ZMEO *must* have modulated the $GABA_A$ receptor activity. (3) Similar protective effects can also be observed by survival analysis, as shown in **Figure 4**.

Synthesized from its precursor L-arginine through the action of NOS enzyme, NO is a gaseous free radical that functions as a neurotransmitter-modulating agent and neural messenger in the CNS.[54] NO is considered an endogenously expressed anticonvulsant agent, because inhibition of its synthesis through administration of NOS inhibitors has been shown to occur together with exacerbation of seizures in experimentally induced rat models of seizure.[55,56] Consistently, administration of L-arginine at concentrations high enough to increase NO levels could reverse proconvulsive effects of NOS inhibitors. In addition, administration of L-arginine could prevent rodents against sound and picrotoxin proconvulsive effects.[57,58] Marangoz

et al.[59] demonstrated that administration of a NO donor, sodium nitroprusside, could reverse epileptiform discharges induced by intracortical administration of penicillin in rats. Administration of L-arginine at concentrations high enough to increase NO levels has been shown to indirectly increase GABA levels through inhibiting the function of GABA transaminase (GABA-T), a specific enzyme involved in metabolization of GABA.[60-62] In addition, NO is capable of increasing the secretion of GABA from different regions of brain, including cortex, striatum, and hippocampus.[63-65] In addition, increased NO and GABA levels through the action of L-arginine could protect rats from picrotoxin-induced seizure.[58] Thus, L-arginine could promote both secretion and synthesis of GABA in the CNS as a function of increased secretion of NO. Consistent with this finding, Paul *et al.* demonstrated an additive function for L-arginine upon coadministration with phenobarbital, a well-known GABA-potentiating agent.[66]

Based on the data obtained, it can be hypothesized that ZMEO pretreatment enhanced production of NO, and this upregulation in CNS levels of NO was enough to protect against the proconvulsive effects of gradually increasing concentrations of PTZ. This happens because synthesized NO increases GABA concentration through enhancing its secretion from different compartments of the CNS and decreasing its metabolism by inhibiting GABA-T. In view of this unique form of administration (i.v. infusion), the initial concentration of PTZ in the mouse body is very low and increases at a slow rate. Therefore, the amount of PTZ will not be high enough to promote secretion of an adequate volume of glutamate required for activating NMDARs and inducing production of proconvulsive concentrations of NO. Instead, an enhanced level of GABA at the site of action ($GABA_A$ receptor) compensates for the noncompetitive antagonizing effect of PTZ. This hypothesis is much strengthened considering the fact that administration of L-arginine, a precursor of NO, at concentrations not sufficient to significantly affect clonic seizure threshold, could significantly enhance the anticonvulsant effects of ZMEO at both submaximal and maximal concentrations. Furthermore, pretreating mice with both of the NOS inhibitors, as done in the present study with L-NAME and aminoguanidine, with concentrations not enough to significantly affect clonic seizure threshold, could completely reverse the anticonvulsant effects seen with administration of ZMEO.

In conclusion, based on the data obtained in the present study, the essential oil obtained from leaves of *Z. majdae* could demonstrate a biphasic anticonvulsant response in both i.v. and i.p. PTZ-induced seizure models and could effectively increase survival rate in i.p. PTZ-administered mice. ZMEO, however, was not effective in the maximal electroshock-induced model of seizure, but showed potential for antiepileptic activity against myoclonic/generalized absence epilepsy. This is very important as absent seizure is mostly refractory to currently existing antiepileptic drugs, with development or discovery of new lead compounds with much higher potency and efficacy being of utmost importance. Applying different pharmacologic agonists and antagonists of $GABA_A$ receptor, precursors of NO, and different inhibitors of NOS revealed that anticonvulsant activity of ZMEO is mediated in part through activation of the $GABA_A$ pathway and NO synthesis. Finally, understanding the underlying mechanism of ZMEO's anticonvulsant activity will help physicians in its rational application as a complementary component in combinational therapies for resistant epilepsies.

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