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Original Article

A Comparison of the Anxiolytic and Hypnotic Effects of *Origanum majorana* Essential Oil and Alprazolam in Male Mice

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Abstract

Background and Aim: Previous studies have reported several pharmacological properties, including anticonvulsant, analgesic, sedative, and antidepressant-like effects, for *Origanum majorana*. Likewise, the aim of the present study was to compare the anxiolytic and hypnotic properties of *Origanum majorana* essential oil (OMEO) and alprazolam in male mice.

Materials and Methods: In this experimental research, 60 male NMRI mice were divided into 10 groups of six mice. In both of the experiments, negative control groups received vehicle (10 ml/kg), positive control groups received alprazolam (0.5 mg/kg), and treatment groups received OMEO (10, 20 and 40 mg/kg). For assessing the anxiolytic effects, the elevated plus maze (EPM) test was used to record the number of entries and the time spent in the open and closed arms. Moreover, the onset and duration of the sleep were recorded by the use of ketamine-induced sleeping time test in order to evaluate the hypnotic effects. Finally, one-way ANOVA, and subsequently *post hoc* Tukey's test were utilized to carry out the statistical analysis.

Results: All doses of OMEO and alprazolam, compared to the control group, raised the number of entries and the time spent in the open arms and, conversely, decreased the number of entries and the time spent in the closed arms. Furthermore, similar to alprazolam, all doses of the essential oil reduced the onset of sleeping and raised the duration of sleep.

Conclusion: The results indicated that the OMEO could have anxiolytic and hypnotic effects. However, further studies are required to determine their exact mechanism of action.

Keywords: *Origanum majorana*, Anxiety, Hypnotic effect, Elevated plus maze, Mice

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Introduction

Anxiety disorders are one of the most common psychological problems that often lead to certain dysfunctions in the human body. This mental disorder has a negative impact on everyday activities, and

increases the risk of other disorders (1). On the other hand, insomnia is a common public health problem whose prevalence is estimated to be 10%-20% around the globe. (2).

Previous studies have indicated that several receptors, such as GABA (gamma-Aminobutyric acid), serotonin (5HT), catecholamines and sex hormones, could be influential in the pathophysiology of anxiety (3, 4). In similar studies, various neurotransmitters, such as noradrenaline (NA), histamine, acetylcholine (Ach), dopamine (DA), GABA and orexin A and B neuropeptides, play pivotal roles in the regulation of the sleep duration in the hypothalamus. (5).

Today, benzodiazepines (e.g. alprazolam) are one of the most important drugs that are used to treat anxiety and sleep disorders. These agents act as benzodiazepine receptor agonists, facilitate the entry or increase of GABA activity, and increase the entry of chloride ion into cells (6).

Alprazolam is one of the most commonly used drugs for the treatment of anxiety, insomnia, and panic disorders. Moreover, it also has muscle relaxant and anticonvulsant effects (7). Nevertheless, several side effects of benzodiazepines, including fatigue, drowsiness, dizziness, blurred vision, gradual decrease in the effects of the drug, and even the addiction and drug dependence have limited their clinical use (6). Hence, the use of drugs with few side effects, particularly alternative approaches, is warranted. Medicinal plants induce few side effects compared to synthetic drugs (8).

The *Origanum majorana* is a herb that reaches the height of 80 cm and is from the *Labiatae* family. One of the characteristics of the plant is its aromatic and strong smell. *Origanum majorana* grows in the Mediterranean region and northern Iran, particularly in Khorasan, Mazandaran, Gilan and Azarbaijan provinces. Previous studies have reported several pharmacological properties for *Origanum majorana*, including anti-Alzheimer, anti-cancer, antimicrobial, anti-inflammatory, antioxidant, antispasmodic, analgesic and hypnotic properties (9). Furthermore, we recently demonstrated the antidepressant-like activity and the effect of OMEO on morphine dependence in mice (10, 11). Apart from these findings, a study indicated the anxiolytic effects of *Origanum vulgare* L. (another species of Origanum) in animal models of anxiety (12).

Given this and due to the lack of studies on the anxiolytic and anxiolytic effects of this plant, the aim of the present research was to compare the anxiolytic and anxiolytic effects of *Origanum majorana* essential oil (OMEO) and alprazolam in male mice.

Materials and Methods

Animals

In this experimental study, adult male NMRI mice weighing 20-30 g (purchased from Pasteur Institute, Tehran, Iran) were used. Animals were kept in cages at 23 ± 2 °C and under 12:12 h light/dark cycle. During this time, water and commercial food (Razi Vaccine and Serum Research Institute) were provided to the animals *ad libitum*. All the experiments were conducted in the light phase. In the present study, all the ethical principles were in accordance with the guidelines of the Faculty of Pharmacy and Pharmaceutical Science, Islamic Azad University, Tehran Medical Sciences Branch (IR.IAU.PS.REC.1396.140) and National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978).

Preparation of OMEO and Chemicals

The OMEO was purchased and prepared from Dr. Soleimani Pharmaceutical Company (Batch no. 006, Gorgan, Iran). Moreover, alprazolam (Rouzdaru Pharmaceutical Company, Tehran, Iran) and ketamine hydrochloride (Aflasan, Netherlands) were used. All drugs and essential oils were injected intraperitoneally (i.p.) at a constant volume of 10 ml/kg.

Elevated Plus Maze (EPM) Test

Briefly, the box consisted of two open arms (25 × 5 × 30 cm), two closed arms (15 × 5 × 30 cm), and a central section (5 × 5 cm). The box was made of Plexiglas and was located 60 cm above the floor. The illuminance of the open arms was 420 Lx. In this test, every mouse was individually placed at the center of the box, and during 5 min the number of entries and the time spent in the arms were recorded by a counter (Hand Tally Counter, Japan) and a chronometer (Citizen, Japan) respectively (13).

Ketamine-Induced Sleeping Time Test

To measure the sedative and hypnotic affects, the ketamine-induced sleeping time test was used. Briefly,

the intervening time between the administrations of ketamine and the loss of the righting reflex was recorded as the beginning of sleep, whereas the interval from the loss to regaining of the righting reflex was considered as the duration of sleep. In this test, the onset and duration of sleeping (time) were recorded by a chronometer in min (14).

Animal Grouping and Experimental Design

This study, in which a total 60 male NMRI mice were used, was divided into two different experiments. In the first set of the experiment, 30 mice were divided into five groups of six mice as follows:

Group I: Negative control or vehicle (normal saline with 10% Tween 80; 10ml/kg).

Groups II: Positive control or alprazolam (0.5mg/kg).

Groups III-V: treatment group or different doses of OMEO (10, 20, and 40mg/kg)

Thirty 30 min after the i.p. administration of the agents, the EPM test was conducted.

In the second set of the experiment similar to EPM test, 30 mice were divided into five groups of six mice as follows:

Group I: Negative control or vehicle (normal saline with 10% Tween 80; 10ml/kg).

Groups II: Positive control or alprazolam (0.5mg/kg).

Groups III-V= treatment group or different doses of OMEO (10, 20, and 40mg/kg).

In this set of the experiment, 30 min after the i.p. administration of the agents, the animals received ketamine (100 mg/kg, i.p.). Immediately following the ketamine injections, the onset (time) and duration of sleep were recorded.

In the present study, all essential oil and drug doses, routes of administrations, and sample size were determined on the basis of the data presented in previous literature (10, 11, 15 and 16).

Data Analysis

The data were presented as mean ± standard deviation [S.D] (n=6) and were analyzed using the one-way analysis of variance (ANOVA) followed by *post hoc* Tukey's test. All the statistical analyses were carried out by the use of SPSS 23 software. The results were considered statistically significant at $P<0.05$.

Results and Discussion

Anxiolytic Effects of OMEO in EPM Test

Our findings demonstrated that all doses of OMEO (10, 20, and 40 mg/kg; P=0.078, P=0.019 and P=0.001, respectively) as well as alprazolam (0.5 mg/kg; P=0.003) compared to the control (vehicle) group significantly increased the number of entries in the open arms. Moreover, there were no significant differences between the OMEO groups and alprazolam group (Figure 1).

Fig. 2 illustrates that the all doses of OMEO increased the time spent in the open arms (P=0.001). Furthermore, the effects (time spent in the open arms) produced by the low dose (10 mg/kg) of the essential were significantly shorter than the alprazolam group (P=0.003).

As it has been indicated in Fig.3, all doses of OMEO (P=0.001) as well as alprazolam (P=0.001) decreased the number of entries in the closed arms. Moreover, all doses of OMEO (P=0.001) as well as alprazolam (P=0.001) reduced the time spent in the closed arms. Nevertheless, there were no significant changes between the OEMO group and alprazolam group (Figure 4).

Hypnotic Effects of OMEO in Ketamine-Induced Sleep Test

The results showed that the onset of the sleeping time of all doses of OMEO were significantly (P=0.001) shorter than the control (vehicle) group, whereas all doses of the essential oil had a longer onset of sleeping than the alprazolam group (P=0.001) (Figure 5).

As it has been exhibited in Fig. 6, the durations of ketamine-induced sleeping time of all doses of OMEO (P=0.023, P=0.000 and P=0.000, respectively) as well as alprazolam (P=0.000) were significantly higher than the control (vehicle) group ($P<0.05$), but all doses of the essential oil had shorter durations of sleep compared to the alprazolam group (P=0.001).

The results of this study indicated that all doses of the essential oil could significantly and dose-dependently increase the number of entries and time spent in the open arms in the EPM. Moreover, all doses of the essential oil could significantly and dose-dependently reduce the number of entries and time spent in the closed arms in the EPM. Hence, OMEO proved to have anxiolytic properties. Consistent with our

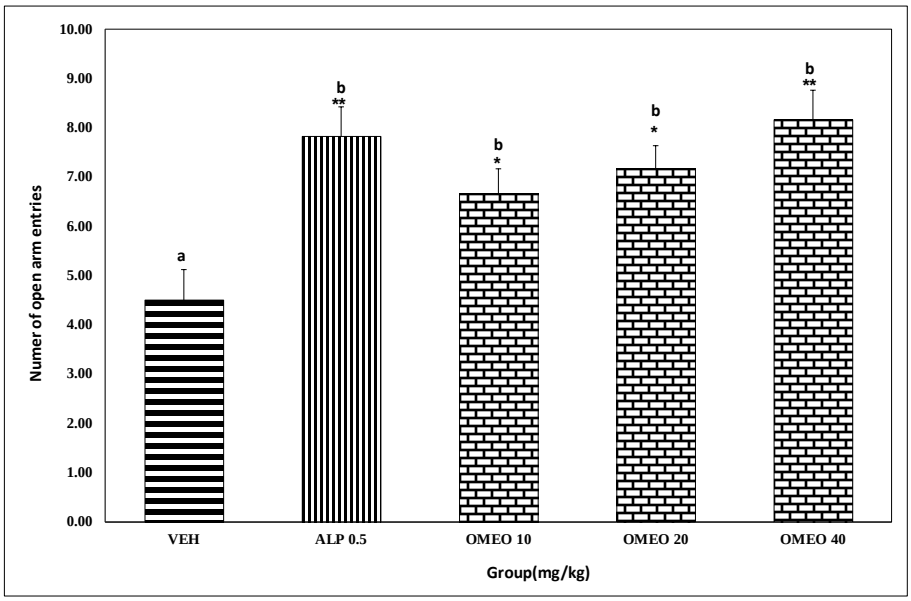

Figure 1. The effect of OMEO on the number of open arm entries in EPM test within 5 minutes. The results are expressed as mean ± S.D for six mice in each group. * and ** $P<0.05$ and $P<0.05$, compared to the control (vehicle) group, respectively. Different letters in each column indicate statistically significant difference between groups ($P<0.05$).
ALP: Alprazolam; OMEO: *Origanum majorana* essential oil; VEH: Vehicle.

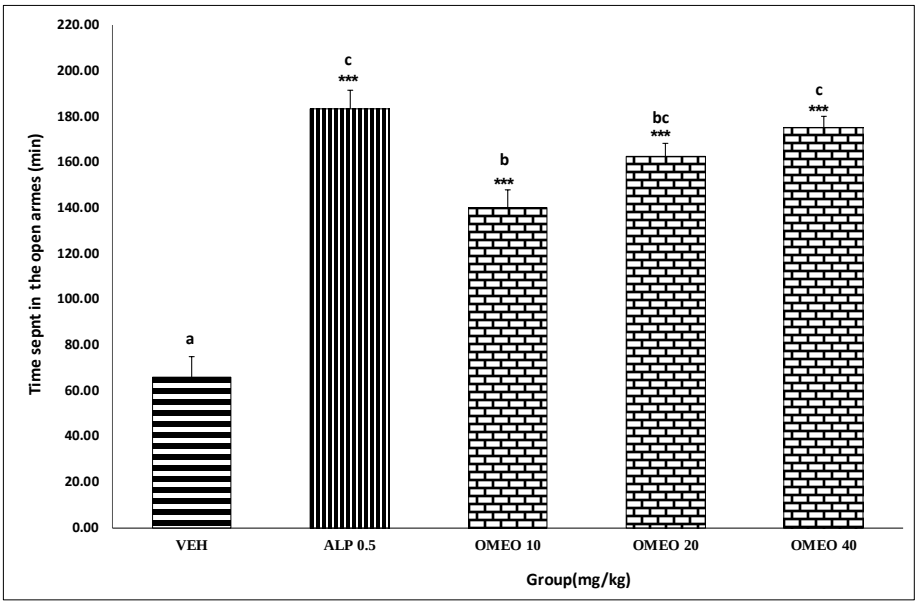

Figure 2. The effect of OMEO on the time spent in the open arms in EPM test within 5 minutes. The results are expressed as mean ± S.D for six mice in each group. *** $P<0.001$ compared to the control (vehicle) group. Different letters in each column indicate statistically significant difference between groups ($P<0.05$).
ALP: Alprazolam; OMEO: *Origanum majorana* essential oil; VEH: Vehicle.

findings, it has been reported that in the EPM test, the control group feel safer in the closed arms but tend to explore in the open arms (17). In addition, the exploration behavior also induces more movements of animals in the open arms of the EPM. On the contrary, animals refuse to remain in the open, illuminated, or elevated arms due to anxiety. Therefore, during anxiety, animals have the most entries into the closed arms or spend the most time in these arms in the EPM test (18). Furthermore, in line with our results, previous studies have demonstrated that the reference drug (alprazolam) could increase the number of entries and the time spent in the open arms, and reduce the number of entries and the time spent in the closed arms (19). Hence, benzodiazepines agents (such as Alprazolam), and GABAergic activity could

increase the time spent in the open arms in the EPM, while the time spent in the closed arm of the test decreased (20).

However, GABA is one of the most significant systems involved in the pathophysiology of anxiety and exertion of hypnotic effects, and even many medicinal plants exert anxiolytic and sedative effects through the GABAergic mechanism (21- 23).

In a study concerning the phytochemical investigation of OMEO by the use of gas chromatography–mass spectrometry (GC-MS), we identified that the 4-terpineol followed by other monoterpene alcohols such as γ-terpinene, trans-sabinene hydrate, α-terpinene and α-terpineol are the major volatile compounds of OMEO (11).

Monoterpenes are the most important components of

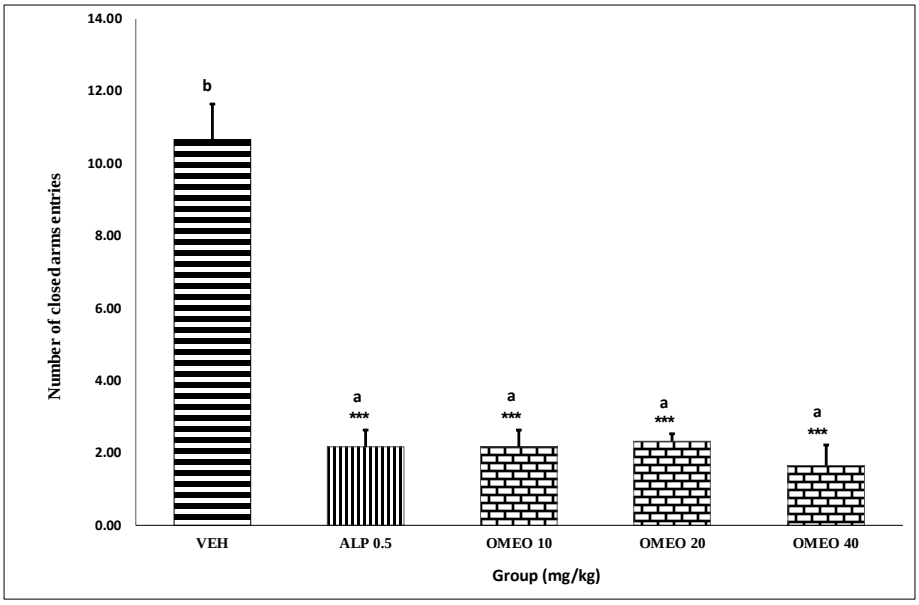

Figure 3. Effect of OMEO on the number of closed arm entries in EPM test within 5 minutes. The results are expressed as mean ± S.D for six mice in each group. *** P<0.001 compared to the control (vehicle) group. Different letters in each column indicate statistically significant difference between groups (P<0.05).
ALP: Alprazolam; OMEO: *Origanum majorana* essential oil; VEH: Vehicle.

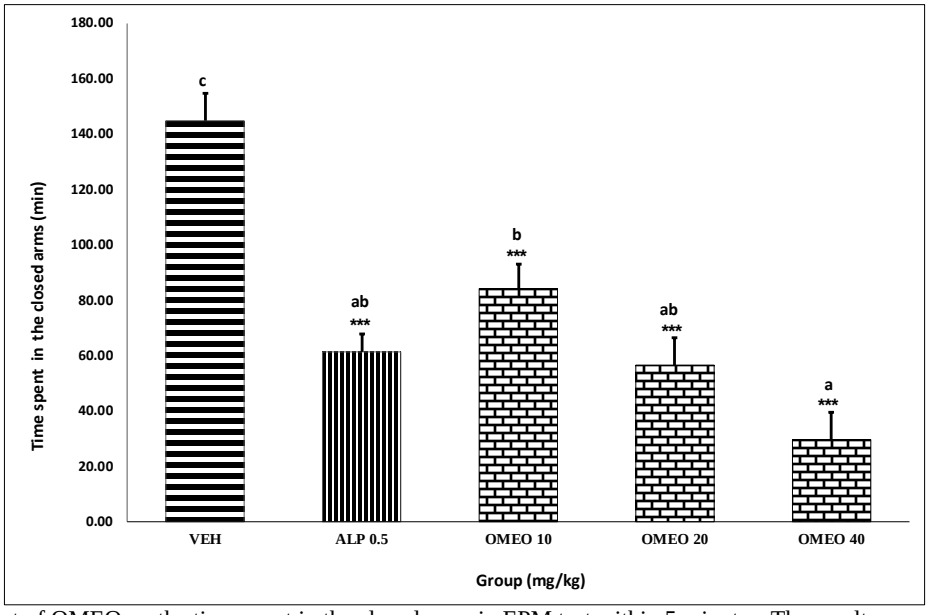

Figure 4. The effect of OMEO on the time spent in the closed arms in EPM test within 5 minutes. The results are expressed as mean ± S.D for six mice in each group. *** P<0.001 compared to the control (vehicle) group. Different letters in each column indicate statistically significant difference between groups (P<0.05).
ALP: Alprazolam; OMEO: *Origanum majorana* essential oil; VEH: Vehicle.

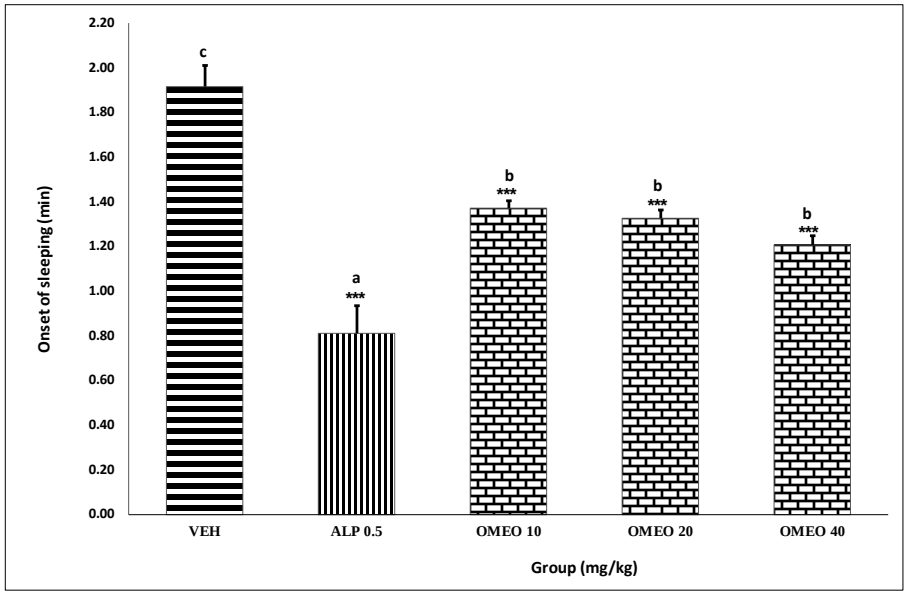

Figure 5. The effect of OMEO on the onset of sleeping-induced by ketamine. The results are expressed as mean ± S.D for six mice in each group; *** P<0.001 compared to the control (vehicle) group. Different letters in each column indicate statistically significant difference between groups (P<0.05).
ALP: Alprazolam; OMEO: *Origanum majorana* essential oil; VEH: Vehicle.

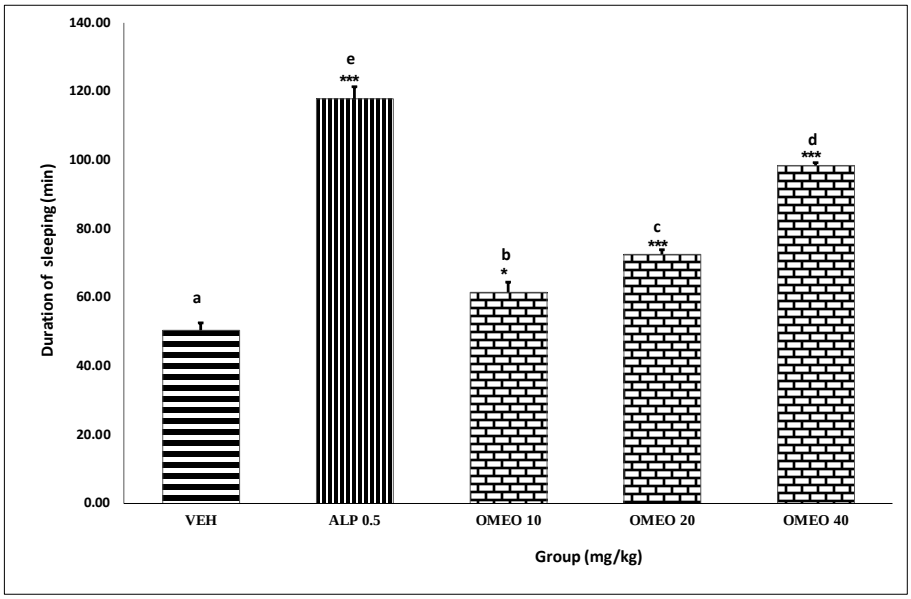

Figure 6. The effect of OMEO on the duration of sleeping-induced by ketamine. The results are expressed as mean ± S.D for six mice in each group; *** P<0.001 compared to the control (vehicle) group. Different letters in each column indicate statistically significant difference between groups (P<0.05).
ALP: Alprazolam; OMEO: *Origanum majorana* essential oil; VEH: Vehicle.

plant essential oils, so that many of the therapeutic and pharmacological properties of the essential oils are attributed to their monoterpenes (24). Moreover, in line with our findings, various studies have indicated that monoterpenes have anticonvulsant effects that could be mediated partly by the GABAergic system (25, 26). In a research, Yousefi *et al*. exhibited that α-pinene is the major component of *Myrtus communis* essential oil (27). In another study, consistent with our findings, α-pinene, present in pine tree oil, was observed to increase the quiescent sleep, namely, non-rapid eye movement (NREM), through the mechanism of benzodiazepine $GABA_A$ receptors. Confirming this view, the α-pinene hypnotic

effects have been reported to be completely blocked by flumazenil (a benzodiazepine $GABA_A$ receptor antagonist) (28). In addition to these findings, and consistent with the results of our study, Satou *et al*. (2004) have also reported the anxiolytic properties of α-pinene (29).

Borneol, a bicyclic monoterpene that exists in many essential oils, is used as an analgesic and anesthetic agent in Chinese traditional medicine. This compound also exerts its effects through the mediation of $GABA_A$ receptors (30). It has also been reported that other monoterpenes, such as verbenol and pinocarveol, are capable of enhancing the effect of GABA on $GABA_A$ receptors (31). Consistent with our findings, it has been indicated that apigenin and limonene, two monocyclic monoterpenes, could penetrate the blood-brain barrier (BBB) and, by binding to GABA receptors, produce anxiolytic effects (32). All of these findings indicate that anxiolytic and hypnotic effects of monoterpenes are partly mediated by the GABAergic system.

Apart from the potential role of the GABAergic system in the anxiolytic and hypnotic effects of OMEO, various studies have revealed that the serotonergic system also plays a pivotal role in the anxiolytic effects of different drugs or medicinal plants. For example, in line with this view, the partial agonist of $5HT1_A$ receptors such as buspirone and ipsaperone are used in the treatment of generalized anxiety disorder (GAD). Buspirone has been reported to be more efficacious than placebo, and its ability to control anxiety is equal to those of diazepam and other benzodiazepines (33). To confirm this issue, we could refer to the fact that $5HT_{1A}$ receptor knockout mice showed anxiety associated with depression in behavioral models (34). Furthermore, $5HT_2$ receptors are also highly influential in the pathophysiology of anxiety. In agreement with this, the $5HT_2$ receptor agonist, or meta-Chlorophenylpiperazine (mCPP), has been reported to exhibit anxiogenic-like effects (35). Studies have also shown that $5HT_3$ receptor antagonists produce anxiolytic effects (36).

Irrespective of the impact of serotonergic system on anxiolytic effects, certain studies have reported that the serotonergic system also plays a key role in the controlling of sleep. In this regard, the activity of these neurons decreases during rapid eye movement (REM) sleep. In other words, blocking $5HT_2$ receptors reduce REM and sleep duration (37). In this regard, our recent study demonstrated that the OMEO effects could be partly attributed to the serotonergic system. On the other hand, we indicated that the antidepressant-like effect of the OMEO could be exerted partly through the mediation of the serotonergic system. Hence, the monoterpenes in this essential oil (e.g., 4-terpineol) are likely to exert their antidepressant effect partly through mediation of the $5HT_{1A}$ receptors. In line with our finding, antagonizing the effect of this receptor with WAY 100135 (a selective $5HT_{1A}$ receptor antagonist) has been reported to block the antidepressant-like effect of the essential oil. Moreover, our recent research attributed the antidepressant-like effects of peppermint extract partly to the presence of monoterpenes, such as menthol. On the other hand, with the antidepressant effects of OMEO, the antidepressant effects of peppermint are blocked by the drug WAY-100135 (38). In a similar study, Komy *et al*. observed that volatile lemon essential oil could induce anxiolytic and antidepressant-like effects in mice. They attributed this effect mainly to the limonene, which is a monoterpene in the herb, and argued that the essential oil exerts its sedative and anxiolytic effects through mediation of $5HT_{1A}$ receptor (39).However, due to the role of $5HT_2$ receptors in sleep pathophysiology, previous studies have attributed the antidepressant-like effects of monoterpenes partly to their effects on $5HT_{2A/C}$ receptors, confirmed by blocking of the antidepressant-like effects of OMEO and the crude extract of peppermint by ketanserin ($5HT_{2A/C}$ receptor antagonist) (12, 37).Therefore, these receptors could also be involved in the essential oil's sedative effects. Nevertheless, the strengths of this study include the determination of the anxiolytic and hypnotic effects of OMEO for the first time, and its limitations include lack of determining the exact mechanism of the essential oil.

Conclusion

The results of the present study indicated the anxiolytic and hypnotic effects of OMEO. Nonetheless, further studies are required to determine their exact mechanism of action.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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