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Original Article

Chemical composition of *Zhumeria majdae* essential oil and its effects on the expression of morphine withdrawal syndrome and tolerance to the anticonvulsant effect of morphine on pentylenetetrazole-induced seizures in mice

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(With 7 figures)

Abstract

Regarding the proven anticonvulsant effect of *Zhumeria majdae* essential oil (ZMEO) in previous studies we were prompted to investigate the ZMEO effects on the tolerance to the anticonvulsant effects of morphine and the morphine withdrawal syndrome. Tolerance to the morphine anticonvulsant effect was induced in mice by subcutaneous injection of 2.5 mg/kg of morphine for 4 days. Subsequent doses of ZMEO (20 mg/kg) were used to study the expression and development of morphine tolerance. Clonidine was used as the standard drug to inhibit the morphine withdrawal syndrome symptoms. To study the ZMEO effect on withdrawal syndrome, mice received appropriate morphine values for 4 days and on the fifth day, 60 min before administration of naloxone. The effective dose of ZMEO was determined and the number of jumps, stands and changes in the dry stool weight, as symptoms of withdrawal syndrome were evaluated. The dose of 20 mg/kg of ZMEO decreased the tolerance in development and expression groups significantly. Counting the number of jumping, standing and defecation were assessed 30 min after morphine and 1 h after the vehicle and clonidine. The dose of 40 mg/kg ZMEO decreased all the signs of withdrawal syndrome significantly. ZMEO was analyzed by GC/MS and linalool (53.1%) and camphor (23.8%) were characterized as the main components. The results suggest that ZMEO possesses constituent(s) that have activity against tolerance to the anticonvulsant effects of morphine and the morphine withdrawal symptoms.

Keywords: *Zhumeria majdae,* volatile oil, opioid withdrawal symptoms, antiseizure.

Composição química do óleo essencial de *Zhumeria majdae* e seus efeitos na expressão da síndrome de abstinência de morfina e tolerância ao efeito anticonvulsivante da morfina em crises induzidas por pentilenotetrazol em camundongos

Resumo

Em relação ao efeito anticonvulsivante comprovado do óleo essencial de *Zhumeria majdae* (ZMEO) em estudos anteriores, fomos instigados a investigar os efeitos do ZMEO em relação à tolerância aos efeitos anticonvulsivantes da morfina e da síndrome de abstinência de morfina. A tolerância ao efeito anticonvulsivante da morfina foi induzida em camundongos por injeção subcutânea de 2,5 mg/kg de morfina por 4 dias. Doses subsequentes de ZMEO (20 mg/kg) foram utilizadas para estudar a expressão e o desenvolvimento da tolerância à morfina. A clonidina foi usada como droga padrão para inibir os sintomas da síndrome de abstinência da morfina. Para estudar o efeito do ZMEO na síndrome de abstinência, os camundongos receberam valores apropriados de morfina por 4 dias e, no 5º dia, 60 minutos antes da administração de naloxona. A dose efetiva de ZMEO foi determinada, e o número de saltos e de permanência e as alterações no peso das fezes secas, conforme os sintomas da síndrome de abstinência, foram avaliados. A dose de 20 mg/kg de ZMEO diminuiu significativamente a tolerância nos grupos de desenvolvimento e expressão. A contagem do número de saltos, permanência e defecação foi avaliada 30 minutos após a morfina e 60 minutos após o veículo e a clonidina. A dose de 40 mg/kg de ZMEO diminuiu significativamente todos os sinais da síndrome de abstinência. O ZMEO foi analisado por GC/MS, e linalol (53,1%) e cânfora (23,8%) foram caracterizados como os principais componentes. Os resultados sugerem que o ZMEO apresenta constituintes que possuem atividade contra a tolerância aos efeitos anticonvulsivantes da morfina e aos sintomas de abstinência da morfina.

Palavras-chave: *Zhumeria majdae*, óleo volátil, sintoma de abstinência de opioide, anticonvulsão.

1. Introduction

Zumeria majdae Rech. f. & Wendelbo (Lamiaceae) is an endemic plant to the south of Iran and only grows wild just in the heights of Genow Mountains in Northern Bandar Abbas (Rechinger, 1989). The analgesic, anti-inflammatory, antibacterial, anti-leishmania, anti-malaria and anti-seizure effects of this plant have been previously proven (Peana et al., 2003; Linck et al., 2009; Elisabetsky et al., 1999; Moein et al., 2008; Hosseinzadeh et al., 2012).

The aerial part of this plant contains essential oil which the major component characterized as linalool (55-65%). Many studies have shown the effective role of linalool in reducing morphine tolerance and dependence (Hosseinzadeh et al., 2012; Peana et al., 2006; Haghparast et al., 2008). Linalool is an alcohol monoterpenoid that several studies have confirmed its anticonvulsant effects (Elisabetsky et al., 1999) which is comparable to drugs such as phenytoin and diazepam. Linalool seems to be able to inhibit the excitability of neurons. Studies have shown the linalool inhibitory effects on the adenylate cyclase enzyme and intracellular mechanisms in inhibiting action on the central nervous system. Some early studies have suggested the presence of direct agonist effects with GABA-A (Gamma Amino Butyric Acid-A) receptors (Aprotosoaie et al., 2014). In 2008, it was shown that linalool affects the morphine tolerance and dependence by suppressing production of NO (Nitric Oxide) (Haghparast et al., 2008). Linalool also regulates a wide range of neurotransmitter and conductive systems such as opioid, dopaminergic, Muscarinic and adenosine receptors as well as KATP canals (K Adenosine Tri Phosphate channel) (Hosseinzadeh et al., 2012).

Regarding the presence of Linalool with high content in ZMEO, we were prompted to evaluate the probabilistic role of it on the tolerance to the anticonvulsant effects of morphine in Pentylenetetrazole (PTZ)-induced seizures Also, due to the possible interaction of this plant with opioid receptors, its effect on morphine withdrawal syndrome is investigated.

2. Materials and Methods

2.1. Drugs, chemicals and plant material

TBEO, Anesthetic ether and Morphine sulfate were purchased from PubChem CID: 3283 and Temad Pharmaceutical Co. (Iran), respectively.

Powdered morphine was dissolved in saline. The solutions were prepared immediately before use and injected intraperitoneally in a volume of 10 mL/kg.

Leaves of *Z. majdae* were collected in March 2015 from the Genow protected area, Bandar Abbas, Hormozgan Province, south of Iran. The leaves were identified by R. Asadpour. A voucher specimen has been deposited at the herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Tehran Medical Sciences, Islamic Azad University, Tehran, Iran, under code number 1091-AUPF.

The leaves were freshly hydro-distillated in a Clevenger tool for 2 h. Afterward the essential oil was extracted, dried using anhydrous Na2SO4, and stored in a set of glass vials at -18°C for further analyses.

2.2. Analysis of ZMEO

Analysis of ZMEO was performed on a HP-6890 gas chromatography (GC) equipped with a FID and a DB-5 capillary column, 30 m × 0.25 mm, 0.25 µm film thickness, temperature programmed as follows: 60 °−240 °C at 4 °C/min. The carrier gas N_2 was at a flow rate of 2.0 mL/min; the temperatures of injector port and detector were 250 and 300 °C, respectively. Sample was injected by splitting and the split ratio was 1:10. The analysis of GC/MS was performed on a Hewlett-Packard 6890/5972 system with a DB-5 capillary column (30 m × 0.25 mm; 0.25 µm film thickness. The operating conditions were the same as described above but the carrier gas was He. Mass spectra were taken at 70 eV. The range of scan mass was from 40-400 m/z at a sampling rate of 1.0 scan/s. Quantitative data were obtained from the electronic integration of the FID peak areas. The components of ZMEO were identified by their retention time, retention indices, relative to C_9-C_{28} n-alkanes, computer matching with the WILEY275.L library and as well as by comparison of their mass spectra with those of authentic samples or with data already available in the literature (Adams, 1995; Swigar and Silverstein, 1981). The percentage composition of the identified compounds was computed from the GC peaks areas without any correction factors and was calculated relatively.

2.3. Experimental animals

In this study, experiments were performed on adult male Swiss Albino mice (20-25 g). They were prepared by the animal house of Tehran University of Medical Sciences. The normal diet was maintained in the animal house at a temperature of 21 ± 1°C and light/dark cycle of 12/12 hours. Groups of eight mice and five rats were chosen randomly for the studies. All procedures were carried out in accordance with the Law 11,794, of October 8, 2008 by the National Council of Animal Experimentation Control (CONCEA). Ethical approval was granted from the Islamic Azad University Research and Ethics Committee (Protocol # 067/16). In all tests on animals, they were euthanized by cervical dislocation after completing the experiments.

2.4. Seizure induction by PTZ

PTZ (0.5%) was prepared in normal saline and injected into the animals at the speed of 1 mL/min, through one of four mice veins. To evaluate the effect of different doses of ZMEO on the clonic seizure threshold, 5 groups of 8 mice were used. In the first group (control), sweet almond oil (10 mL/kg as the vehicle) was injected. In the second to fifth groups the doses of 20, 40, 80, and 160 mg/kg of ZMEO were injected respectively. To evaluate the effect of the administration of different doses of morphine on the clonic seizure threshold, 5 groups including the control group and 4 groups were administered with different doses of morphine (1, 2.5, 5, and 10 mg/kg, respectively) (Roshanpour et al., 2009).

In order to evaluate the effect of chronic administration of morphine and tolerance to its anticonvulsant effects, a group of morphine (2.5 mg/kg, subcutaneous), twice daily for four days and the fifth day, before the PTZ test Injected. PTZ test

was performed in all groups 45 min after the last injection. To evaluate the effect of essential oil on expression of this tolerance, on the day of the test, an essential oil (20 mg/kg) was used prior to administration of PTZ. In order to evaluate the effect of essential oil on development, essential oil (20 mg/kg) and morphine (2.5 mg/kg) was administered for 4 days and performed on day 5 of the test.

The interval between the start of PTZ injection and the first clonic seizure (foot cycling) was obtained for each mice. Finally the clonic seizure threshold was calculated for each mice.

2.5. Morphine withdrawal syndrome induction

Mice were assigned to the test room for 24 h prior to the start of the experiment, and the morphine was injected the next day. The mice received three daily doses of morphine (50, 50, and 75 mg/kg) subcutaneously received at 8:00 am, 12:00 am and 4:00 pm, respectively (the third dose was increased due to the prevention of withdrawal syndrome during night). This was done for 4 days. On fifth day, the control group received clonidine (10 mg/kg, ip).

In sweet almond oil group, we injected 0.1 mL of almond oil (i.p) per 10 grams of mice. One group received the essential oil (20 mg/kg, i.p). Another group receiving the essential oil (40 mg/kg, i.p).after 30 min, all groups received morphine 50 mg/kg and received naloxone 30min afterwards and immediately inserted into the glass cylinder in order to observe the symptoms. In all groups, the test was performed immediately after injection of naloxone (Hosseinzadeh et al., 2012). During the test, for 30 min, the number of rearing, jumping, standing and dry stool weight was examined. For more accurate recording, each group was tested using the camera.

2.6. Statistical analysis

In PTZ administration, all data are expressed as Mean ± S.E.M One-way ANOVA was used for statistical analysis of data and Tukey-Kramer test for comparison between groups. In all analyzes, the values of $P<0.05$ showed a significant difference between the groups in each test. Graph pad prism 6 was used for this purpose.

3. Results

3.1. Effect of different doses of ZMEO on the clonic seizure threshold induced by PTZ

The effect of doses of 20, 40, 80, and 160 mg/kg of the essential oil compared to the control group showed that the doses of 40, 80 and 160 mg/kg compared to the control group (sweet almond oil) significantly increased the clonic seizure threshold ($P<0.001$) (Figure 1).

Effect of different doses of morphine on the clonic seizure threshold induced by PTZ.Acute administration of 1, 2.5, 5, and 10 mg/kg doses of morphine compared to the control group showed that just the doses of 2.5 and 5 mg/kg significantly increased the clonic seizure threshold ($P<0.05$) (Figure 2).

3.2. Effect of chronic morphine administration on the clonic seizure threshold Induced by PTZ

Chronic administration of morphine significantly reduces the clonic seizure threshold induced by PTZ ($P<0.001$) (Figure 3).

3.3. Effect of ineffective dosage of ZMEO on the expression and development tolerance to the morphine

ZMEO administration in both groups of expression and Development significantly increased the clonic seizure threshold Induced by PTZ ($P<0.01$) (Figure4).

3.4. Effect of different dosages of ZMEO on morphine withdrawal syndrome

Doses of 20 and 40 mg/kg of essential oil on the reduction of the number of jumps caused by the withdrawal syndrome are significant. ($P<0.05$) (Figure 5), while the only dose of 40 mg/kg of the essential oil was significantly decreased the excretion of dry stool from the withdrawal syndrome. ($P<0.001$) (Figure 6). This dose also significantly reduces the number of standing due to withdrawal syndrome. ($P<0.01$) (Figure 7).

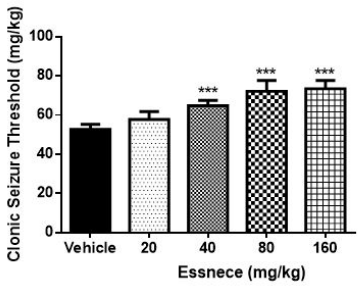

Figure 1. Effect of different doses of essential oil on clonic seizure threshold induced by PTZ compared to control group ($P<0.001$) (***).

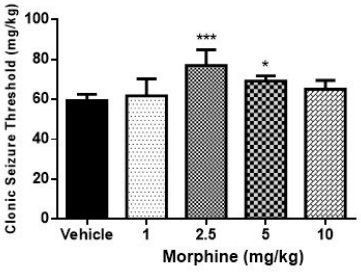

Figure 2. The effect of acute administration of different doses of morphine on clonic seizure threshold induced by PTZ in comparison with the control group (*** $P<0.001$), (* $P<0.05$).

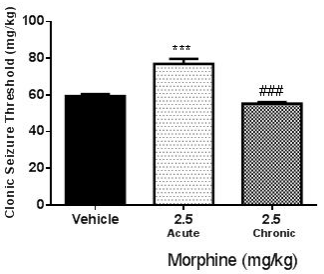

Figure 3. The effect of chronic morphine administration on clonic seizure threshold induced by PTZ. Compared to the control group (*** $P<0.001$) and in comparison with the morphine administration group (###$p<0.001$).

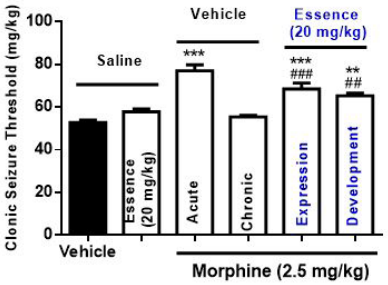

Figure 4. Effect of administration of an ineffective dose of essential oil (20 mg / kg) with administration of an effective dose of morphine (2.5 mg / kg) in chronic form in two groups: Expression and Development. Compared with the control group (** P <0.01), (***P <0.001) and compared with the morphine administration group (##p <0.01), (###P <0.001)

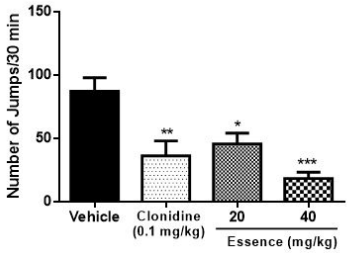

Figure 5. Effect of doses of essential oil on the number of jumps caused by withdrawal syndrome compared with the control group (*P <0.05), (**P <0.01), (***P <0.001)

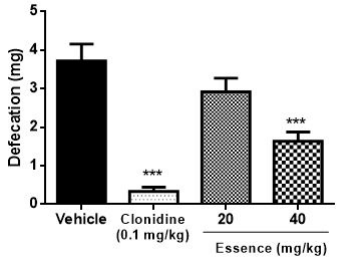

Figure 6. Effect of doses of essential oil on defecation weight from the withdrawal syndrome compared with the control group (*** P <0.001).

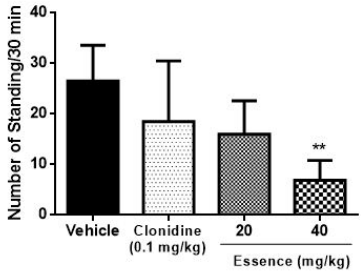

Figure 7. Effect of doses of essential oil on the number of standing from the withdrawal syndrome in comparison with the control group (**P <0.01).

Table 1. GC-MS analysis of *Z. majdae* essential oil.

Compound[a]	KI[b]	KI[c]	Percentage
1. α-Pinene	941	939	0.7
2. Camphene	958	954	2.2
3. 3-Octanone	987	984	0.6
4. Myrcene	995	991	0.3
5. ρ-Cymene	1028	1026	0.6
6. Limonene	1030	1031	3.3
7. γ-Terpinene	1064	1060	1.2
8. *trans*-Sabinene hydrate	1102	1098	0.2
9. Linalool oxide	1083	1087	0.3
10. α-Terpinolene	1091	1089	0.6
11. Linalool	1101	1098	53.1
12. Camphor	1151	1146	23.8
13. Borneol	1173	1169	1.8
14. Terpinene-4-ol	1182	1177	0.2
15. α-Terpineol	1187	1189	0.8
16. Neral	1240	1238	0.6
17. Geraniol	1255	1253	2.7
18. Geranial	1271	1267	0.9
19. *cis*-Jasmone	1398	1393	0.2
20. Caryophyllene	1422	1418	0.2
21. Caryophyllene oxide	1578	1583	0.4
22. β-Eudesmol	1648	1651	0.2
Total			94.9

[a]Compounds listed in order of elution; [b]KI (Kovats index) measured relative to *n*-alkanes (C_9-C_{28}) on the non-polar DB-5 column under condition listed in the Materials and Methods section; [c]KI (Kovats index) from literature.

The hydrodistillation of Z. majdae leaves gave colorless oil with pleasant odor and yield of 12.3% (v/w) based on the fresh weight. As shown in Table 1, twenty two components were identified in this oil which presented about 94.9% of the total chromatographical material. The major constituents of the oil were linalool (53.1%) and camphor (23.8%).

4. Discussion

Several studies have shown that opioid receptor agonists such as morphine with dual and dose-dependent behavior can have anticonvulsant or proconvulsant effects (Shafaroodi et al., 2007; Zhang & Ko, 2009). Generally, opioids at lower doses (0.5-5 mg/kg) induce anticonvulsant effects, while at higher doses they have proconvulsant effects (Homayoun et al., 2002).

In this study, the effects of acute administration of different doses of morphine (1, 2.5, 5, and 10 mg/kg) on the clonic seizure threshold were investigated, according to doses of 2.5 and 5 mg/kg increased the seizure threshold significantly. After repeated administration of morphine,

tolerance occurred in some effects of morphine, such as its analgesic effects. Among the mechanisms involved in the phenomenon of tolerance and dependence on opioid compounds, neurotransmitter systems such as nitric oxide (Ozdemir et al., 2011), glutamate (Capone et al., 2008), dopamine (Zarrindast et al., 2002) and stimulatory amino acid receptors, especially the receptor NMDA (Noda and Nabeshima, 2004) have a significant role. In this study, using 2.5 mg/kg as a chronic dose, results tolerance to the anticonvulsant effects of morphine.

4.1. Effects of different doses of ZMEO on seizure activity

Essential oil bearing plants are used to treat a wide range of diseases, including cardiovascular disease, cancers, diabetes, and many neurological diseases, such as epilepsy and Alzheimer's, due to essential oils and other chemical compounds. The therapeutic use of aromatic herbs is mainly due to the extensive pharmacological effects of the essential oils of these native plants. Many of these aromatic herbs can only affect the central nervous system. Of these, the group has anticonvulsant effects and is considered in the treatment of epilepsy (Peana et al., 2003).

Studies have shown the anticonvulsant effects of the extract and essential oil of *Z. majdae* by the doses higher than 250 mg/kg (Mandegary et al., 2012).

In this study, the effects of acute administration of different doses of essential oil (20, 40, 80, and 160 mg/kg) on the clonic seizure threshold were investigated, according to which doses of 40, 80, and 160 mg/kg increased the seizure threshold significantly.

Z. majdae is one of the plants belonging to the Lamiaceae family, whose dispersion is limited to certain areas of Hormozgan province and has been considered as a treatment for epilepsy and seizure since ancient times. The aerial parts of this plant are capable of producing high levels of essential oils, which are contain linalool and camphor, the main components have different biological effects (Naghibi et al., 2010).

In this study, co-treatment of 20 mg/kg of essential oil and 2.5 mg/kg of morphine, in both Expression and Development groups, has a significant decrease in tolerance.

4.2. Linalool effects on reducing morphine dependence

Long-term administration of the opioids causes tolerance and dependence and discontinuation of them, leading to symptoms such as restlessness, anxiety, aggression and irritability, which in the term of these symptoms are called withdrawal syndrome (Koob, 2009).

In this study, the effect of essential oil on the morphine withdrawal syndrome symptoms on mice was also assessed and doses of 20 and 40 mg/kg of essential oil were administered. According to the results, doses of 20 and 40 mg/kg of essential oil, both reduced the number of jumps caused by the withdrawal syndrome. A significant reduction in dry stool content due to exclusion syndrome was observed at 40 mg/kg dose. Also, this dose reduced the number of standing due to withdrawal syndrome significantly.

Factors and systems such as adenosine (Michalska & Malec, 1993), adrenergic system (Ambrosio et al., 1997), stimulatory amino acid (Gonzalez et al., 1997) and PKC inhibitors (Tokuyama et al., 1995) can play a role in withdrawal syndrome. In a study that investigated the effect of the *Z. majdae* extract on withdrawal syndrome, it was stated that the methanol extract of this plant, with its effect on the opioid system and stimulation of its receptors (agonists), or triggering the release of endogenous peptides in the supine spinal cord, It produces an analgesic effect and improves the symptoms of exclusion syndrome. The extract also contains linalool, which reduces the tonicity of skeletal muscle with effect on the cAMP system, which causes muscle relaxation and ultimately reduces the number of jumps from the withdrawal syndrome (Hosseinzadeh et al., 2007).

Another possible mechanism that improves the symptoms of withdrawal syndrome is probably the inhibition of the stimulatory amino acids by the plant extract. The linalool in the plant modulates glutamate activity in external testing (as a competitive antagonist of L-glutamate) and in in vitro tests (blocking the seizure caused by NMDA and Quin) and decreasing glutamate release (Elisabetsky et al., 1999; Lis-Balchin & Hart, 1999; Re et al., 2000).

5. Conclusion

In conclusion, the results of this study confirm that the linalool rich essential oil of *Z. majdae* plays a major role in the reduction of all the signs of morphine withdrawal syndrome and decrease of tolerance in development and expression groups. The results also suggest that ZMEO possesses biologically active constituent (linalool) that has significant activity against tolerance to the anticonvulsant effects of morphine and the morphine withdrawal symptoms.

Acknowledgements

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