Journal of Ethnopharmacology 279 (2021) 113627

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

Sedative and hypnotic effects of *Perilla frutescens* essential oil through GABAergic system pathway

Yu Zhong [a], Qin Zheng [a,*], Pengyi Hu [a], Xiaoying Huang [a], Ming Yang [a,**], Guilin Ren [b], Jing Li [a], Qing Du [a], Shanshan Liu [a], Kenan Zhang [a], Ling Wu [a], Liyun Zhu [a], Yuanyuan Guo [a], Wenjing Li [a], Shuai Xiao [a], Shuyuan Shuai [a], Mingxia Zhang [a]

[a] Key Laboratory of Modern Preparation of Traditional Chinese Medicine, Ministry of Education, State Key Lab.of Innovation Drug and Effcient Energy-Saving Pharmaceutical Equipment, Jiangxi University of Traditional Chinese Medicine, Nanchang, 330004, China
[b] Traditional Chinese Medicine Hospital Affliated to Southwest Medical University, Luzhou, 646000, China

ARTICLE INFO

Keywords:
Sedative and hypnotic effects
Perilla frutescens essential oil
Inhalation
GABAergic system pathway

ABSTRACT

Ethnopharmacological relevance: Traditional Chinese medicine believes that depression syndrome has become one of the core pathogenesis of insomnia. The pharmacology of traditional Chinese medicine points out that *Perilla frutescens* has the effect of regulating Qi and relieving depression, promoting Qi circulation to relieve pain, so *Perilla frutescens* may have the potential therapeutic effect on insomnia. Related studies have reported the sedative and hypnotic effects of *Perilla frutescens*, but these studies have not yet explored the mechanism of sedative and hypnotic effects of *Perilla frutescens* essential oil (PFEO) through inhalation administration.

Aim of the study: The purpose of this study is to explore the underlying sedative and hypnotic mechanisms of PFEO through the GABAergic system pathways.

Materials and methods: Established the PCPA insomnia model of mice, The open field test, pentobarbital-induced falling asleep rate, latency of sleeping time, and duration of sleeping time experiments were used to evaluate the behavior of mice, the enzyme-linked immunosorbent assay was used to analyze the content of 5-HT and GABA in hypothalamus and cerebral cortex. Immunohistochemical experiment, Western blot experiment and RT-PCR experiment were used to study the mechanism of PFEO through GABAergic pathway to regulate insomnia. The main volatile constituents of PFEO were analyzed by gas chromatography-mass spectrometry (GC-MS).

Results: The inhalation of PFEO has sedative and hypnotic effects, which reduce significantly the autonomic activity of PCPA insomnia mice, increase falling asleep rate, shorten latency of sleeping time, and prolong duration of sleeping time; the results of enzyme-linked immunosorbent assay show that PFEO increase the content of 5-HT and GABA in hypothalamus and cerebral cortex. The results showed that inhalation of PFEO increase the expression of GABAAα1 and GABAAα2 positive cells, increase the level of GABAAα1 and GABAAα2 protein and also increase the level of GABAAα1 mRNA and GABAAα2 mRNA in the hypothalamus and cerebral cortex. The highest content of PFEO is Perillaldehyde (54.37%), followed by 1,4-Cineole (7.42%), Acetaldehyde diethyl acetal (6.61%), D-Limonene (5.09%), Eucalyptol (4.94%), etc.

Conclusion: The inhalation of PFEO has sedative and hypnotic effects, it is speculated that the mechanism of which may be the sedative and hypnotic effects through the GABAergic pathway.

1. Introduction

Insomnia is a common sleep disorder that can be caused by psychological stress, chronic pain, and medications (Oh et al., 2019). A WHO study shows that about 27% of people worldwide suffer from sleep disorders (Taylor et al., 2003). Drugs used to treat insomnia include benzodiazepine receptor agonists, non-benzodiazepine receptor agonists, selective melatonin receptor agonists, and sedative

Abbreviations: PFEO, *Perilla frutescens* essential oil.

* Corresponding author.
** Corresponding author.
E-mail addresses: zhengqin912006@163.com (Q. Zheng), yangming16@126.com (M. Yang).

https://doi.org/10.1016/j.jep.2020.113627
Received 21 May 2020; Received in revised form 16 September 2020; Accepted 23 November 2020
Available online 25 November 2020

antidepressants (Winkler et al., 2014). However, taking these drugs will be accompanied by side effects such as the "hangover" effect during the day, psychomotor disorders, drug dependence, addiction, tolerance, amnesia, and rebound insomnia, and its clinical efficacy remains controversial (Shi et al., 2014). Therefore, research on finding new sedative and hypnotic drugs with fewer side effects and better efficacy is continuing (Askari et al., 2016).

Now emotional factors have become the main cause of insomnia, insomnia patients are usually accompanied by different degrees of depression, anxiety symptoms (Johnson et al., 2006). Traditional Chinese medicine believes that depression syndrome has become one of the core pathogenesis of insomnia (Xu et al., 2019). The traditional Chinese medicine that regulating Qi and relieving depression, promoting Qi circulation to relieve pain, most of which is acrid, bitter, pungent in flavor and warm in property, and normally used qi-regulating and digestive system disease treatment. (Lou et al., 2018). In the prescription of traditional Chinese medicine, traditional Chinese medicine with the functions of regulating Qi and relieving depression, promoting Qi circulation to relieve pain is commonly used in the compatibility of tranquilizers, including orange peel, agarwood, *Platycodon grandiflorum*, chuanxiong and so on (Yin, 2009). Sancao Anshenfang, a Chinese medicine prescription that has the functions of regulating Qi and relieving depression, promoting Qi circulation to relieve pain, has also been shown to have sedative, hypnotic and antidepressant effects (Long et al., 2018). The study found that Liqi Ningshen Fang, which has the functions of regulating Qi and relieving depression, promoting Qi circulation to relieve pain, was used to treat patients with insomnia, and its clinical effect is considerable, no less than that of western medicine zopiclone. At the same time, it can significantly improve the traditional Chinese medicine syndrome of insomnia patients, effectively relieve the patients' bad emotions, and no adverse reactions have been seen (Chen, 2018). The Sini Suanzaoren Decoction, which has the functions of regulating Qi and relieving depression, promoting Qi circulation to relieve pain, has also been shown sedative and hypnotic effect. Its mechanism is to exert sedative and hypnotic effect through the GABAergic system (Lin et al., 2018). Related studies have reported the sedative and hypnotic effects of traditional Chinese medicine essential oil through inhalation administration, which has the functions of regulating Qi and relieving depression, promoting Qi circulation to relieve pain. Studies have found that the inhalation of agarwood essential oil has sedative and hypnotic effects (Takemoto et al., 2008). Inhalation of patchouli essential oil reduce significantly the motor ability of mice and reduce spontaneous activity of mice (Ito and Ito, 2011); In addition, Chuanxiong essential oil also has sedative and hypnotic effects (Guo et al., 2010).

Perilla frutescens (L.) Britton. is rich in essential oils, fatty acids, flavonoids and other ingredients. The pharmacology of traditional Chinese medicine points out that *Perilla frutescens* has the effect of regulating Qi and relieving depression, promoting Qi circulation to relieve pain (He et al., 2018). Related research reports have confirmed that *Perilla frutescens* has potential sedative and hypnotic effects. Jin Jianming found that water extract of *Perilla frutescens* reduce the spontaneous activity of normal mice, and has certain synergistic effect on pentobarbital sodium to promote animal sleep (Jianming and Zhengshan, 2012). Honda found that methanol extract from *Perilla frutescens* leaves can prolong the sleep time of mice induced by cyclohexene barbiturate (Honda et al., 1988). Studies have also shown that water extract of *Perilla frutescens* and perillaldehyde prolong significantly the sleep time of mice induced by cyclohexyl barbiturate, and water extract of *Perilla frutescens* also inhibit rats movement (Changge, 1982). However, these studies have not yet explored the mechanism of sedative and hypnotic effects of PFEO.

Therefore, the purpose of this study is to explore the underlying sedative and hypnotic mechanisms of PFEO through the GABAergic system pathway, so as to provide theoretical basis for the treatment and improvement of insomnia by inhalation of PFEO.

2. Methods and materials

2.1. Animals

ICR mice aged 6–8 weeks of either sex in the ratio of 1:1, weighing 25–35 g, provided by Jiangsu Jicui Yaokang Biotechnology Co., Ltd. License number: SCXK (su) 2018–0008. The temperature of the feeding environment was 25 °C ± 1 °C, the humidity was 55% ± 5%, the light and dark alternate for 12 h, and the test was started after 1 week of adaptive feeding (Zhong et al., 2019).

The experiments were approved by the Institutional Animal Ethics Committee of Jiangxi University of Traditional Chinese Medicine. All animals were maintained in accordance with the guidelines outlined by the legislation on the ethical use and care of laboratory animals.

2.2. Main drugs and reagents

The leaf of *Perilla frutescens* (Jiangxi Zhangshu Tianqitang Traditional Chinese Medicine Pieces Co., Ltd. batch number: 1812008); Diazepam Tablets (Beijing Yimin Pharmaceutical Co., Ltd., batch number: H11020898); Sodium pentobarbital (Merck, Germany, batch number: 20171230); 5-HT ELISA kit (Shanghai Yuchun Biotechnology Co., Ltd., batch number: 20190521); GABA ELISA kit (Shanghai Yuchun Biotechnology Co., Ltd., batch number: 20190408); PBS buffer (Solarbio, batch number: 20190505); Paraffin (Shanghai Sinopharm Group, batch number: 69018961); Formaldehyde (Shanghai Sinopharm Group, batch number: 10010018); Xylene (Shanghai Sinopharm Group, batch number: 10023418); 30% H_2O_2 (Shanghai Sinopharm Group, batch number: 10011218); Broad-spectrum secondary antibody (Shanghai Long Island Biotechnology Co., Ltd., batch number: D-3004); DAB concentrated kit (Shanghai Long Island Biotechnology Co., Ltd., batch number: FL-6001); Hematoxylin (BASO, batch number: 714094); Neutral resin (Beijing Solibao, batch number G8590); RIPA tissue cell rapid lysate (Solarbio, batch number: R0020); BCA protein quantification kit (Thermo, batch number: PICPI23223); Tris-HCl, pH = 8.8 electrophoresis buffer (Solarbio, batch number: T1010); Tris-HCl, pH = 6.8 electrophoresis buffer (Solarbio, batch number: T1020); 10% SDS (Solarbio: S1010); 10% ammonium persulfate (Solarbio, batch number: A1030); TEMED (Solarbio, batch number: T8090); Protein loading buffer (Solarbio; batch number: P1015); Protein prestained (Marker Fermentas, batch number: SM1811); NC membrane (millipore, batch number: HATF00010); Skimmed milk powder (Solarbio, batch number: D8340); Luminescent liquid (Millipore, batch number: WBKLS0100); GABAAα1 antibody (Abbexa, batch number: abx112662); GABAAγ2 (Abbexa, batch number: abx001446); β-Actin antibody (abcam, batch number: ab179467); Goat anti-rabbit HRP-labeled secondary antibody (Biyuntian, batch number: A0208), PCR kit (SYBR Green Thermo, batch number: K0223); Reverse transcription Kit (Fermentas, batch number: K1622); Trizol (invitrogen, batch number: 1596026).

2.3. Main equipment and instruments

Ultrasonic atomizing aromatherapy machine (Shenzhen Kangmeitai Industrial Co., Ltd.); Animal aromatherapy room (50 × 50 × 40cm large box structure made of plexiglass, which contains 4 20 × 20 × 20cm gas-permeable small box structures, and an ultrasonic fragrance machine can be placed in the middle); SMART Behavior Recording Video Analysis System (Panlab); Agilent 7890 A Gas Chromatograph-5975 Mass Spectrometer (Agilent, USA); High-throughput Tissue Grinder (Ningbo Xinzhi Biotechnology Co., Ltd., SCIENTZ-192); Low temperature High-speed centrifuge (2K15C, German SIGMA); microplate reader (Gene Co., Ltd., Elx800); Upright microscope (CX4, product of OLYMPUS); Paraffin microtome (SQ2125, Leike); Digital Camera: (D5100, NIKON Corporation); Electrophoresis Instrument (mini protean 3 cell, BIO-RAD Corporation); Electron Transducer (TE77XP, HOEFER Corporation); Imaging System (Tanon-5200, Tanon); Real-time detector (ABI

company, ABI-7300); Vortex oscillator (Qingpu Yunxi Instrument Factory, K30).

2.4. Preparation of Perilla frutescens essential oil

Precisely weighed amount of Perilla leaf was taken and the volatile oil was extracted by steam distillation according to the volatile oil method in 2015 edition of Chinese pharmacopoeia, and the same was collected.

2.5. Establishing mice models of insomnia

The experimental animals (except the control group) were injected with PCPA into the abdominal cavity to create an animal insomnia model. Each mouse was injected with a suspension of PCPA weakly alkaline saline at a dose of 300 mg/kg for 2 consecutive days, with the first intraperitoneal injection After 28–32 h of administration, the animal's circadian rhythm disappeared, and the animals were kept active during the day and night, indicating that the model was successful.

2.6. Grouping and treatment

ICR mice were randomly divided into 6 groups. This experiment consisted of control group, model group, diazepam group, and PFEO groups (low-dose group, medium-dose group, high-dose group). After the establishment of the PCPA insomnia model, the model group did not implement the inhalation of essential oil intervention, in which the low-dose, medium-dose, and high-dose groups were daily aromatherapy for 7 consecutive days, 60 min per day. The essential oil was diluted with distilled water. The concentration of low, medium, and high doses of essential oil was 1.5×10^{-3}, 3×10^{-3}, 6×10^{-3}, and the inhalation time was set at 8:00 daily. Diazepam was prepared into a solution of 0.1 ml/10 g with distilled water. The diazepam group was given diazepam solution by gavage for 7 consecutive days, and the dose was 1 mg/kg (Zhong et al., 2019).

2.7. wt changes in mice

Mice were weighed before the establishment of the PCPA insomnia model and at the end of the experiment, and the weight gain was calculated (Zhong et al., 2019).

2.8. Open field test

After the last aromatherapy or the last 30 min of administration in each group of mice, open field test were performed on each group of mice. After the mice were acclimatized for 5 min, Distance moved, Average velocity and Rest time of mice were counted within 5 min to compare the differences between the groups of mice (Zhong et al., 2019).

2.9. Subthreshold dose of pentobarbital sodium induced sleep rate in mice

Mice in each group were injected intraperitoneally with sodium pentobarbital at subthreshold dose of 35 mg/kg 30 min after the last aromatherapy or last administration. After the injection, the mice were placed on a warm pad and the time to sleep was recorded. With the 1 min disappearance of the righting reflex after administration as the index of falling asleep, the number of mice falling asleep in each group of mice within 30 min was recorded, and the sleep rate of the mice in each group was calculated.

2.10. Sleep latency and sleep duration induced by threshold dose of pentobarbital sodium

Mice in each group were intraperitoneally injected with a threshold dose of pentobarbital sodium of 45 mg/kg after the final aromatherapy or 30 min of administration. The time from the injection of the drug to the disappearance of the righting reflex was latency of sleeping time, the time that the righting reflex disappeared until the recovery was duration of sleeping time, and latency of sleeping time and duration of sleeping time were recorded (Zhong et al., 2019).

2.11. Analysis of neurotransmitters in hypothalamus and cerebral cortex

The mice in each group were sacrificed by cervical dislocation, and the hypothalamus and cerebral cortex was completely removed and taken, thoroughly homogenized, centrifuged (3000 r/min, 20 min), and the supernatant was taken. After treatment with the mouse 5-HT and GABA ELISA kits, the 5-HT and GABA contents were measured (Zhong et al., 2019).

2.12. Immunohistochemistry

The hypothalamus and cerebral cortex were separated, repaired with sodium citrate buffer heat antigen, S–P immunohistochemical staining, developed with DAB/H_2O_2, and counterstained with hematoxylin. According to the instructions of the kit, the known positive sections were used as the positive control, and the primary antibody was replaced by PBS as the negative control. The sections were then put into the microscope Image acquisition system for Image acquisition, and the integrated optical density (IOD) of the expression of GABAAα1-positive cells and GABAAγ2-positive cells was analyzed by image-proplus Version 5.1 software.

2.13. Western blot

The hypothalamus and cerebral cortex were separated and total protein was extracted. Tissue protein concentration was determined by BCA method, and GABAAα1 protein and GABAAγ2 protein were separated by 10% SDS-PAGE gel electrophoresis. After 30 min electrotransfer to the PVDF membrane, remove the membrane and rinse it in Tris buffer (TBS-T) for 5 min, then block with blocking solution for 1 h (TBS-T buffer containing 5% skimmed milk powder). After incubation at room temperature, the blocking solution was discarded, and primary antibodies GABAAα1 (1: 1000) and GABAAγ2 (1: 1000) were added. After incubation at 4 °C overnight, TBS-T was washed 3 times for 5 min each. Add secondary antibody (1: 1000) and incubate for 1 h, wash TBS-T 3 times for 5 min each time, and finally add ECL color development solution to develop color.

2.14. RT-PCR

The hypothalamus and cerebral cortex were separated, and mRNA was extracted for RT-PCR experiments. 10 μL PCR products were subjected to gel electrophoresis, the results were observed and photographed with a gel imaging system, and the photographs were scanned for density with a gel imaging system. The ratio of the optical density values of GABAAα1 mRNA and GABAAγ2 mRNA to β-actin was used to express the level of GABAAα1 mRNA and GABAAγ2 mRNA.

2.15. Analysis of chemical composition of Perilla frutescens essential oil

Gas chromatography conditions: An Agilent DB-624 (30 m × 320 μm x 1.8 μm) capillary column was used. The carrier gas was high-purity He (99.999%), the injection volume was 1 μL, the split ratio was 40: 1, and the flow rate was 1 mL/min. Heating program: Initial temperature is 50 °C (maintain for 2 min), and heat up to 300 °C (maintain for 5min) at 10 °C/min. Mass spectrometry conditions: EI ion source, electron energy 70eV, ion source temperature 230 °C, MS quadrupole temperature 150 °C; interface temperature 250 °C, solvent delay 3.0min, full scan in mass scanning mode, scanning range 29–650amu. Standard library

NIST11 search, peak area normalization method to calculate the relative percentage content of each component.

2.16. Statistical analysis

The test results were expressed as Mean ± SD. SPSS 21.0 software was used for statistical analysis, single factor ANOVA method was used for analysis, LSD method was used for homogeneity of variance, Games-Howell method was used for heterogeneity of variance, and $P < 0.05$ was taken as the level of test.

3. Results

3.1. Measurement results of body weight changes in mice

Compared with the model group (Fig. 1), the body weight of the medium-dose group increased significantly ($P < 0.05$), and the body weight of the high-dose group ($P < 0.01$) and the diazepam group ($P < 0.01$) extremely increased significantly. It is observed that the weight gain of the mice in the PFEO high-dose group was closer to the level of the control group, which proved that inhalation of the high-dose PFEO had a smaller effect on body weight and tended to a normal level.

3.2. Open field test results

Compared with the model group (Fig. 2), the male mice in the diazepam group had a significant decrease in distance moved and average velocity ($P < 0.05$), and the rest time of the male mice in the diazepam group was significantly increased ($P < 0.05$); The distance moved and average velocity of the male mice in the medium-dose group of PFEO were significantly reduced ($P < 0.05$); The distance moved and average velocity of the female mice in the high-dose group of PFEO were significantly reduced ($P < 0.05$), and rest time of the female mice in the high-dose group of PFEO was significantly increased ($P < 0.05$). In addition, The distance moved and average velocity of male mice are greater than that of female mice, and the resting time of male mice is shorter than that of female mice. Therefore, from the perspective of the overall data trend, male mice have more autonomous activities than female mice. The results indicate that inhalation administration of PFEO can significantly reduce the autonomous activity of insomniac mice.

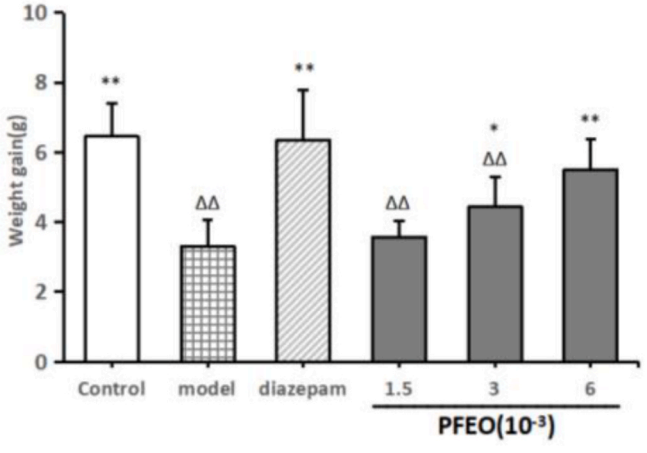

Fig. 1. Comparison of weight gain in each group at the end of the experiment (Mean ± SD, n = 12). The weight gain at the end of the experiment was shown Fig. 1. Note: compared with control group, $^{\Delta}P<0.05$,$^{\Delta\Delta}P<0.01$, compared with model group,$^{*}P < 0.05$,$^{**}P < 0.01$.

3.3. Effect of Perilla frutescens essential oil on the falling asleep rate of mice induced by sodium pentobarbital

Compared with the model group, the number of falling asleep extremely significantly increased in the diazepam group and the high-dose group ($P < 0.01$), and the number of falling asleep significantly increased in the medium-dose group ($P < 0.05$). In addition, the number of falling asleep and the falling asleep rate in male mice were more than which in female mice. Therefore, from the perspective of the overall data trend, the falling asleep rate in male mice more than which in female mice. It can be concluded that inhalation administration of PFEO can significantly increase the falling asleep rate (Tables 1 and 2).

3.4. Effect of Perilla frutescens essential oil on latency of sleeping time and duration of sleeping time in pentobarbital-induced mice

Compared with the model group (Fig. 3), the latency of sleeping time of the male mice in the diazepam group, low-dose group, medium-dose group, and high-dose group decreased extremely significantly ($P < 0.01$), the duration of sleeping time of the male mice in the diazepam group, low-dose group, medium-dose group, and high-dose group increased extremely significantly ($P < 0.01$). Compared with the model group (Fig. 3), the latency of sleeping time of the female mice in the diazepam group, medium-dose group, and high-dose group decreased extremely significantly ($P < 0.01$); the latency of sleeping time of the female mice in low-dose group decreased significantly ($P < 0.01$); the duration of sleeping time of the female mice in the medium-dose group and the diazepam group increased extremely significantly ($P < 0.01$); the duration of sleeping time of the female mice in the high-dose group increased significantly ($P < 0.05$). It serves to show inhalation administration of PFEO can significantly shorten the latency of sleeping time and prolong the duration of sleeping time.

3.5. Effect of Perilla frutescens essential oil on neurotransmitters in hypothalamic and cerebral cortical

Compared with the model group (Fig. 4), the level of 5-HT and GABA in hypothalamus and cerebral cortical of the diazepam group, medium-dose group, and high-dose group increased extremely significantly ($P < 0.01$), the level of 5-HT in hypothalamus and the level of GABA in cerebral cortical of low-dose group increased significantly ($P < 0.05$). It shows that inhalation administration of PFEO can significantly increase level of 5-HT and GABA in the hypothalamus and cerebral cortex.

3.6. Effect of Perilla frutescens essential oil on the expression of GABAAα1 and GABAAγ2 in the hippocampus and cerebral cortex

GABAAα1-positive cells and GABAAγ2-positive cells in the hypothalamus and cerebral cortex of each group were brown-yellow, and the positive cells' bodies were round, oval, and triangular and other shapes (Fig. 5). From the perspective of the expression of GABAAα1-positive cells and GABAAγ2-positive cells in hypothalamus and cerebral cortex (Fig. 5), compared with the control group, the expression of GABAAα1-positive cells and GABAAγ2-positive cells in the model group was extremely significantly reduced ($P < 0.01$). Compared with the model group, the expression of GABAAα1-positive cells and GABAAγ2-positive cells in the hypothalamus and cerebral cortex was extremely significantly increased in the low-dose group (except the expression of GABAAα1-positive cells in cerebral cortex), diazepam group, the medium-dose group, and the high-dose group ($P < 0.01$); the expression of GABAAα1-positive cells in cerebral cortex was extremely significantly increased in the low-dose group ($P < 0.05$).

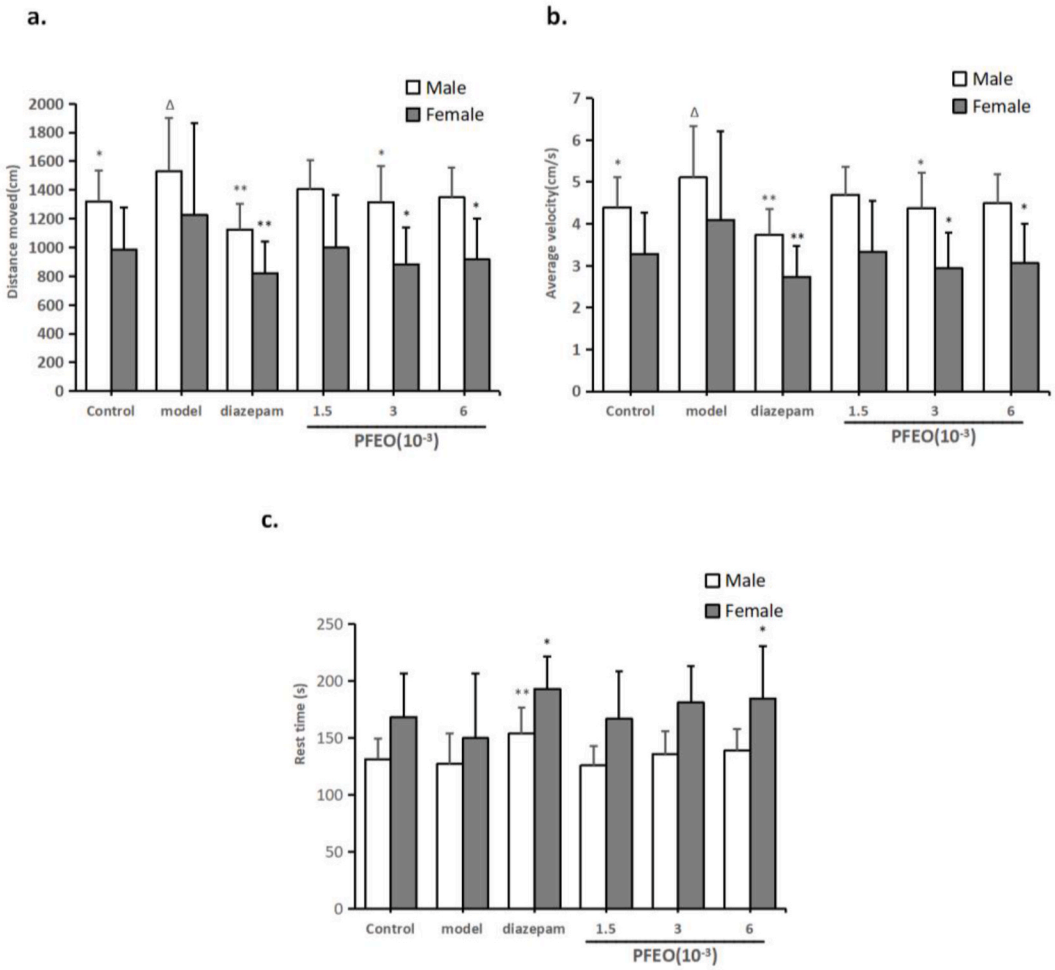

Fig. 2. Effect of PFEO on Distance moved (a), Average velocity (b) and Rest time (c) of mice (Mean ± SD, n = 12). The results of the open field test are shown in Fig. 2. Note: Compared with the control group, $^{\Delta}P<0.05$, $^{\Delta\Delta}P<0.01$, compared with the model group, *P < 0.05, **P < 0.01.

Table 1
Effects of PFEO on the falling asleep rate of male mice induced by sodium pentobarbital (Mean ± SD, n = 12). The experimental results of pentobarbital-induced falling asleep rates in male mice are shown in Table 1. Note: Compared with the control group, $^{\Delta}P<0.05$, $^{\Delta\Delta}P<0.01$, compared with the model group, *P < 0.05, **P < 0.01.

Male group	Number of mice falling asleep	falling asleep rate (%)
Control group	8	66.67
Model group	2^{Δ}	16.67
Diazepam group	10**	83.33
Low-dose group	4	33.33
Medium-dose group	7*	58.33
High-dose group	9**	75

Table 2
Effect of PFEO on the falling asleep rate of female mice induced by sodium pentobarbital (Mean ± SD, n = 12). The experimental results of male pentobarbital-induced fall asleep rates in female mice are shown in Table 2. Note: Compared with the control group, $^{\Delta}P<0.05$, $^{\Delta\Delta}P<0.01$, compared with the model group, *P < 0.05, **P < 0.01

Female group	Number of mice falling asleep	falling asleep rate (%)
Control group	7	58.33
Model group	$1^{\Delta\Delta}$	8.33
Diazepam group	9**	75
Low-dose group	4	33.33
Medium-dose group	6*	50
High dose group	8**	66.67

3.7. Effects of Perilla frutescens essential oil on the level of GABAAα1 protein and GABAAα2 protein in hypothalamus and cerebral cortex

From the perspective of the level of GABAAα1 protein and GABAAγ2 protein in hypothalamus and cerebral cortex (Fig. 6), compared with the control group, the level of GABAAα1 protein and GABAAγ2 protein in the model group was extremely significantly reduced (P < 0.01); compared with the model group, the level of GABAAα1 protein of the diazepam group was extremely significantly increased (P < 0.01); the level of GABAAα1 protein in hypothalamus and cerebral cortex of the high-dose group was extremely increased significantly (P < 0.01); the level of GABAAα1 protein in hypothalamus of the medium-dose group was extremely increased significantly (P < 0.01); the level of GABAAγ2 protein in cerebral cortex of the medium-dose group was increased significantly (P < 0.05); the level of GABAAγ2 protein in hypothalamus and cerebral cortex of the high-dose group was increased significantly (P < 0.05).

3.8. Effects of Perilla frutescens essential oil on the level of GABAAα1 mRNA and GABAAγ2 mRNA in hypothalamus and cerebral cortex

From the perspective of the level of GABAAα1 mRNA and GABAAγ2 mRNA in hypothalamus and cerebral cortex (Fig. 7), compared with the control group, the level of GABAAα1 mRNA and GABAAγ2 mRNA in the

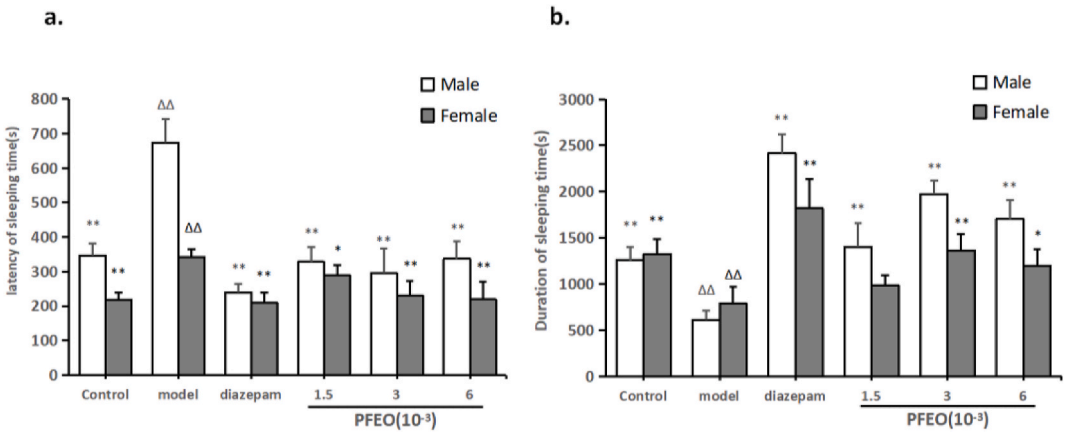

Fig. 3. Effect of PFEO on pentobarbital-induced latency of sleeping time (a) and duration of sleeping time (b) in mice (Mean ± SD, n = 12). The experimental results of latency of sleeping time and duration of sleeping time induced by pentobarbital sodium after inhalation of PFEO in mice were shown Fig. 3. Note: Compared with the control group, $^{\Delta}P<0.05$, $^{\Delta\Delta}P<0.01$, compared with the model group, *P < 0.05, **P < 0.01.

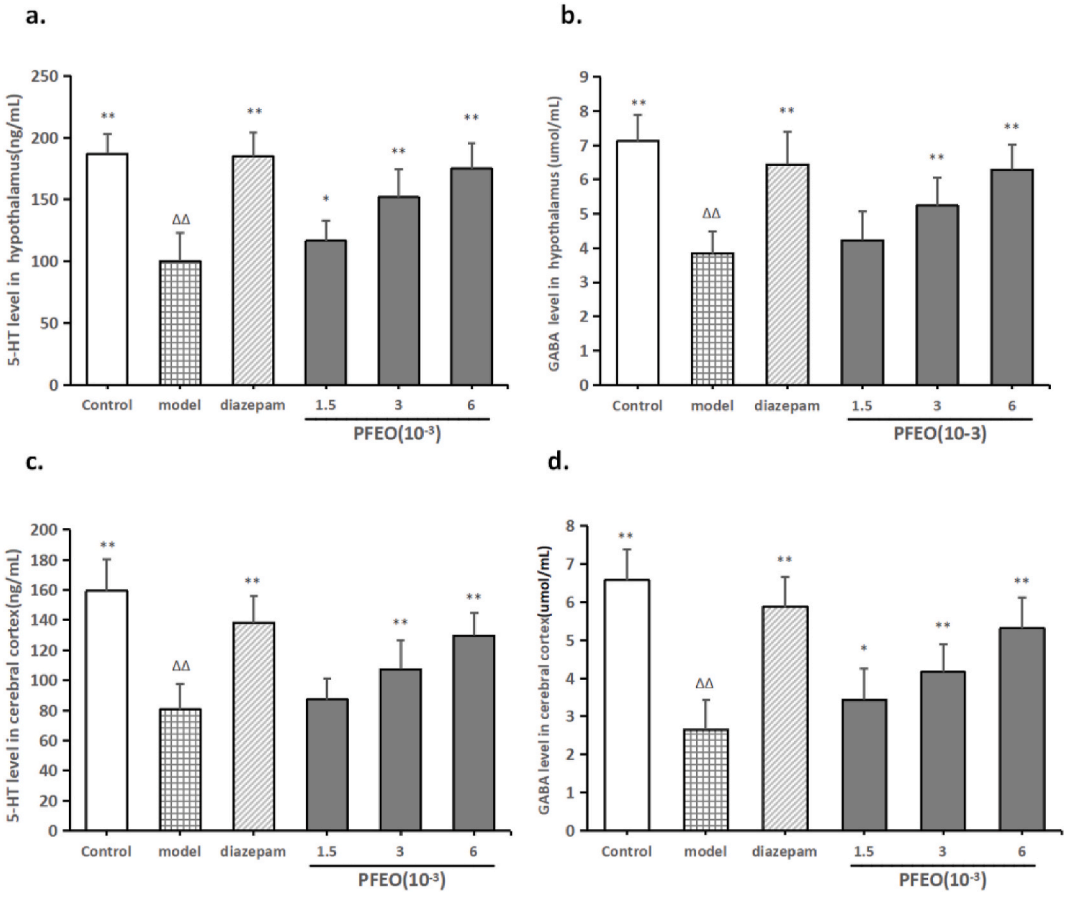

Fig. 4. Effects of PFEO on neurotransmitters in hypothalamic (a,b) and cerebral cortical (c,d) of mice (Mean ± SD, n = 12). The experimental results of the PFEO on hypothalamic and cerebral cortical neurotransmitters are shown in Fig. 4. Note: Compared with the control group, $^{\Delta}P<0.05$, $^{\Delta\Delta}P<0.01$, compared with the model group, *P < 0.05, **P < 0.01.

model group was extremely significantly reduced (P < 0.01); compared with the model group, the level of GABAAα1 mRNA and GABAAγ2 mRNA in the diazepam group, medium-dose group (except the level of GABAAγ2 mRNA in cerebral cortex), and high-dose group was extremely significantly increased (P < 0.01); the level of GABAAγ2 mRNA in the low-dose group and medium-dose group was significantly increased (P < 0.05).

3.9. The analysis of Perilla frutescens essential oil component

The 16 chemical components of PFEO were identified, accounting for 96.31% of the total volatile oils (Fig. 8, Table 3), the highest content of which is Perillaldehyde (54.37%), followed by 1,4-Cineole (7.42%), Acetaldehyde diethyl acetal (6.61%), D-Limonene (5.09%), Eucalyptol (4.94%), etc.

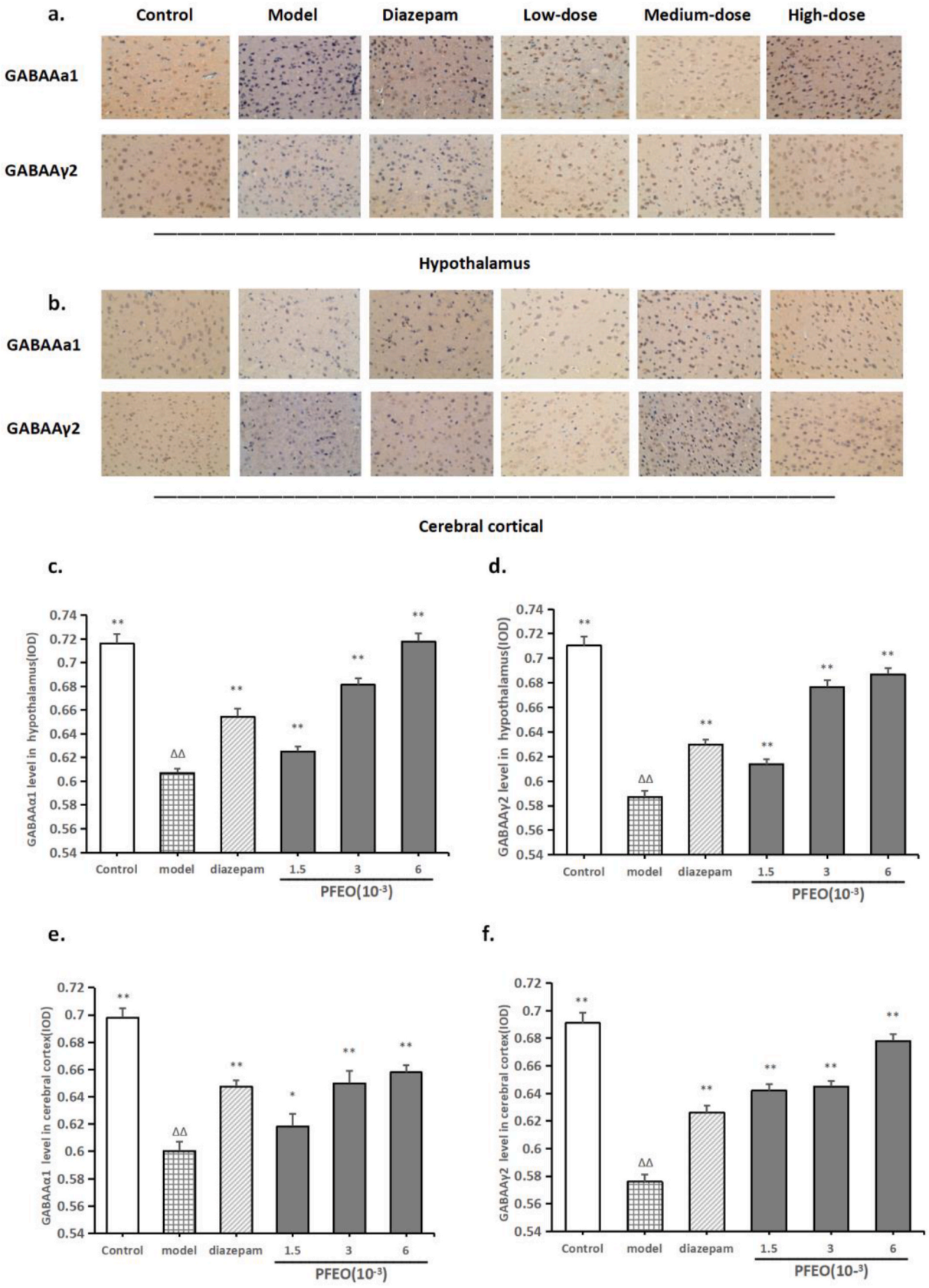

Fig. 5. Effect of PFEO on the expression of GABAAα1 and GABAAγ2 in the hippocampus (a,c,d) and cerebral cortex (b,e,f) (Mean ± SD, n = 3). The results of immunohistochemical staining are shown in Fig. 5 (IOD: integral optical density). Note: Compared with the control group, $^{\Delta}P<0.05$, $^{\Delta\Delta}P<0.01$, compared with the model group, *P < 0.05, **P < 0.01.

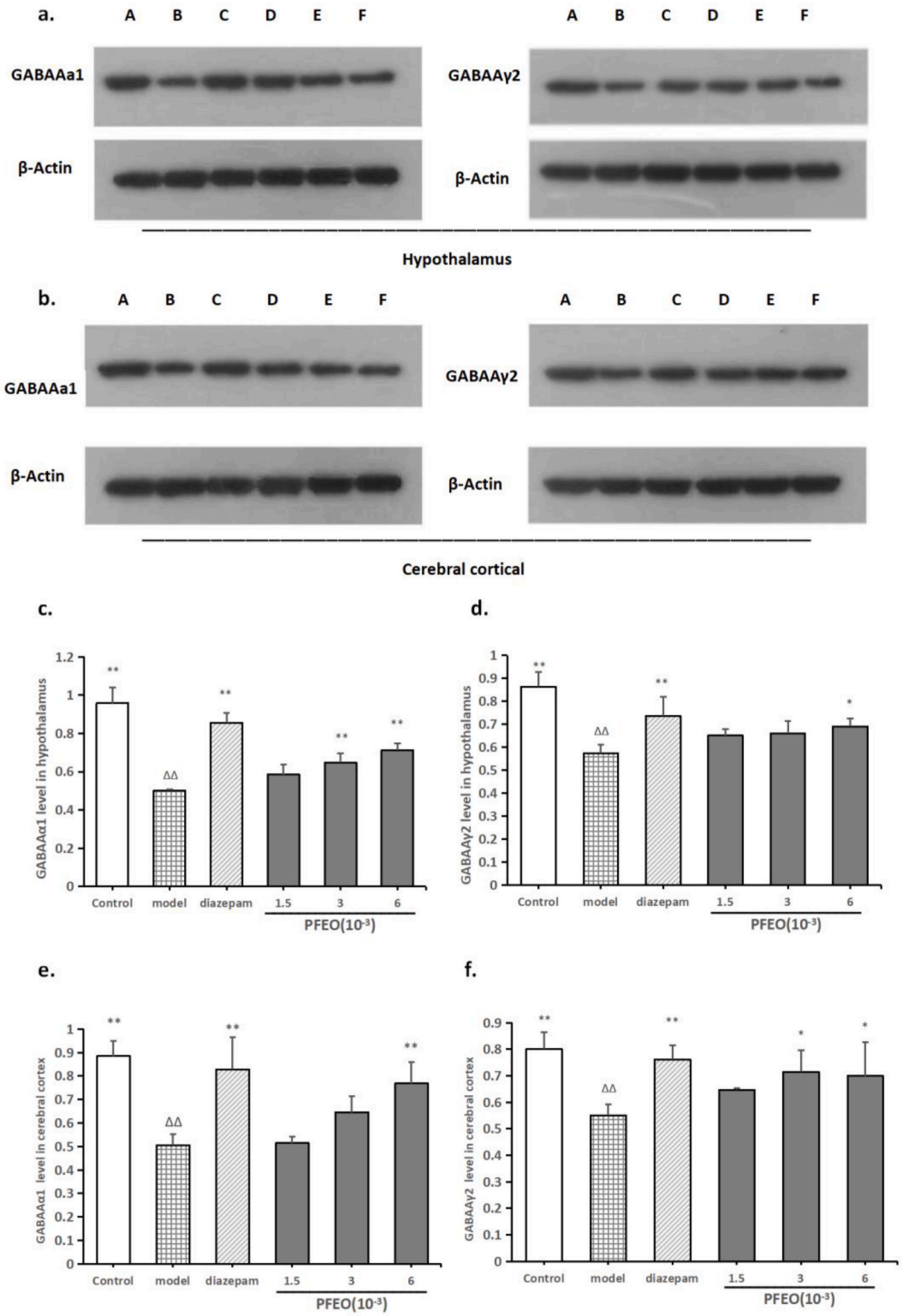

Fig. 6. Effects of PFEO on the level of GABAAα1 protein and GABAAα2 protein in the hippocampus (a,c,d) and cerebral cortex (b,e,f) (Mean ± SD, n = 3). The results of Western blot are shown in Fig. 6 (A: control group, B: model group, C: diazepam group, D: low-dose group, E: medium-dose group, F: high-dose group). Note: Compared with the control group, $^{\Delta}P<0.05$, $^{\Delta\Delta}P<0.01$, compared with the model group, *P < 0.05, **P < 0.01.

4. Discussion

The result of research shows that the mice in the PFEO medium-dose group, the high-dose group, which had moist hair, the circadian rhythm returned to normal, the mental state improved, and the body weight increased significantly. On the contrary, in the PCPA model group, the hair of the mice was dry and dull, and the amount of drinking water increased significantly, showing that they were sensitive to external sound and light stimuli, the circadian rhythm of sleep disappeared, and the body weight was significantly reduced. It can be seen that inhalation

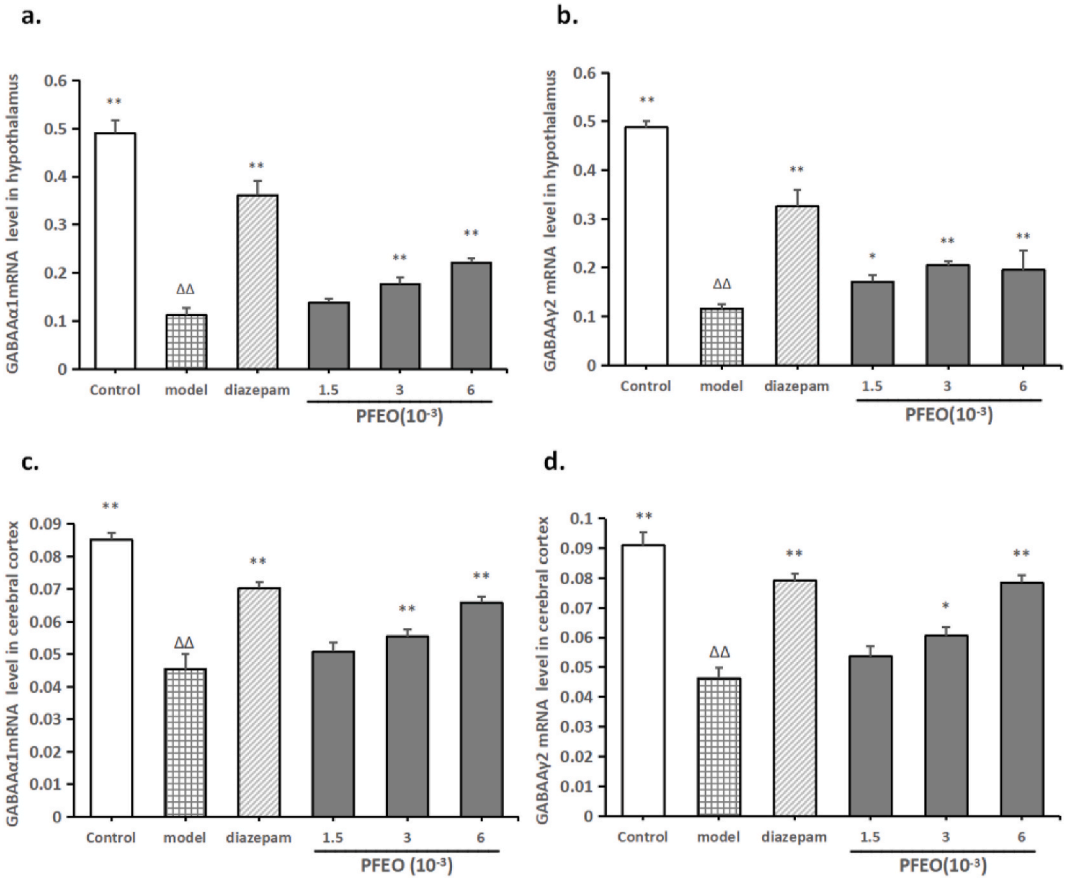

Fig. 7. Effects of PFEO on the level of GABAAα1 mRNA and GABAAγ2 mRNA in hypothalamus and cerebral cortex (Mean ± SD, n = 3). The results of the RT-PCR are shown in Fig. 7. Note: Compared with the control group, $^{\Delta}P<0.05$, $^{\Delta\Delta}P<0.01$, compared with the model group, *P < 0.05, **P < 0.01.

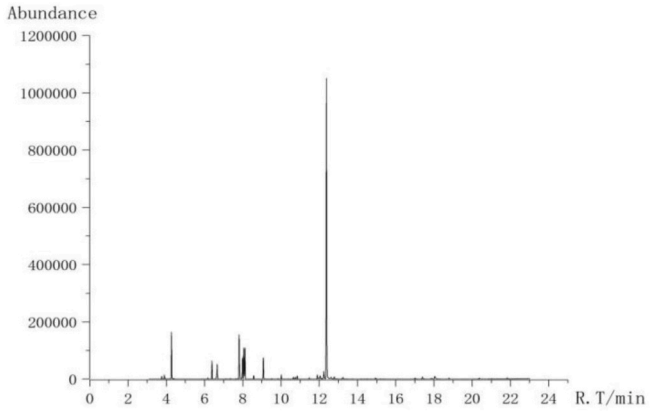

Fig. 8. GC-MS analysis of the TIC component of PFEO.

administration of PFEO has a tendency to restore normal weight to insomniac mice. The behavioral observation method is to use instruments or artificial visual observation to investigate the decrease or increase of the animal's autonomous activity when the drug causes the central nervous system to inhibit or excite. Open field test is a classic and the most common method, which was used to evaluate the changes in the autonomous activity of mice after administration in this experiment. In general, a decrease in autonomic activity in mice indicates that the sedative effect of the drug, and this change in behavior is thought to reflect the decrease in central nervous system excitability. Inhibition of autonomic activity indicates that PFEO has sedative and anti-excitatory effects. Most sleep experiments are based on the synergistic effect of

Table 3
Main chemical components and percentage content of PFEO.

No.	Retention Time (min)	Compound	Relative Amount (%)
1	3.903	2,3-Butanediol	0.78
2	4.276	Acetaldehyde diethyl acetal	6.61
3	6.391	α-Pinene	2.89
4	6.667	Camphene	2.84
5	7.816	1,4-Cineole	7.42
6	7.985	p-Cymene	3.42
7	8.059	D-Limonene	5.09
8	8.117	Eucalyptol	4.94
9	8.573	3-Carene	0.56
10	9.081	Terpinolene	3.63
11	10.855	Myrtenol	0.61
12	11.906	Citral	0.68
13	12.382	Perillaldehyde	54.37
14	12.586	β-elemene	0.29
15	12.633	Caryophyllene	0.43
16	17.010	cis-ligustilide	0.16
17	17.402	Asarone	0.53
18	18.049	Linalyl propionate	1.06

sedative drugs and barbiturates. These methods mainly observe the falling asleep rate, latency of sleeping time and duration of sleeping time of animals. Experimental investigation shows that PFEO has synergistic effect with pentobarbital sodium, the increase of the falling asleep rate, shorten the latency of sleeping time and prolong the duration of sleeping time show the hypnotic effect of PFEO.

Serotonin (5-HT) plays a key role in sleep-wake regulation (Monti, 2011). 5-HT neurons are mainly distributed in the medulla oblongata,

pons and midbrain of the central nervous system. In addition, there were a small amount of 5-HT positive cells in the ventral and dorsal part of the blue spot. 5-HT can regulate various physiological responses of the central nervous system and the peripheral nervous system. Changes in the content of 5-HT can cause changes in the body's physiological functions, such as sleep, sexual behavior, and appetite. Among them, it plays a key role in regulating NREM sleep (Jo et al., 2018; Monti, 2011). In addition, the PCPA is a tryptophan hydroxylase inhibitor and consumes 5-HT, which leads to insomnia, the detection of 5-HT level in the hypothalamus and cerebral cortex can reflect whether the insomnia model was successfully established (Pujol et al., 1971; Foltran et al., 2020). GABA is the most important inhibitory monoamine neurotransmitter widely present in the mammalian central nervous system, and plays an important role in the process of excitation-inhibition regulation. GABA is considered to be closely related to sleep, and is a unique substance in the central nervous system. The tissues outside the brain and spinal cord are very low in content, which has a general inhibitory effect on neurons in the nervous system. Abnormal content of GABA in the human brain is often accompanied by emotional problems such as depression and anxiety (Benson et al., 2015). At the same time, GABAergic neurons in the basal forebrain are related to the regulation of REM, mainly by reducing the REM regulation neurons in the pons network structure directly related to the inhibition of REM (Jones, 2005). In addition, the spontaneous firing of GABAergic neurons in the anterior visual area of the hypothalamus and the activation of GABAA receptors are involved in the regulation of sleep in the hypothalamus, especially rapid eye movement sleep (Ali et al., 1999). The enzyme-linked immunoassay was used to detect the level of 5-HT and GABA in this study, the results of which showed that the low-dose, middle-dose and high-dose groups of PFEO showed 5-HT up-regulation and GABA up-regulation in different degrees and were dose-dependent.

The inhibitory effect of γ-aminobutyric acid (GABA) is mainly through the formation of ligand-gated chloride ion channels through the GABAA receptor (Laurén et al., 2005). GABAA receptor, as an oligomer formed by pentagonal isomeric peptide, and 5 units (7-unit series named origin α1-6, β1-4, γ1-4, δ1, ε1, π1, and ρ1-3) installed in the lipid bilayer of the nerve cell membrane. By inhibiting neuron activity by opening the chloride ion channel, the chloride ion of the nerve cell membrane increases, and the chloride ion along concentration difference enter in the cell, resulting in hyperpolarization of the cell membrane and a corresponding decrease in excitability, thus manifesting as sedative and anxiolytic effects (Wang et al., 2015). The GABAA receptor is a commonly used indicator in the study of insomnia (Sun et al., 2019). The A receptor is an important functional group of the GABAA receptor, which has the most of GABAAα1 and GABAAγ2 receptors. The effects of sedative and hypnotic drugs are mainly mediated by receptors containing the α1 subunit. The expression of α1 subunit is activity-dependent, at the inhibitory synapse, the α1 subunit may regulate the intensity of the inhibitory synapse by controlling the duration of the inhibitory post-synaptic current (Swanson et al., 2015). GABAAα1 has the characteristics of rapid inhibition, and the decreased expression and function of GABAAα1 is related to depression and other chronic stress-related psychopathology (Alexander and Petes, 1998). It is believed that the sites of action of benzodiazepines are on the α and γ subunits, and the presence of γ2 subunits is beneficial to the positive regulation of benzodiazepines (Draguhn and Hartmann, 2006; Vergnes et al., 2001). The results indicate that PFEO can up-regulate the expression of GABAAα1-positive cells, GABAAγ2-positive cells in the hypothalamus and cerebral cortex, at the same time can up-regulate the expression of GABAAα1 protein, GABAAγ2 protein in the hypothalamus and cerebral cortex, and up-regulate the expression of GABAAα1 mRNA, GABAAγ2 mRNA in hypothalamus and cerebral cortex. Therefore, inhalation of PFEO can regulate insomnia through GABAergic pathway.

5. Availability of data and materials

The data sets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Authors' contributions

Designed the experiments, analyzed the data, and wrote the first draft of the manuscript: YZ and QZ. Conceived the research hypotheses and ideas, drafted the manuscript: QZ and MY. Conducted the drug preparation, essential oil preparation, related data analyze: PYH, XYH. Conducted the gas chromatography-mass spectrometry analysis, related data analysis and interpretation, and discussion on the main conception: YZ, GLR and KNZ. All authors have been involved in reviewing and approving the final manuscript.

6. Ethics approval and consent to participate

The experiments were approved by the Institutional Animal Ethics Committee of Jiangxi University of Traditional Chinese Medicine. All animals were maintained in accordance with the guidelines outlined by the legislation on the ethical use and care of laboratory animals.

7. Code availability

Not applicable.

8. Consent to participate

Not applicable.

9. Consent for publication

Not applicable.

Funding

This work was supported by the 5511 Science and Technology Innovation Talent Project of Jiangxi Province (No.20171BCB18001), Key technology of Chinese medicine essential oil and R&D and industrialization project of Chinese medicine aromatherapy health products (No.20194ABC28009), which supported design, analysis, and the interpretation of data in this study, and the Jiangxi Province First-class Subject Project (No.JXSYLXK-ZHYA0091), and the Jiangxi Province First-class Subject Project (No.JXSYLXK-ZHYA0092), which both provided the animals, medicine, and other materials needed in the study.

Conflicts of interest/Competing interests

The authors declare that they have no competing interests.

Acknowledgements

We wish to thank Key Laboratory of Modern Preparation of Traditional Chinese Medicine, Ministry of Education, Jiangxi University of Chinese Medicine for support.

References

Alexander, S., Petes, J.A., 1998. GABAA receptor. TIPS (Trends Pharmacol. Sci.) 1, 35–37.

Ali, M., Jha, S.K., Kaur, S., Mallick, B.N., 1999. Role of GABA-A receptor in the preoptic area in the regulation of sleep-wakefulness and rapid eye movement sleep. Neurosci. Res. 33, 245–250.

Askari, V.R., Baradaran Rahimi, V., Ghorbani, A., Rakhshandeh, H., 2016. Hypnotic effect of ocimum basilicum on pentobarbital-induced sleep in mice. Iran. Red Crescent Med. J. 18 https://doi.org/10.5812/ircmj.24261.

Benson, C., Mifflin, K., Kerr, B., Jesudasan, S.J., Dursun, S., Baker, G., 2015. Biogenic amines and the amino acids GABA and glutamate: relationships with pain and depression. Mod Trends Pharmacopsychiatry 30, 67–79.
Changge, L., 1982. Effects of perilla leaf water extract and perillaldehyde on nervous system. World Notes Plant Med 2, 39.
Chen, D.D., 2018. Clinical Study on Modification of Liqi Ningshen Prescription for Treatment of Insomnia Due to Liver Depression and Qi Stagnation. Beijing University of Chinese Medicine, Beijing, China.
Draguhn, A., Hartmann, K., 2006. GABAergic synaptic transmission. Adv. Mol. Cell. Biol. 38, 215–240.
Foltran, R.B., Stefani, K.M., Hocht, C., Diaz, S.L., 2020. Neurochemical, behavioral, and neurogenic validation of a hyposerotonergic animal model by voluntary oral consumption of para-chlorophenylalanine. ACS Chem. Neurosci. 11 (6), 952–959.
Guo, J., Duan, J.A., Tang, Y., Jia, N., Li, X., Zhang, J., 2010. Fast onset of action and the analgesic and sedative efficacy of essential oil from Rhizoma Chuanxiong after nasal administration. Pharmazie 65, 296–299.
He, Y.P., Hao, E.W., Xie, J.L., Wei, W., Qin, J.F., Hou, X.T., Deng, J.G., 2018. Research process on pharmacological effect and substance basis of Perilla frutescens. Chin. Tradit. Herb. Drugs 49, 3957–3968.
Honda, G., Koezuka, Y., Tabata, M., 1988. Isolation of dillapiol from a chemotype of Perilla frutescens as an active principle for prolonging hexobarbital-induced sleep. Chem. Pharm. Bull. 36, 3153–3155.
Ito, K., Ito, M., 2011. Sedative effects of vapor inhalation of the essential oil of Microtoena patchouli and its related compounds. J. Nat. Med. 65, 336–343.
Jianming, J., Zhengshan, W., 2012. Experimental study on sedative and hypnotic effects of perilla water extract on mice. J Taizhou Polytechnic College 12, 102–104.
Jo, K., Suh, H.J., Choi, H.S., 2018. Polygonatum sibiricum, rhizome promotes sleep by regulating non-rapid eye movement and GABAergic/serotonergic receptors in rodent models. Biomed. Pharmacother. 105, 167–175.
Johnson, E.O., Roth, T., Breslau, N., 2006. The association of insomnia with anxiety disorders and depression: exploration of the direction of risk. J. Psychiatr. Res. 40 (8), 700–708.
Jones, B.E., 2005. From waking to sleeping: neuronal and chemical substrates. Trends Pharmacol. Sci. 26, 578–586.
Laurén, H.B., Lopez-Picon, F.R., Korpi, E.R., Holopainen, I.E., 2005. Kainic acid-induced status epilepticus alters GABA A receptor subunit mRNA and protein expression in the developing rat hippocampus. J. Neurochem. 94, 1384–1394.
Lin, B.Q., Ma, J., Liu, Y., Yu, X., Fan, A.R., Zhang, W.Y., Dai, N., Zhang, W., Liu, M., Wen, J.Y., Li, F., 2018. Effects of sini suanzaoren soup recipe on GABAARα1, γ2 and NKCC1 in hippocampus of insomnia rats. J Chin Med Pharmacol 46, 49–53.
Long, Q.H., Zhao, B.B., Wang, P., 2018. Effect of sancao anshen recipe on behavior and neurotransmitter content of depressive insomnia rats. Lishizhen Med Mater Med Res 29, 64–66.
Lou, Z.H., Wang, J.W., Zhang, G.J., 2018. Research progress on effects and mechanisms of traditional Chinese medicine for qi-regulating and their components on digestive system disease. China J Traditional Chinese Med Pharmacy 33, 1004–1007.
Monti, J.M., 2011. Serotonin control of sleep-wake behavior. Sleep Med. Rev. 15, 269–281.
Oh, D.R., Kim, Y., Jo, A., Choi, E.J., Oh, K.N., Kim, J., Kang, H., Kim, Y.R., Choi, C.Y., 2019. Sedative and hypnotic effects of Vaccinium bracteatum Thunb. through the regulation of serotonegic and GABAergic systems: involvement of 5-HT receptor agonistic activity. Biomed. Pharmacother. 109, 2218–2227.
Pujol, J.F., Buguet, A., Froment, J.L., Jones, B., Jouvet, M., 1971. The central metabolism of serotonin in the cat during insomnia. A neurophysiological and biochemical study after administration of P-chlorophenylalanine or destruction of the Raphé system. Brain Res. 29, 195–212.
Shi, Y., Dong, J.W., Zhao, J.H., Tang, L.N., Zhang, J.J., 2014. Herbal insomnia medications that target GABAergic systems: a review of the psychopharmacological evidence. Curr. Neuropharmacol. 12, 289–302.
Sun, Y., Qiao, T., Zhang, L., Liu, S., 2019. Effects of ziyin yangxue anshen decoction on content of GABA signaling pathway and expression of receptor in PCPA-induced insomnia mice. Chin Arch Tradi Chin Med 37, 1925–1928.
Swanson, A.M., Allen, A.G., Shapiro, L.P., Gourley, S.L., 2015. GABAAα1-Mediated plasticity in the orbitofrontal cortex regulates context-dependent action selection. Neuropsychopharmacology 40, 1027–1036.
Taylor, D.J., Lichstein, K.L., Durrence, H.H., 2003. Insomnia as a health risk factor. Behav. Sleep Med. 4, 227–247.
Takemoto, H., Ito, M., Shiraki, T., Yagura, T., Honda, G., 2008. Sedative effects of vapor inhalation of agarwood oil and spikenard extract and identification of their active components. J. Nat. Med. 62, 41–46.
Vergnes, M., Boehrer, A., He, X., Greney, H., Dontenwill, M., Cook, J., Marescaux, C., 2001. Differential sensitivity to inverse agonists of GABAA/benzodiazepine receptors in rats with genetic absence-epilepsy. Epilepsy Res. 47, 43–53.
Wang, X., Ma, G., Xie, J., Pang, G., 2015. Influence of JuA in evoking communication changes between the small intestines and brain tissues of rats and the GABAA and GABAB receptor transcription levels of hippocampal neurons. J. Ethnopharmacol. 159, 215–223.
Winkler, A.1., Auer, C., Doering, B.K., Rief, W., 2014. Drug treatment of primary insomnia: a meta-analysis of polysomnographic randomized controlled trials. CNS Drugs 28, 799–816.
Xu, X.W., L, W., Xiao, P.Q., Sun, Q., Liu, F.L., 2019. Overview of theoretical and clinical research on insomnia with Chinese medicine. World J Sleep Med 7, 1001–1008.
Yin, Z.X., 2009. Studies on the Law of Synergy of Medicines for Regulating Qi Flow in Prescriptions. Nanjing University of Chinese Medicine, Nanjing,China.
Zhong, Y., Zheng, Q., Hu, P., Huang, X.Y., Yang, M., Ren, G.L., Du, Q., Luo, J., Zhang, K. N., Li, J., Wu, H.X., Guo, Y.Y., Liu, S.S., 2019. Sedative and hypnotic effects of compound Anshen essential oil inhalation for insomnia. BMC Compl. Alternative Med. 19, 306.