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文献类型:Article
标题:Raw Pinelliae Rhizoma: examination of sedative and hypnotic effects in mice and chemical analysis
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来源出版物:SLEEP AND BREATHING 卷:27 期:3 页:1143-1153
DOI:10.1007/s11325-022-02714-y 出版时间:JUN 2023 在线发表:SEP 2022 已索引:2022-10-10
入藏号:WOS:000860398000001　　PubMed ID:36166133
来自 Web of Science 核心合集的被引频次:0 所有数据库的被引频次:0
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电子邮件地址：794082446@qq.com;wzyzz2008@126.com
ISSN:1520-9512 eISSN:1522-1709
期刊《SLEEP AND BREATHING》2022 年的 JCR 影响因子为：2.5，五年期刊影响因子:2.7。
2022 年该刊在中科院分区(升级版)：

| | 学科名称 | 分区 | Top 期刊 |
|---|---|---|---|
| 大类 | 医学 | 4 | 否 |
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温州医科大学图书馆（盖章）
检索日期：2023-10-18

Sleep and Breathing
https://doi.org/10.1007/s11325-022-02714-y

PSYCHIATRICS • ORIGINAL ARTICLE

# Raw *Pinelliae Rhizoma*: examination of sedative and hypnotic effects in mice and chemical analysis

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Received: 20 March 2021 / Revised: 21 July 2022 / Accepted: 16 September 2022


## Abstract

**Purpose** Raw "Pinelliae Rhizoma" (RPR) is widely used in Chinese clinics to treat insomnia. This study investigated its underlying sedative and hypnotic mechanisms and main active components.

**Methods** A locomotor activity test was used to evaluate the sedative effects of RPR at three dosages (0.2 g/mL, 0.4 g/mL, and 0.8 g/mL) in mice. Polysomnography was used to assess its ability to improve sleep. Ultra-performance liquid chromatography/time of flight/mass spectrometry (UPLC-TOF–MS) analysis was used to identify the potential active components of RPR.

**Results** Mice in the RPR groups were less active than mice in the vehicle group; this difference was greatest in the 0.8 g/mL RPR group. Compared with the vehicle, 0.8 g/mL RPR increased the duration of rapid eye movement (REM) sleep in the dark phase. In addition, the duration of wakefulness in the 0.8 g/mL RPR group decreased with increasing durations of nonrapid eye movement (NREM) and REM sleep. Compared with diazepam, 0.8 g/mL RPR increased REM sleep duration in both the light and dark phases and increased the number of transitions both from NREM sleep to REM sleep and from REM sleep to wakefulness. A total of 33 RPR constituents, including 15 alkaloids, were identified.

**Conclusion** The results preliminarily indicated that RPR exerts sedative and hypnotic effects in mice, mainly leading to improvements in REM sleep. These effects are possibly due to the alkaloid constituents of RPR.

**Keywords** *Pinellia ternata* · Locomotion · Polysomnography · Liquid chromatography · Hypnotics · Sedatives

## Introduction

Insomnia presents not only a large health care challenge but also major social and economic consequences. Cognitive-behavioral therapy for insomnia (CBT-I) and pharmacotherapy are the main therapies at present [1]. Many obstacles prevent the full implementation of CBT-I as the first-line treatment for insomnia, such as limited access to treatment and the lag between therapy initiation and the observed effects (typically 5–8 weeks). Moreover, current drug therapies have issues with side effects. The Food and Drug Administration (FDA) labels carry specific warnings and precautions for drugs approved for insomnia. Thus, the development of cost-effective treatments with fewer side effects has become a focus worldwide.

For over 2000 years, Pinelliae Rhizoma (PR) has been used alone or as a key constituent in many effective formulations for insomnia [2]. Wu reported that ethanol fractions of PR preparations had sedative and hypnotic effects [3]. Our previous studies have also confirmed the hypnotic effect of PR products [4, 5]. Nevertheless, most of

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Published online: 27 September 2022

the identified chemical markers of PR were significantly decreased by 10- to 100-fold after alum processing [6]. Fang reported that Raw Pinelliae Rhizoma (RPR) has a better sedative effect than its preparations [7]. Indeed, RPR seems to be an ideal treatment for insomnia. However, its mechanism remains poorly understood. Thus, we aimed to confirm the sedative and hypnotic effects of RPR and determine its role in sleep/wake regulation. We also analyzed the chemical components of RPR to identify possible sedative/hypnotic compounds for further study.

## Materials and methods

### Preparation of drugs and reagents

PR is the dried tuber of *Pinellia ternata* (Thunb.) Breit. RPR is a PR product that has been washed and dried but does not undergo any additional processing and thus retains as many effective ingredients as possible. The RPR used in this study was obtained from the traditional Chinese medicine dispensary of the First Affiliated Hospital of Wenzhou Medical University (Zhejiang, China) and identified by the deputy director of the traditional Chinese medicine (TCM) pharmacy Xiaochen Chen. Diazepam (DZP, batch number AH120701; Shanghai Xudong Haipu Pharmaceutical Co. Ltd., Shanghai, China) was used as a positive control. Sterile water for injection (batch number M11073107; Fuzhou Haiwang Pharmaceutical Co., Ltd., Fujian, China) was used as a vehicle. All drugs were stored at 4℃.

For the animal experiments, 80 g of RPR was soaked in 300 mL of the vehicle and decocted for 1 h. The filtered liquid was then concentrated to a volume of 100 mL (drug concentration, 0.8 g/mL RPR liquor; administration dose, 16 g/kg of the crude drug). A total of 50 mL of 0.8 g/mL RPR stock solution was then diluted with 50 mL of the vehicle to yield a 0.4 g/mL stock solution (8 g/kg of the crude drug). A further dilution using the same procedure yielded a 0.2 g/mL RPR stock solution (4 g/kg of the crude drug). In addition, one DZP tablet (2.5 mg) was dissolved in 25 mL of the vehicle to yield a DZP stock solution at a concentration of 0.1 mg/mL. Mice were treated with intragastric (i.g.) administration of 0.2 mL of stock solution per 10 g of body weight once daily for 14 days.

For ultra-performance liquid chromatography/time of flight/mass spectrometry (UPLC-TOF–MS) analysis, RPR was powdered and subjected to a cold soak overnight in acetonitrile: water (3:1, v/v) was extracted for 45 min by ultrasonication and then centrifuged for 10 min at 11,180 g. The supernatant was filtered through a 0.22-μm microporous membrane to obtain samples suitable for testing.

### Animals and housing

Male C57BL/6 J mice (only male mice were used to eliminate the influence of sex) weighing 22–24 g were purchased from the Laboratory Animal Centre of the Chinese Academy of Sciences (Shanghai, China). The mice were provided with ad libitum food and water and were entrained to an automatically controlled 12:12-h light/dark cycle (lights on from 7:00 AM to 7:00 PM, illumination intensity approximately 100 lx). The ambient temperature was set to 22 ± 0.5℃, and the relative humidity was controlled in the range of 60 ± 2%.

### Sedative effects: locomotion activity test

The mice were randomly divided into five groups ($n = 4$) as follows: the placebo (vehicle, 0.5 mL i.g.), positive control (DZP, 0.1 mg/mL i.g.), low-dose RPR (0.2 g/mL i.g.), moderate-dose RPR (0.4 g/mL i.g.), and high-dose RPR (0.8 g/mL i.g.). The method of administration and dosages used were based on the results of a preliminary experiment [7].

Mice were housed in individual 28 × 16.5 × 13 cm cages, and each cage was equipped with a passive infrared sensor (Biotex; Kyoto, Japan) mounted 17.5 cm above the floor to record the mouse movements during tests, as previously reported [8]. Sensors in all cages were connected to a computer in parallel. The locomotion data were transferred to the computer every 5 min. All mice were habituated to their cage for 24 h before the start of behavior recording.

The hypnotic effect of drugs can be more clearly observed during the dark phase because mice sleep during the daytime and are active at night. Drugs were administered i.g. at 7:00 PM each day (the time the lights were turned off) for 14 days. Mouse locomotor activity was recorded. The dark phase was defined as lasting from 7:00 PM to 7:00 AM the next day, and the light phase was defined as lasting from 7:00 AM to 7:00 PM.

### Hypnotic effects: polysomnographic recordings and sleep/wake state analysis

The mice were randomly divided into three groups ($n = 3$): the placeo (vehicle, 0.5 mL i.g.), positive control (DZP, 0.1 mg/mL i.g.), and high-dose RPR group (0.8 g/mL i.g.). The optimal dose of RPR was based on the results of the locomotor activity test.

After anesthesia with chloral hydrate (360 mg/kg, i.p.), the mice were implanted with electrodes for electroencephalography (EEG) and electromyography (EMG) in preparation for polysomnographic recording. EEG electrodes with two stainless-steel screws (1 mm in diameter) were inserted through the skull (anteroposterior, + 1.0 mm;

mediolateral, − 1.5 mm from bregma or lambda) based on the Franklin and Paxinos atlas [9]. EMG electrodes with two insulated stainless-steel Teflon-coated wires were placed bilaterally into the trapezius muscles. All electrodes were attached to a microconnector and fixed to the skull using dental cement [10]. After surgery, each mouse was allowed to recover for 7 days.

The mice were habituated to the recording cable for 24 h before polysomnographic recording and then individually housed in the corresponding recording room. The drugs were administered i.g. for 14 days at 7:00 PM each day. EEG and EMG recordings were performed by means of a slip-ring designed to allow for free movement of the mice [11]. Sleep and wakefulness states were monitored for a period of 24 h after 14 days of intervention, beginning at 7:00 PM.

After completion of the recordings, sleep/wake states were automatically classified offline using SleepSign (based on 4-s epochs) into three categories, namely, wakefulness, rapid eye movement (REM) sleep, and nonrapid eye movement (NREM) sleep, according to standard criteria. Finally, the extracted episodes ("bouts") of the three stages were verified visually and corrected if necessary.

### Chemical composition: UPLC-TOF–MS analysis

UPLC-TOF–MS analysis was performed using a Waters Acquity™ Ultra Performance LC system (Waters, USA) equipped with a Waters SYNAPT G1 Q-TOF mass spectrometer. Chromatographic separation was carried out at 40℃ using Acquity™ UPLC HESS C18 columns (100 mm × 2.1 mm, 1.8 μm). The mobile phase consisted of acetonitrile (A) and water (B) with a linear gradient. Separation was performed from 0 to 11.5 min (5–95% A) in positive ionization mode and from 0 to 17 min (5–95% A) in negative ionization mode. The flow rate was 0.4 mL/min, and the sample injection volume was 2 μL. Mass spectra were simultaneously acquired using electrospray ionization with positive and negative ionization modes (ES + and ES −) under the following conditions: capillary voltages, 3 kV and 2.5 kV (ES + and ES −, respectively); cone voltage, 40 V; ion source temperature, 100 ℃; desolvation gas, 800 L/h; cone gas volume flow, 50 L/h; precursor ion collision energy, 6 eV; quality scanning range, 50–1,000 Da; and scan time, 0.2 s. All data acquisition and analyses were performed using Waters Mass Lynx v4.1 software.

### Statistical analysis

All data are expressed as means ± SEMs. Statistical analysis was performed with SPSS 22.0. The main effects of time and group and the interaction of time × group on locomotor activity were analyzed by repeated-measures analyses of variance (ANOVAs). The locomotion levels of different groups during the same time, the duration of sleep/wake stages, number of stage transitions, and number of bouts were analyzed by one-way ANOVAs followed by post hoc least significant difference (LSD) tests. In all cases, $P$ values < 0.05 were considered statistically significant.

## Results

### Sedative effects of RPR in mice

Figure 1a shows the locomotion levels in the dark phase over the 14-day experiment. An interaction of group × time was observed ($F = 7.662$, $P < 0.001$). Subsequent analysis revealed a significant effect of time within the 0.8 g/mL RPR group ($F = 5.831$, $P = 0.031$) and the vehicle group ($F = 17.684$, $P = 0.001$).

Further analysis showed that mice in the 0.2 g/mL RPR group showed less locomotion than mice in the vehicle group on days 1, 2, and 6 ($P < 0.05$) and 7–14 ($P < 0.01$) and less locomotion than mice in the DZP group on days 2, 5, 6, 7, and 14 ($P < 0.05$) and 12–13 ($P < 0.01$). Mice in the 0.4 g/mL RPR and 0.8 g/mL RPR groups showed less locomotion than mice in the vehicle group on days 7 ($P < 0.05$) and 8–14 ($P < 0.01$) and less locomotion than mince the DZP group on days 12 ($P < 0.05$) and 13–14 ($P < 0.01$).

Figure 1b presents the locomotor activity of mice during the light phase over 14 days. A significant main effect of time was observed ($F = 13.572$, $P < 0.001$). The 0.2 g/mL RPR group exhibited a similar effect of time ($F = 5.792$, $P = 0.031$). Mice in the 0.2 g/mL RPR group were more active than mice in the vehicle group on day 10 ($P < 0.05$) but were less active on day 14 ($P < 0.05$). Mice in the 0.2 g/Ml RPR group were more active than those in the DZP group on days 1 and 10 ($P < 0.05$). Compared with the vehicle group, the locomotion of the 0.4 g/mL RPR group was higher on days 1 ($P < 0.01$), 2, and 10 ($P < 0.05$). In the 0.8 g/mL RPR group, locomotion decreased on day 14 ($P < 0.05$) when compared with the vehicle group.

Within the dark phase, DZP and all RPR dose treatments reduced locomotor activity compared with administration of the vehicle. This difference increased over time and peaked on day 10 (Fig. 1c). The locomotor activity of the RPR and DZP groups was significantly decreased during the initial 10 h after administration compared with that of the vehicle group, especially at hours 1–5 (hours 1–4, $P < 0.01$; hour 5, $P < 0.05$). Compared with DZP, 0.2 g/mL RPR treatment reduced locomotion by 83.42% at hour 9 ($P < 0.05$), 0.4 g/mL RPR treatment reduced locomotion by 48.37% at hour 11 ($P < 0.05$), and 0.8 g/mL RPR treatment reduced locomotion by 79.38% at hour 7 ($P < 0.05$) and 86.54% at hour 10 ($P < 0.05$).

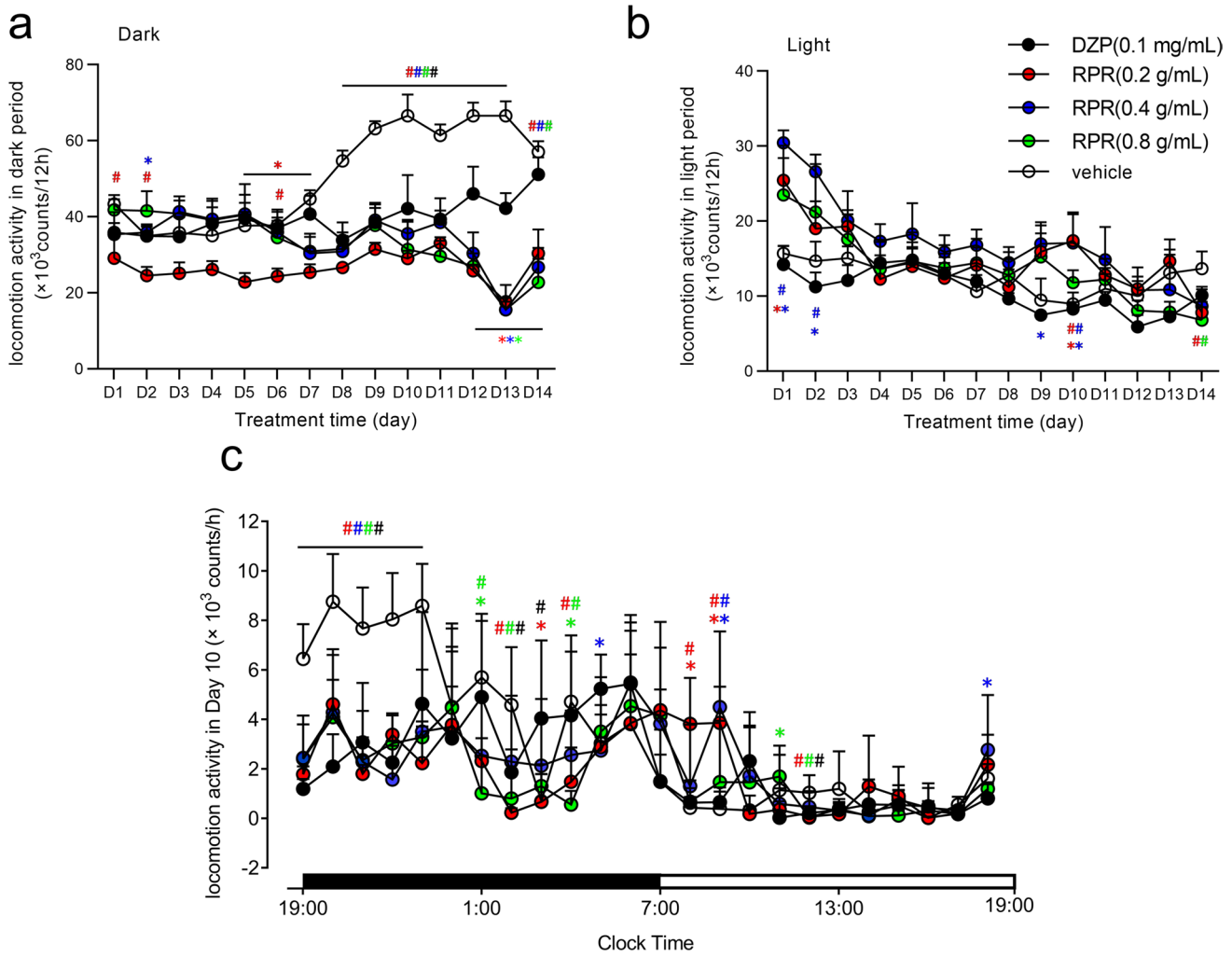



**Fig. 1** RPR decreased the locomotor activity of mice. **a** Amount of locomotor activity in the 12-h dark phase. **b** Amount of locomotor activity in the 12-h light phase. **c** Hourly locomotor activity on day 10 of treatment. Drugs were administered i.g. at 7:00 PM each day (the time lights were turned off) for 14 days. The white, red, blue, green, and black circles show the profiles for the vehicle, 0.2 g/mL RPR, 0.4 g/mL RPR, 0.8 g/mL RPR, and DZP treatments, respectively. Values are means ± SEMs. * and # indicate significant differences compared with DZP and vehicle treatments, respectively. Data were analyzed with repeated-measures ANOVAs or one-way ANOVAs followed by post hoc least significant difference (LSD) tests. Single symbols, $P < 0.05$; double symbols, $P < 0.01$; DZP, diazepam; RPR, raw Pinelliae Rhizoma; vehicle, sterile water for injection

## Hypnotic effects of RPR in mice

### Duration of the three sleep/wake stages

Although the 0.2 g/mL RPR and 0.8 g/mL RPR doses both exhibited sedative effects, we found that the 0.8 g/mL RPR dose produced better sleep continuity and exhibited a greater cumulative effect. The activity under 0.8 g/mL RPR treatment decreased with time and was less than that of the 0.2 g/mL RPR group in the last 4 days. We believe that sleep continuity is a very important feature in the treatment of chronic insomnia and therefore selected the 0.8 g/mL RPR dose for the hypnotic effect experiments. Time-course changes are illustrated in Fig. 2a–c. Compared with the DZP group, 0.8 g/mL RPR treatment increased the duration of REM sleep at hour 8 ($P < 0.05$) and hour 15 ($P < 0.01$). Compared with the vehicle group, 0.8 g/mL RPR treatment decreased the duration of wakefulness at hours 4, 8, and 23 ($P < 0.05$). In this RPR group, the duration of REM sleep increased at hours 4, 5, and 23 ($P < 0.01$), as well as hours 8 and 21 ($P < 0.05$), and the duration of NREM sleep increased at hours 4 and 8 ($P < 0.05$). Moreover, the duration of wakefulness in the DZP group was increased at hour 11 ($P < 0.05$) and decreased at hour 23 ($P < 0.05$), while the duration of NREM sleep was decreased at h 11 ($P < 0.05$) and increased at h 23 ($P < 0.05$).

To further investigate the influence of 0.8 g/mL RPR treatment on mouse sleep/wake stage, we calculated the amount of time spent in each vigilance state during the 12-h dark and

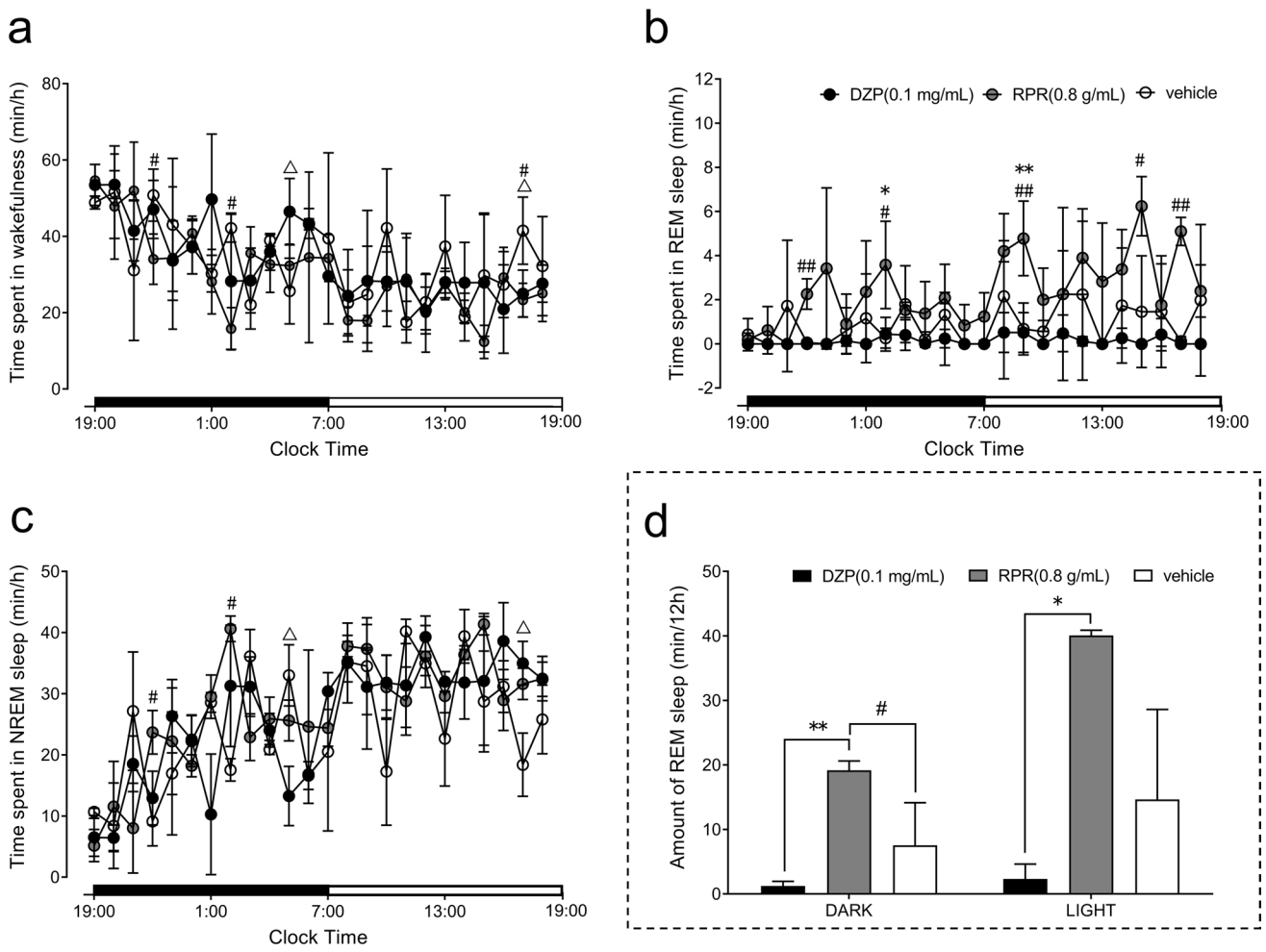


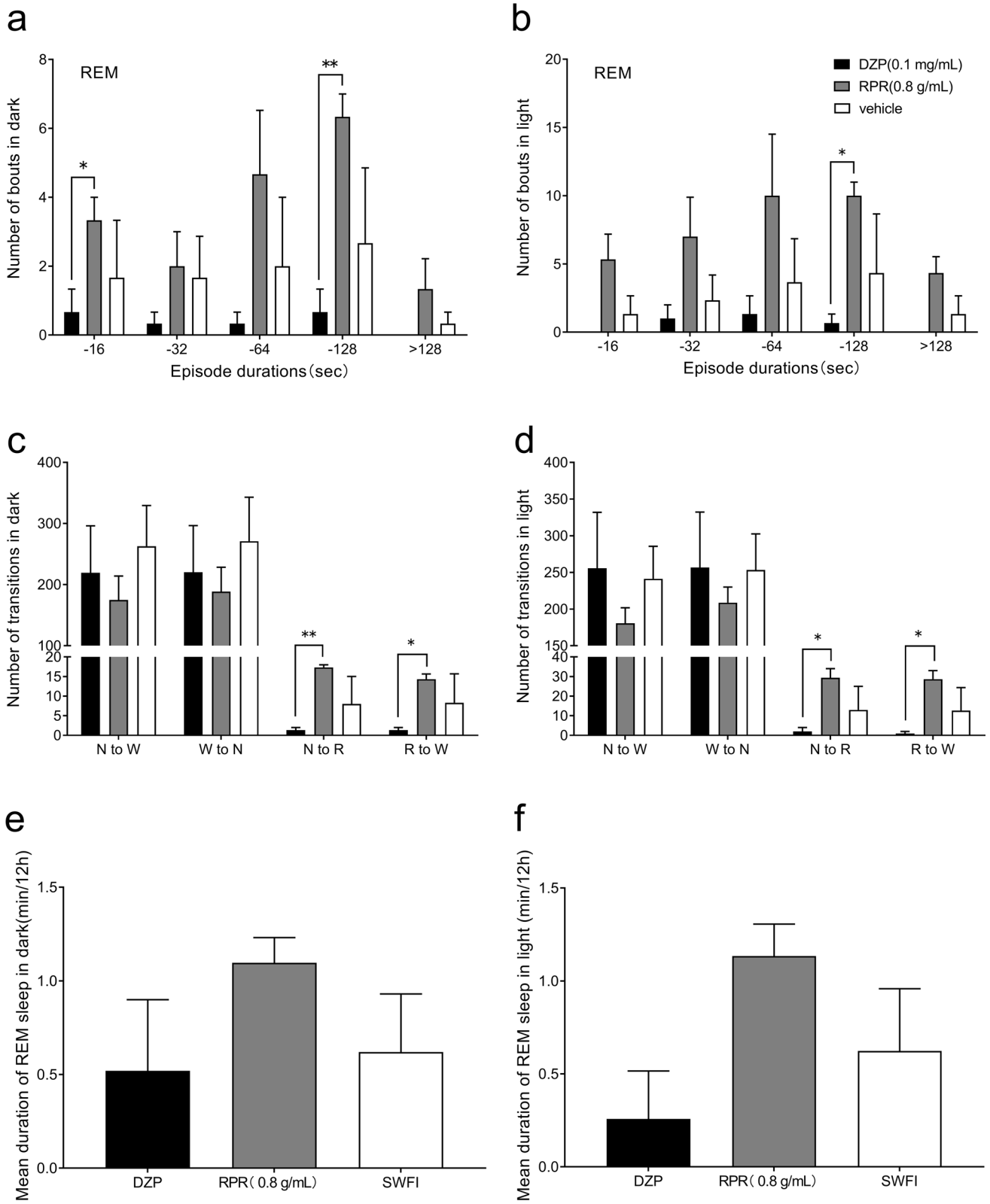

**Fig. 2** RPR increased the REM sleep duration of mice. **a** Time course of changes in wakefulness. **b** Time course of changes in REM sleep. **c** Time course of changes in NREM sleep. **d** Total REM sleep duration during the dark and light phases. Drugs were administered i.g. at 7:00 PM each day (the time that lights were turned off) for 14 days. Each circle represents the hourly average duration in each sleep/wake stage. The horizontal open and filled bars on the *x*-axes indicate the dark phases and light phases, respectively. The white-, black-, and gray-filled circles/bars represent the vehicle, DZP, and 0.8 mg/mL RPR treatments, respectively. Values are means ± SEMs. * and # indicate significant differences compared with DZP and vehicle treatment, respectively, and △ indicates significant differences between DZP and vehicle treatment, all assessed by two-way ANOVAs followed by LSD tests. Single symbols, $P<0.05$; double symbols, $P<0.01$; DZP, diazepam; RPR, raw Pinelliae Rhizoma; vehicle, sterile water for injection; NREM, nonrapid eye movement; REM, rapid eye movement

light phases. The 0.8 g/mL RPR treatment increased REM sleep duration in both the light phase ($P<0.05$) and the dark phase ($P<0.01$) compared with that in the DZP group. The 0.8 g/mL RPR treatment also increased REM sleep duration ($P<0.05$) in the dark phase compared to that of the vehicle group (Fig. 2d).

### RPR-induced alterations in sleep architecture

To assess changes in the sleep–wake profile induced by 0.8 g/mL RPR treatment, we recorded the number of bouts of each sleep/wake state and transitions between sleep/wake states. As shown in Fig. 3a, 0.8 g/mL RPR treatment increased the number of bouts of REM sleep with durations of 0–16 s ($P<0.05$) and 64–128 s ($P<0.01$) during the dark phase and increased the number of bouts of REM sleep with durations of 64–128 s ($P<0.05$) during the light phase, compared with those of the DZP group (Fig. 3b).

The numbers of stage transitions are shown in Fig. 3c, d. During both the dark and light phases, 0.8 g/mL RPR treatment increased the number of stage transitions from NREM sleep to REM sleep (dark, $P<0.01$; light, $P<0.05$) and from REM sleep to wakefulness (dark, $P<0.05$; light, $P<0.05$), compared with those of the DZP group. As shown in Fig. 3e, f, 0.8 g/mL RPR treatment increased the mean duration of REM sleep during both the dark phase and the light phase. However, this difference was not significant, which may be due to the small sample size.


a
REM
Number of bouts in dark
Episode durations (sec)
-16
-32
-64
-128
>128
b
REM
DZP(0.1 mg/mL)
RPR(0.8 g/mL)
vehicle
Number of bouts in light
Episode durations (sec)
c
Number of transitions in dark
N to W
W to N
N to R
R to W
d
Number of transitions in light
e
Mean duration of REM sleep in dark(min/12h)
DZP
RPR( 0.8 g/mL)
SWFI
f
Mean duration of REM sleep in light (min/12h)

◂**Fig. 3** RPR increased the number of bouts of REM sleep and transitions from NREM sleep to wakefulness. **a** RPR increased the number of bouts of REM sleep with durations of 0–16 s and 64–128 s in the dark phase. **b** RPR increased the number of bouts of REM sleep with durations of 64–128 s in the light phase. **c** RPR increased the numbers of transitions from NREM sleep to REM sleep and from REM sleep to wakefulness in the dark phase. **d** RPR increased the numbers of transitions from NREM sleep to REM sleep and from REM sleep to wakefulness in the light phase. **e** Mean duration of REM sleep in the dark phase. **f** Mean duration of REM sleep in the light phase. Drugs were administered i.g. at 7:00 PM each day (the time that the lights were turned off) for 14 days. The white, black, and gray colors represent the vehicle, DZP, and 0.8 mg/mL RPR treatments, respectively. N, R, and W indicate non-REM, REM, and wakefulness, respectively. Values are means ± SEMs. * indicates significant differences compared with DZP treatment, as assessed by ANOVAs followed by LSD tests. Single symbols, $P < 0.05$, double symbols, $P < 0.01$; DZP, diazepam; RPR, raw Pinelliae Rhizoma; vehicle, sterile water for injection

### Characteristics of the chemical constituents of RPR

Multistage MS and high-resolution liquid chromatography were performed in both negative- and positive-ion modes to completely elucidate the chemical composition of RPR. The negative-base-peak MS spectrum and the positive-base-peak MS spectrum are shown in Fig. 4a, b. We identified 33 constituents based on precise mass and relative ion-abundance analyses of the target peaks (Table 1), including 15 alkaloids, 8 fatty acids, 3 phenylpropanoids, 2 flavonoids, 1 saccharide, 1 cerebroside, and 3 other compounds.

## Discussion

The locomotor activity test is typically used to assess the activity of animals administered specific drugs. Inhibition of locomotion after drug administration may suggest a sedative effect. Our research found a temporal effect of receiving the vehicle and 0.8 g/mL RPR) treatments, during the dark phase. The locomotion level in the vehicle group decreased significantly starting on day 8 and peaked on day 10, while the DZP group did not show such decrease. In our opinion, intragastric administration and single-cage housing are two possible reasons for the increase in the amount of mouse locomotion in the vehicle group. Mice are sensitive in nature. Previous studies have reported that intragastric

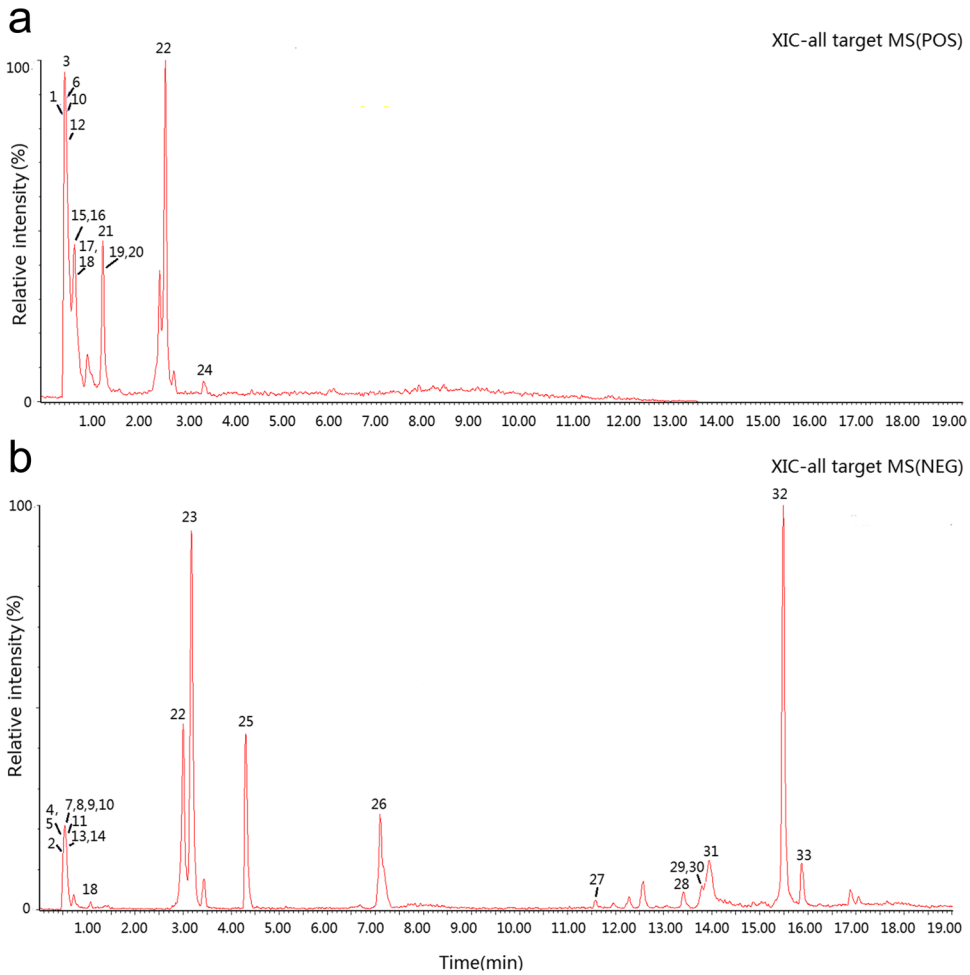



**Fig. 4** High-performance liquid chromatography-electrospray (ES) ionization/time-of-flight (TOF) mass spectrometer (MS) ion chromatograms of RPR extract. **a** ES + TOF MS spectrum. **b** ES-TOF MS spectrum. RPR, raw Pinelliae Rhizoma

**Table 1** Identified chemical components in RPR and their biological activities

| Peak No | Rt (min) | [M + H]/[M-H] (m/z)[1] | Relative abundance[2] (%) | Formula | Identification | Component type |
|---|---|---|---|---|---|---|
| 1 | 0.49 | 116.0694 | 0.876804 | $C_5H_9NO_2$ | Proline* | Alkaloid |
| 2 | 0.50 | /111.0134 | /1.916323103 | $C_4H_4N_2O_2$ | Uracil* | Alkaloid |
| 3 | 0.51 | 175.1184 | 28.89012 | $C_6H_{14}N_4O_2$ | L-arginine* | Alkaloid |
| 4 | 0.52 | /188.0600 | /1.351351351 | $C_7H_{11}NO_5$ | *N*-acetylglutamate | Other |
| 5 | 0.53 | /165.0464 | /0.755840586 | $C_9H_{10}O_3$ | 3,4-Dihydroxy cinnamylalcohol | Phenylpropanoid |
| 6 | 0.53 | 156.0820 | 0.443951 | $C_6H_9N_3O_2$ | Histidine* | Alkaloid |
| 7 | 0.54 | /131.0868 | /0.328294396 | $C_5H_{12}N_2O_2$ | Ornithine* | Alkaloid |
| 8 | 0.54 | /149.0492 | /0.313024889 | $C_9H_{10}O_2$ | E-p-coumarylalcohol | Phenylpropanoid |
| 9 | 0.54 | /146.0486 | /1.114673996 | $C_5H_9NO_4$ | Glutamic acid | Alkaloid |
| 10 | 0.55/0.54 | 176.1151/174.0924 | 28.91232/0.419911437 | $C_6H_{13}N_3O_3$ | Citrulline | Alkaloid |
| 11 | 0.55 | /117.0237 | /0.656588792 | $C_4H_6O_4$ | Succinic acid* | Fatty acid |
| 12 | 0.56 | 118.0852 | 1.109878 | $C_5H_{11}NO_2$ | Valine | Alkaloid |
| 13 | 0.57 | /341.1109 | /0.847457627 | $C_{16}H_{22}O_8$ | Coniferin | Phenylpropanoid |
| 14 | 0.57 | /341.1007 | /0.847457627 | $C_{12}H_{22}O_{11}$ | Sucrose | Saccharide |
| 15 | 0.68 | 244.0946 | 1.243063 | $C_9H_{13}N_3O_5$ | Cytidine | Alkaloid |
| 16 | 0.68 | 182.0803 | 0.344062 | $C_9H_{11}NO_3$ | Tyrosine | Alkaloid |
| 17 | 0.71 | 136.062 | 1.342952 | $C_5H_5N_5$ | Pedatisectine B | Alkaloid |
| 18 | 0.71/1.06 | 284.099/282.0878 | 1.021088/0.259581616 | $C_{10}H_{13}N_5O_5$ | Guanosine | Alkaloid |
| 19 | 1.28 | 120.0809 | 0.710322 | $C_4H_9NO_3$ | L-threonine | Alkaloid |
| 20 | 1.28 | 166.0859 | 2.27525 | $C_9H_{11}NO_2$ | Phenylalanine | Alkaloid |
| 21 | 1.29 | 268.1058 | 6.304107 | $C_{10}H_{13}N_5O_4$ | Adenosine* | Alkaloid |
| 22 | 2.57/3.00 | 565.1574/563.1415 | 25.99334/8.344785463 | $C_{26}H_{28}O_{14}$ | 6-C-Xylopyranosyl-8-C-galactopyranosylapigenin | Flavonoid |
| 23 | 3.17 | /563.1414 | /16.49106734 | $C_{26}H_{28}O_{14}$ | 6-C-Galactopyranosyl-8-C-xylopyranosylapigenin | Flavonoid |
| 24 | 3.35 | 696.5405 | 0.532741 | $C_{40}H_{73}NO_8$ | 1-O-Glucosyl-N-2′-palmitoyl-4,8-sphingodienine | Cerebroside |
| 25 | 4.31 | /740.4995 | /13.87234692 | $C_{42}H_{79}NO_9$ | 1-O-β-D-Glucopyranosyl-(2S,3R,4E,-8E)-2-[2'(R)-hydroxyl-octadecanoyl-amino]-4,8-octadecadiene-1.3-diol | Other |
| 26 | 7.10 | /329.2361 | /8.306611696 | $C_{18}H_{34}O_5$ | Pinellic acid | Fatty acid |
| 27 | 11.57 | /277.2230 | /0.641319285 | $C_{18}H_{30}O_2$ | α-Linolenic acid | Fatty acid |
| 28 | 13.42 | /255.2334 | /1.229195297 | $C_{16}H_{32}O_2$ | Hexadecanoic acid/palmitic acid | Fatty acid |
| 29 | 13.80 | /281.2520 | /0.45045045 | $C_{18}H_{34}O_2$ | 8-Octadecenoic acid | Fatty acid |
| 30 | 13.80 | /281.2538 | /0.45045045 | $C_{18}H_{34}O_2$ | Oleic acid | Fatty acid |
| 31 | 13.97 | /293.1832 | /7.924874027 | $C_{17}H_{26}O_4$ | Gingerol | Other |
| 32 | 15.49 | /339.3356 | /30.94365552 | $C_{22}H_{44}O_2$ | Docosanoic acid | Fatty acid |
| 33 | 15.86 | /279.2349 | /2.534738128 | $C_{18}H_{32}O_2$ | Linoleic acid | Fatty acid |

*Component exerts sedative and hypnotic effects

1. Data on the left side of the slash ("/") are m/z of [M + H]. Data on the right side of the slash ("/") are m/z of [M-H]

2. The relative abundance value was obtained by a normalization method

*RPR* raw Pinelliae Rhizoma

administration of the drug can increase the locomotion level of mice [12–14]. These studies only examined the data collected 30 min after intragastric stimulation, we believe that long-term intragastric stimulation can increase the baseline level of locomotor activity in mice. Manouze reported housing mice in isolation produced a marked increase in anxiety index scores, and Namrata Joshi reported that an absence of social or physical stimulation increased some measures of anxiety-like behavior in the EOFT, but decreased anxiety-like behavior in the EPM task [15]. These results indicate individual housing likely induces anxiety and that one of the manifestations of anxiety in mice is increased activity. DZP is commonly used as a sedative drug in animal experiments. Marina Pádua-Reis reported that DZP can reduce the total

duration of animal movement [16]. Our results are consistent with this finding.

Moreover, the locomotion level in the 0.8 g/mL RPR group decreased steadily during the experimental period, reaching a minimum on day 10; similar effects were not observed for the other two doses of RPR, namely 0.2 g/mL RPR and 0.4 g/mL RPR. Thus, at sufficiently high concentrations, intragastric administration of RPR may offset the increases in mouse activity via its sedative effect. In the light phase, there were fewer active mice in the 0.8 g/mL RPR group than in the 0.2 g/mL RPR and 0.4 g/mL RPR groups, also suggesting that RPR at higher concentrations may have a stronger sedative effect. The experimental results indicate a cumulative effect of high concentrations of RPR; therefore, continuous dosing over at least 7 days might be a reasonable treatment.

Hourly locomotion data on day 10 showed that within 12 h of administration, locomotion in the RPR group decreased significantly compared with that in the vehicle group. In addition, from hour 7 to hour 11 after administration, the locomotor activity of the RPR group was lower than that of the DZP group, indicating that RPR mainly exerts effects in the 12 h after administration. Overall, the results indicated that RPR has the potential to be an ideal complement to sedative therapy. These results are in agreement with those of Fang's study [7].

Sleep can be classified into the following stages: wakefulness, NREM sleep, and REM sleep. Sleep always starts with NREM sleep and then turns into REM sleep, and one can wake from either NREM sleep or REM sleep. Our time-course data showed that 0.8 g/mL RPR reduces wakefulness and increases sleep durations, especially REM sleep duration. In our study, the hypnotic effect began 3 h after administration and continued until 23 h. This pattern suggests that RPR does not take effect quickly but has a long-lasting effect, which may be suitable for patients with sleep continuity issues. However, this pattern is not completely consistent with our results regarding the locomotor activity. We speculate that the sedative effect and the hypnotic effect of RPR have different mechanisms.

Further analysis showed that 0.8 g/mL RPR increased the total duration of REM sleep over 12 h, increased the number of REM-sleep bouts, and promoted the transition from NREM to REM sleep. These results suggest that RPR mainly acts by regulating REM sleep, which is the key finding of this experiment. These results indicate that RPR is potentially an ideal supplementary drug for the treatment of insomnia.

In recent years, liquid chromatography−mass spectrometry (LC−MS) has become an essential tool for the analysis of the constituents of herb constituents [17, 18]. In the present study, negative- and positive-base-peak spectra of RPR were obtained and 33 constituents were identified including 15 alkaloids, 8 fatty acids, 3 phenylpropanoids, 2 flavonoids, 1 saccharide, 1 cerebroside, and 3 other compounds, as shown in Table 1. This result is similar to those of other studies on PR [6, 19, 20], which also reported alkaloids, such as L-arginine, adenosine, proline, uracil, histidine, glutamic acid, cytidine, tyrosine, guanosine, L-threonine, and phenylalanine, as well as other components such as succinic acid. In this study, the content of succinic acid met the requirements of the Chinese Pharmacopoeia for PR. However, alkaloids such as ephedrine, trigonelline, and hypoxanthine were not found in our study. This is consistent with the current understanding that the components of PR vary significantly among populations, but that the main components are consistent with those described in the latest review [21].

Deng confirmed that *Pinellia* total alkaloids regulate the GABAergic system in epileptic rats [22], this system also plays an important role in sleep. Thus, alkaloids in PR may also regulate sleep by modulating the GABAergic system. Specifically, L-arginine accounted for 28.89% of the RPR components in this study and is a common substrate of endothelial nitric oxide synthase and arginases. Hess reported that serum L-arginine levels decreased in major depression [23]. Additionally, Phuong V. Tran reported that intracerebroventricular injection of L-arginine attenuated acute isolation stress by inducing sleep-like behavior [24]. Adenosine was the second most abundant ingredient in this study, accounting for 6.3% of the components, and its hypnotic effects are well established [25]. In light of these findings, we speculate that alkaloids are the main active constituents of RPR and act on multiple targets to produce synergistic therapeutic effects against insomnia.

Some limitations of the present study should be noted. Due to the small sample size of this experiment, the results are preliminary, and subsequent experiments with a greater sample size are needed. Although we have provided preliminary confirmation of the sedative and hypnotic effects of RPR, the pharmacokinetics and molecular mechanism remain elusive. However, the glutamatergic neurons in the caudal laterodorsal tegmental-sublaterodorsal nucleus (cLDT-SLD) and theγ-aminobutyric acid (GABA)ergic system may be potential targets. Furthermore, considering the cytotoxicity and its reproductive toxicity of PRR, which acts as a strong stimulant of mucous membranes [26, 27], determining a safer way to use RPR is of great clinical importance.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s11325-022-02714-y.

**Acknowledgements** We appreciate the support from the Department of Pharmacology, School of Basic Medical Sciences, Fudan University, Shanghai, China.

**Authors' contributions** Conceptualization: Zhengzhong Yuan; methodology: Zhengzhong Yuan; formal analysis and investigation: Sisi Lin, Haipeng Chen, and Chengrou Jiang; data curation: Sisi Lin, Haipeng Chen, and Hui Yang; writing—original draft preparation: Sisi Lin, Haipeng Chen, and Bo Nie; writing—review and editing: Bo Nie and Qinglai Wang; Funding acquisition: Zhengzhong Yuan and Sisi Lin; Resources: Zhengzhong Yuan and Qinglai Wang; and supervision: Zhengzhong Yuan and Qinglai Wang. The authors read and approved the final manuscript.

**Funding** This work was supported by the Outstanding Youth Fund Project for Chinese Medicine of Zhejiang Province, China (Grant No. 2011zq014), and the 2019 incubation project of the First Affiliated Hospital of Wenzhou Medical University (Grant No. FHY2019041).

**Data availability** The data analyzed during the current study are available from the corresponding author on reasonable request.

## Declarations

**Ethics approval** The present study was approved by the experimental laboratory animal Ethics Committee of the Laboratory Animal Center at Whenzhou Medical University (wydw 2016–026) and was performed in accordance with the ethical standards provided in the 1964 Declaration of Helsinki and its later amendments.

**Conflict of interest** The authors declare no competing interests.

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