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journal homepage: <https://www.sciopen.com/journal/2097-0765>Essential oil and saponin from *Semen Platycladi* exert sedative-hypnotic effect on ICR mice by regulating multi-targets of serotonergic and GABAergic systemRuifang Zhong^{a,1}, Chunyan Shen^{b,1}, Wei Zhu^{c,d,*}, Jianguo Jiang^{a,*}^a School of Food Science and Engineering, South China University of Technology, Guangzhou 510640, China^b Southern Medical University, School of Traditional Chinese Medicine, Guangzhou 510515, China^c The Second Affiliated Hospital, Guangzhou University of Chinese Medicine, Guangzhou 510120, China^d State Key Laboratory of Dampness Syndrome of Chinese Medicine, The Second Affiliated Hospital of Guangzhou University of Chinese Medicine, Guangzhou 510120, China

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ABSTRACT

Semen Platycladi (SP) is the dried ripe kernel of *Platycladus orientalis* (L.) Franco, has been used for insomnia treatment for bimillennium in China, which mechanism is not fully understood. The aim of this study was to explore the sleep-promoting mechanism of essential oil and saponins from SP. As 2 main bioactive constituents of SP, essential oil (SPO) and saponin (SPS) were extracted, then was given to *p*-chlorophenylalanine (PCPA)-induced insomnia mice by intragastric administration. Then, the behavioral tests, neurotransmitter receptors, cytokines and hormone in brain were investigated. Behavior test showed that SPO and SPS exhibited sleep-enhancing effect through suppressing depression, shortening the onset time while prolonging the sleep duration in insomnia mice. Also SPO and SPS up-regulated serotonin (5-HT) receptors in serotonergic neurons, increased glutamic acid decarboxylase (GAD) content in GABAergic neurons, and stimulated γ -aminobutyric acid (GABA) receptors expression to enhance the synaptic inhibition. Moreover, they could down-regulated the cytokines and rebalanced hormone expressions. Although both SPO and SPS exerted sleep-promoting, they had different focusing targets. SPS had stronger effect on neurotransmitter receptors regulation while SPO had better hormone rebalanced ability. Briefly, SPO and SPS exerted sedative-hypnotic effect in insomnia mice through modulating multi-targets in serotonergic and GABAergic system.

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1. Introduction

Sleep is a vital biological process that is regulated through complex interactions between multiple brain regions and neuromodulators^[1]. Sleep disorders or insomnia is one of the psychiatric problems in global. The prevalence of insomnia symptoms is as high as 50% in the general population, and even higher prevalence of older adults^[2-3]. Sleep deprivation has serious consequences on executive function,

non-declarative and declarative memory, sustained attention and cognitive performance^[4-6], which is closely associated with the increased risk of many diseases including heart failure^[7], depressive disorder^[8], anxiety disorder^[9], type 2 diabetes mellitus^[10-11].

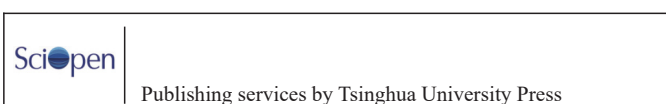
To date, medications for insomnia are widely used in practice. The 2 most commonly prescribed medications for insomnia are benzodiazepines and non-benzodiazepines, whereas they might have potential side effects, especially for older adults^[12-15]. Therefore, natural medicine became another reliable option, which has the synergistic effect of various chemical components with multiple targets and multiple pharmacological effects, thereby avoid the side effects caused by the single link of chemical drugs^[16]. Traditional

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Chinese medicine (TCM) has a long history in treating insomnia and has accumulated abundant clinical experience^[17]. The application of TCM is of particular interest^[18]. Pharmacological studies show that many natural constituents could alleviate insomnia and may prove to be a valuable source for the development of novel hypnotics, such as essential oil from *Perilla frutescens* (L.) Britton^[19], jujubosides from *Semen Ziziphi Spinosa*^[20-21], organic extraction from *Opuntia ficus indica* (L.) Mill.^[22], schisantherin A from *Schisandra*^[23], alkaloid of *Pinelliae Rhizoma*^[24] and so on.

Semen Platycladi (SP) is the dry and ripe kernel of the *Platycladus orientalis* (L.) Franco, known as Baiziren by Chinese, has been used for the treatment of insomnia and constipation in China for 2 000 years^[25-26]. Previous studies showed that the essential oil from SP have high medicinal value due to its sedative-hypnotic, antioxidant, antimicrobial and insecticidal activity^[27-30]. However, the mechanism of its sleep promoting activity is still unavailable. Moreover, if saponin, as the other main bioactive compounds in SP, have contributed to the sleep promoting activity is also unclear.

Therefore, in this study essential oil and saponin, the 2 main bioactive compounds of SP, were extracted. And their possible contribution to sedative-hypnotic activity in insomnia mice was evaluated. Furthermore, their inferential molecular mechanism of sleep promoting was also determined, which provide more available information on the utilization of SP in pharmacology.

2. Materials and methods

2.1 Materials and reagents

SP were purchased from Guangzhou Qingping chinese herbal medicine wholesale market (Guangzhou, China) in February 2018, and have been identified by Professor Gang Hao (College of Life Science, South China Agriculture University). The voucher specimen of SP (No. SP180227) has been deposited in College of Food Science and Engineering, South China University of Technology. The *p*-chlorophenylalanine (PCPA) and diazepam were obtained from Sigma-Aldrich (MO, USA). Mouse interleukin-6 (IL-6), tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β) ELISA kits were purchased from Cusabio Biotech CO., Ltd. (Wuhan, China). The mouse polyclonal antibody of serotonin 1A receptor (5-HT_{1A}), γ -aminobutyric acid type A receptor α -2 subunit (GABA_AR α 2), glutamic acid decarboxylase (GAD) and glyceraldehyde-3-phosphate dehydrogenase (GADPH) were all purchased from Cell Technology (MA, USA). All the other chemical reagents used in this study were analytical grade.

2.2 Preparation of saponin and essential oil

SP were dried to constant weigh, smashed into powder, passed through 60-mesh screen and stored with sealed bag. Then, the powder was mixed with 75% alcohol (*m*:*V* = 1:15) and heated at 80 °C for 3 h, which was repeated for 2 times. The alcohol liquid was collected and concentrated into paste, which was dried at 45 °C to obtain the alcohol extracts. Following, the alcohol extracts was re-dissolved with distilled water, and then partitioned with petroleum ether (aqueous phase), ethyl acetate (aqueous phase) and *n*-butyl alcohol (organic phase) in sequence, to remove the impurities. The extract liquor was concentrated with reduced pressure distillation and then dried to obtain saponins, which was named as SPS.

As the method in the previous study^[31], the essential oil was extracted through heating reflux extraction. The powder was soaked in petroleumether (*m*:*V* = 1:10), and then extracted by heating and reflux at 80 °C for 6 h. Subsequently, the essential oil, named as SPO, was collected with reduced pressure distillation (SHZ-D Rotary Evaporator, Shanghai, China).

2.3 Animal administration

Male ICR mice weighing 22–25 g were purchased from Jinan Pengyue Laboratory Animal Breeding Co., Ltd. (Jinan, China, Licence No. SCXK 20140007), housed in a controlled environment at a constant temperature ((24 ± 1) °C) with a relative humidity of $(50 \pm 10)\%$ under a normal light/dark (12 h/12 h) cycle (lights on from 7:00 a.m. to 7:00 p.m.). All mice had free access to standard laboratory granular diets and distilled water during the study period. Under the above conditions, behavioral testing was conducted from 8 a.m. to 3 p.m. in a quiet place. All mice were randomly divided into different groups (*n* = 10 per group). All operations on animals (including the mice euthanasia procedure) complied with the regulations and guidelines of Jinan University's animal care institutions (Permission No. 20180611-02). Every effort was made to minimize the number of animals used and their suffering.

2.4 Animal experiment design

For intragastric (i.g.) administration and intraperitoneal (i.p.) injection, diazepam, SPS, SPO, and PCPA were all suspended in physiological saline. After 5–7 days of adaptive feeding, 90 ICR mice were intraperitoneally injected with PCPA (300 mg/(kg·day)) on day 1–2 to establish insomnia model, and then stochastically assigned to 9 groups, including normal control group (Group 1), PCPA-induced model group (Group 2), diazepam group (Group 3), SPO (Groups 4–6) and SPS groups (Groups 7–9). On days 3–12, mice in different group were operated as follow: Groups 1–2 were treated with physiological saline (i.g.); Group 3 was operated with 0.9 mg/kg diazepam (dissolved in physiological saline, i.g.); Groups 4–6 were administrated with 20, 40, 80 mg/kg SPO (dissolved in physiological saline, i.g.), respectively; and Groups 7–9 were given 20, 40, 80 mg/kg SPS (dissolved in physiological saline, i.g.), respectively. At the end of the experiment, all mice were fasted overnight and sacrificed by cervical dislocation under deep chloral hydrate anesthesia to collect biological materials for pharmacokinetic studies. The scheme of the experimental procedure is summarized in Fig. 1.

2.5 Tail suspension test

On day 8, 60 min after intragastric administration, the tail suspension test was conducted to test the emotional behaviors according to previous report with minor modifications^[18]. Mice were suspended 5 cm away from the bottom of the suspension support for 6 min by fixing approximately 2 cm from the very end of the tail on the suspension bar. Mice were considered immobile when ceased moving and remained motionless^[32]. The immobility time during the last 4 min of the total 6 min was recorded.

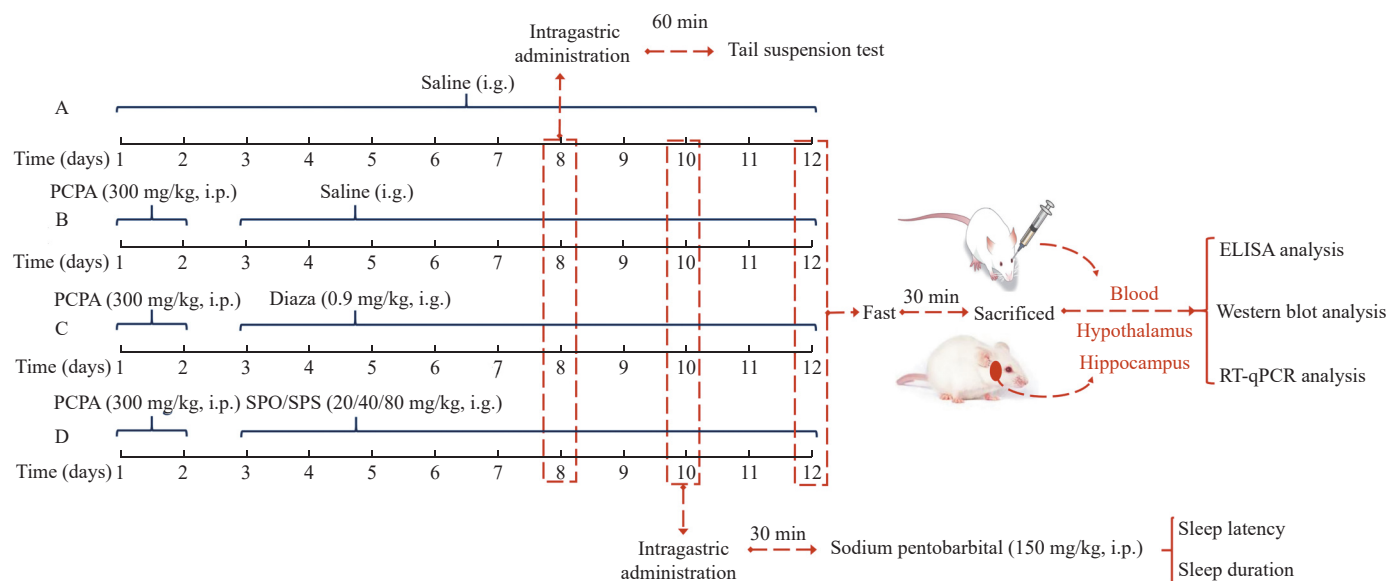


Fig.1 Scheme of the experimental procedure. (A) Normal control group (Group 1), (B) PCPA-induced model group (Group 2), (C) diazepam group (Group 3), (D) SPO (Groups 4–6) and SPS groups (Groups 7–9).

2.6 Sodium pentobarbital-induced sleeping test

According to previous literature with minor modification^[33]. On day 10, 30 min after treatment by intragastric administration with SPO, SPS or diazepam, all mice were intraperitoneally injected with sodium pentobarbital (150 mg/kg). Observation for sleep onset began immediately after the sodium pentobarbital injection by placing each mouse on its back. The mice were considered asleep when they lost their righting reflex over 60 s. The sleep latency was recorded as the elapsed time between sodium pentobarbital injection and sleep onset, and the sleep time was recorded from the loss of righting reflex to that of recovery.

2.7 Analysis of the cytokines in plasma

At the end of the experiment, the blood was obtained by eyeball extirpation and plasma separation was performed by centrifugation at 5 000 r/min for 10 min at 4 °C. Concentrations of IL-6, IL-1 β and TNF- α in plasma were evaluated using the commercial mouse ELISA kits (Cusabio Biothch CO., Ltd., Wuhan, China) strictly following the manufacturer's instructions.

2.8 Quantitative real-time PCR (RT-qPCR) and Western blot analysis

The brain tissue was prepared and tested by RT-qPCR analysis as reported previously^[34]. The total RNA was extracted with TRIzol reagent, chloroform and isopropanol. The concentration and purity of RNA was determined by Nanodrop 2000 (Thermo Fisher Scientific Co., Ltd., Shanghai, China). Then were reverse transcribed into cDNA using a reverse transcription kit. Finally, the PCR reaction was performed using a light cycler instrument with the Fast Star DNA Master SYBR Green I kit and the results were expressed as the ratio of optimal density to *GAPDH*. The expression levels with respect to the control were estimated by calculating $\Delta\Delta C_t$ and subsequently

analyzed using the $2^{-\Delta\Delta C_t}$ method. Subsequently, the protein was determined by Western blot analysis. Briefly, the proteins were extracted using RIPA lysis buffer and were quantitatively analyzed by PierceTM BCA Protein Assay Kit (Thermo Fisher Scientific, Co., Ltd., Shanghai, China). Then calculated the loading volume of samples, conducted the Western blot analysis according to the method reported^[35].

2.9 Statistical analysis

Each experiment was analyzed in triplicate and the results were expressed as the mean $\pm$ standard deviation (SD). The differences between groups were analyzed by One-way ANOVA followed by LSD as post hoc test using SPSS 13.0 software (IBM spss Inc., New York, USA). Values of $P < 0.05$ was considered statistically significant.

3. Results

3.1 Essential oil and saponin content of SPO and SPS

The SPO and SPS was weighed and their yield was calculated by the ratio of the weight of SPO/SPS and the weight of SP powders. The results showed that the yield of SPO and SPS were $(1.24 \pm 0.04)\%$ and $(0.38 \pm 0.02)\%$, respectively (data not shown). The essential oil content of SPO was $(44.23 \pm 1.37)\%$ and the saponin content of SPS was $(43.72 \pm 1.05)\%$, respectively.

3.2 Antidepressant effect on PCPA-induced insomnia mice

The antidepressant effect of SPO and SPS was tested by tail suspension experiment. As shown in Fig. 2A, the duration of immobility of PCPA-induced model group $((123.00 \pm 5.57) s)$ was significantly longer than that of the normal control group $((96.50 \pm 2.18) min)$. The diazepam group has shorter time of immobility $((114.86 \pm 10.19) min)$, suggesting diazepam could help insomnia mice to relieve their depression. Compared to insomnia group, SPO and SPS were also

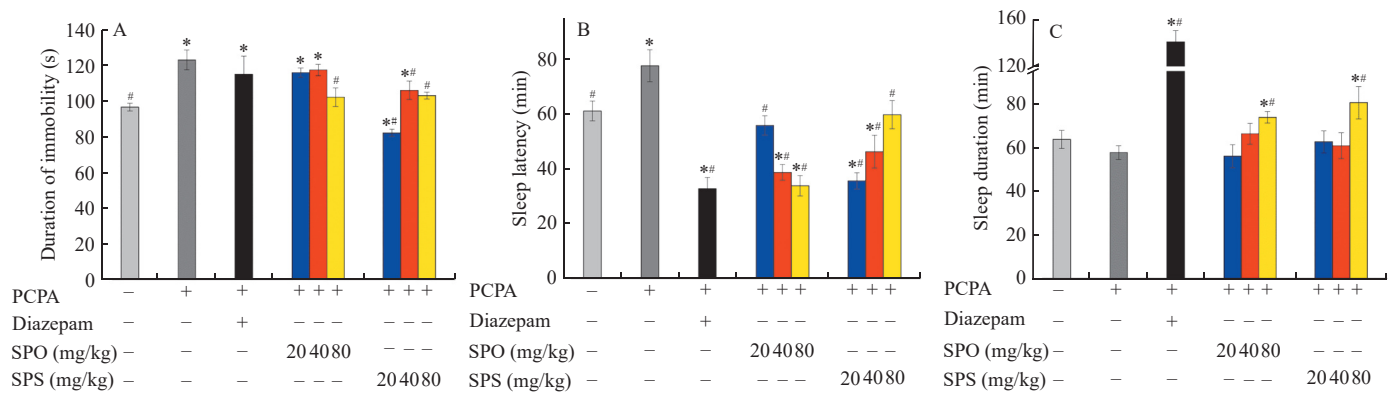


Fig. 2 Effects of SPO and SPS on behavioristics of ICR mice. (A) Effect on duration of immobility in tail suspension test. (B) Effect on sleep latency in sodium pentobarbital-induced sleeping test. (C) Effect on sleep duration in sodium pentobarbital-induced sleeping test.

*P < 0.05 vs. control group, #P < 0.05 vs. PCPA-induced model group.

helpful to relieve the depression, especially the SPS group. Low-dose group SPS (20 mg/kg) exhibited the strongest effect on insomnia mice, which was even better than that of diazepam group. These data showed that SPO and SPS form SP might alleviate insomnia by suppressing depression.

3.3 Potentiating effect on sleep induced by sodium pentobarbital

As shown in Figs. 2B and C, the sleep latency of PCPA-induced model group was longer while the sleep duration was shorter than normal control group, suggesting the presence of sleep disorders in model group. After treatment of SPO and SPS, all dose of SPO and SPS shorten the latency time compared to model group. And high and middle-dose SPO (40, 80 mg/kg) while low and middle-dose SPS (20, 40 mg/kg) significantly reduced the onset sleep time of insomnia mice compared to normal control group. Moreover, longer sleep duration was observed under the treatment of high-dose SPO and SPS.

3.4 Neurotransmitter receptors and their transcriptional levels in the brain

To investigate the mechanism of sleep-promoting action of SPO and SPS, the level of neurotransmitters' receptors and their mRNA

expression were determined, which was shown in Fig. 3. Compared with PCPA-induced model group, the mRNA expressions of *5-HT_{1A}* and *5-HT_{2C}* were up-regulated after treatment of SPO and SPS, while the mRNA expression of *5-HT_{2A}* was down-regulated. Furthermore, the sensitive of 5-HT receptors activation to SPO and SPS were different. *5-HT_{1A}* and *5-HT_{2C}* were more sensitive to SPO, while *5-HT_{2A}* was more sensitive to SPS. As shown in Figs. 3D-F, the mRNA expressions of *GABA_ARα2*, and *GABA_ARα3* in SPO and SPS groups were increased compared to PCPA-induced model group, which those of *GABA_ARα5* was lower slightly than PCPA-induced model group. This confounding results might be caused by the different optimum sampling time of different receptors, so the expression of *GABA_ARα5* might in the upswing period during sampling period.

Additionally, 2 typical neurotransmitters' receptors in hippocampus and hypothalamus, *5-HT_{1A}* and *GABA_ARα2*, were determined by Western blot to determine if the neurotransmitters' receptors were modulated by the mRNA expressions successfully. The result showed that the content of *5-HT_{1A}* in hippocampus and hypothalamus was increased similar to their mRNA expressions (Figs. 3G and H). While the content of *GABA_ARα2* in hippocampus was increased under the treatment of middle and high-dose SPS, and in hypothalamus was all aggrandized under the treatments of SPO and SPS. Besides, SPO enhanced *GABA_ARα2* expression in hypothalamus while has no enhancement of *GABA_ARα2* expression in hippocampus. Moreover, SPS promoted *GABA_ARα2* expression in both hippocampus and hypothalamus

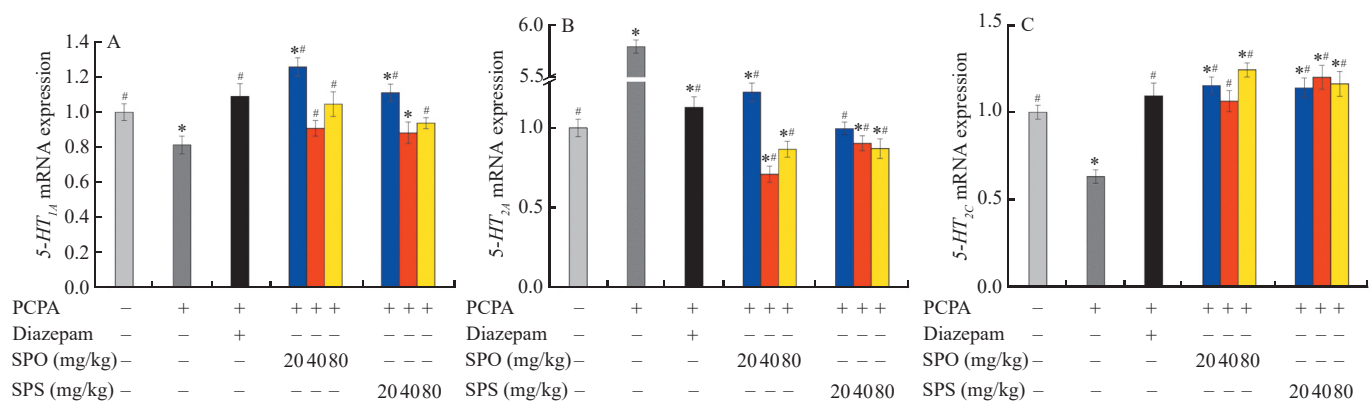


Fig. 3 Effects of SPO and SPS on mRNA expression of (A) *5-HT_{1A}*, (B) *5-HT_{2A}*, (C) *5-HT_{2C}*, (D) *GABA_ARα2*, (E) *GABA_ARα3* and (F) *GABA_ARα5* in the hypothalamus; and protein expression of *5-HT_{1A}*, *GABA_ARα2*, GAD and in the (G) hippocampus and (H) hypothalamus.

*P < 0.05 vs. control group, #P < 0.05 vs. PCPA-induced insomnia group.

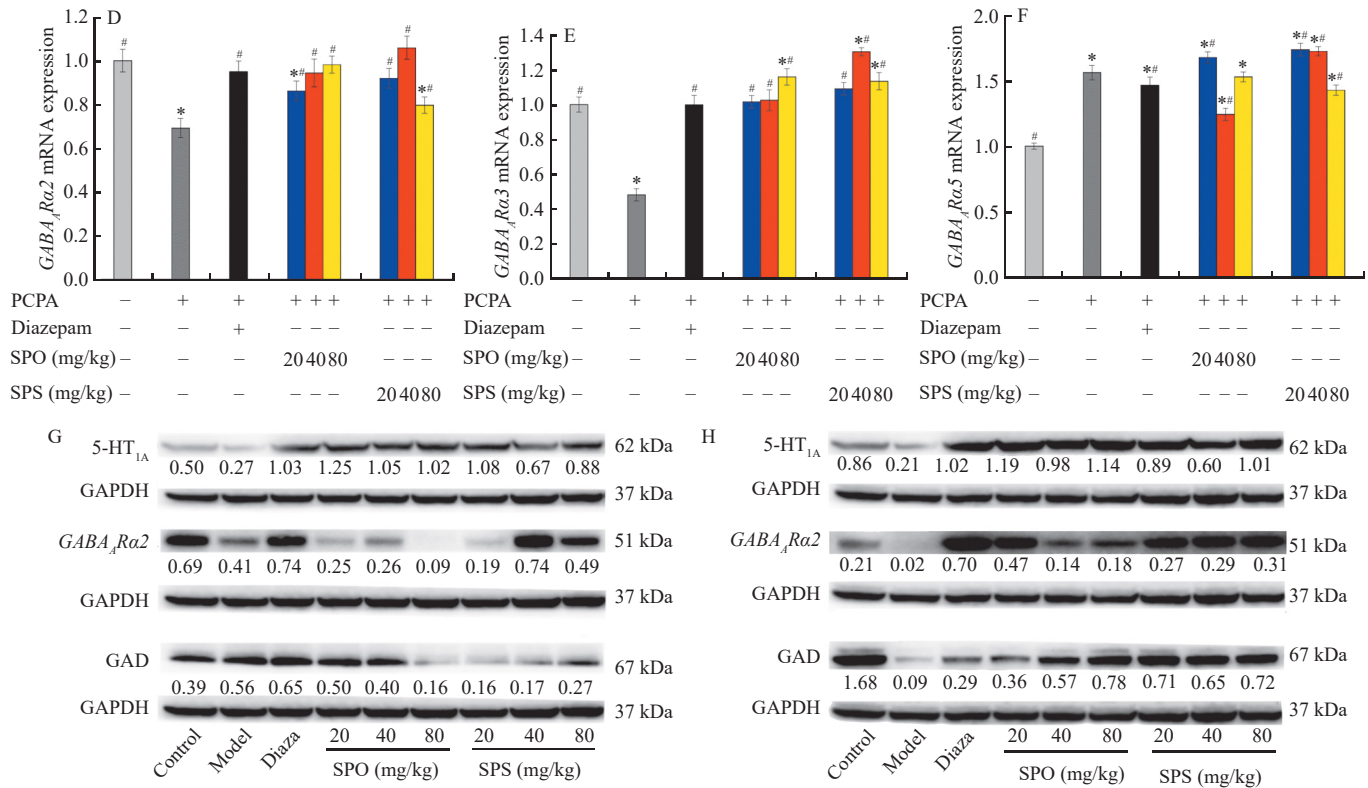


Fig. 3 (Continued)

The protein expression of GAD was investigated (Figs. 3G and H). The content of GAD in hypothalamus were increased with the treatments of SPO and SPS, and SPS exerted better enhancement. While SPS and SPO have no significant effect on GAD content in hippocampus.

3.5 Level of proinflammatory cytokines in plasma

The levels of proinflammation cytokines in plasma were detected by ELISA assay. In PCPA-induced model group, the secretion of IL-1 β was lower, and the IL-6 and TNF- α were higher than normal control group (Fig. 4). After treatments with the SPO and SPS, the secretion of these 3 cytokines have been inverted. The secretion of IL-1 β in plasma increased which was comparable with the diazepam group. While the accumulation of IL-6 and TNF- α decreased, especially for the low-dose SPO (20 mg/kg). Also, the mRNA

expression was tested (Figs. 4D-F). The mRNA expressions of IL-1 β was down-regulated compared to model control, except to the high dose of SPO and middle dose of SPS, which was inconsistent with the increased accumulation of IL-1 β . This may be caused by the different sampling time of IL-1 β in plasma, which may in the declining phase. Otherwise, the decreased accumulation of IL-6 and TNF- α were consistent with the down-regulated mRNA expressions.

3.6 Transcriptional levels of PGD2 and DPR

The mRNA expression of PGD2 and DPR were determined (Fig. 5). Unlike diazepam group, low and middle-dose of SPO and high-dose of SPS could increase the mRNA expression of PGD2. While the mRNA expression of DPR were lower than model group under the treatment of SPO and SPS, which was similar to diazepam group.

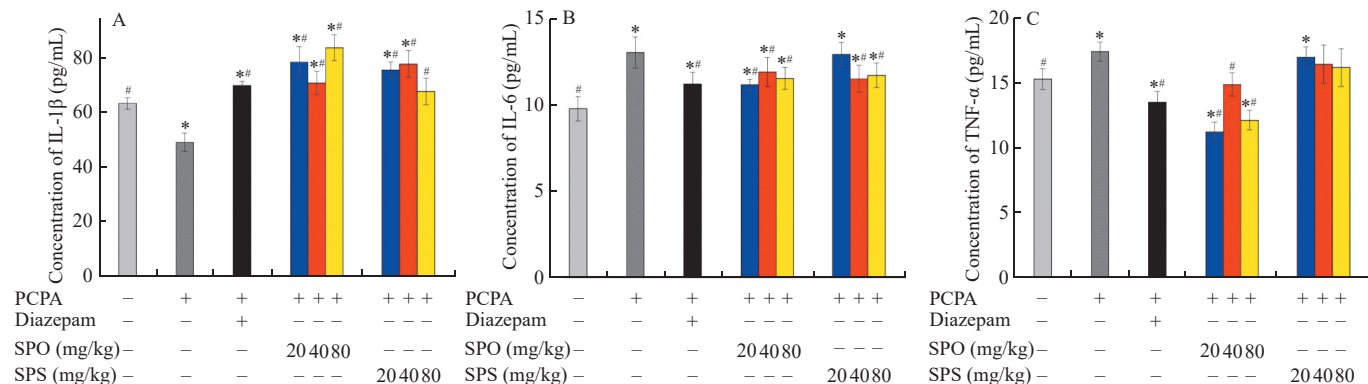


Fig. 4 Effects of SPO and SPS on cytokines in plasma and their mRNA expression in hypothalamus. Concentration of (A) IL-1 β , (B) IL-6, (C) TNF- α ; (D) IL-1 β , (E) IL-6, (F) TNF- α mRNA expression. * P < 0.05 vs. control group, # P < 0.05 vs. PCPA-induced insomnia group.

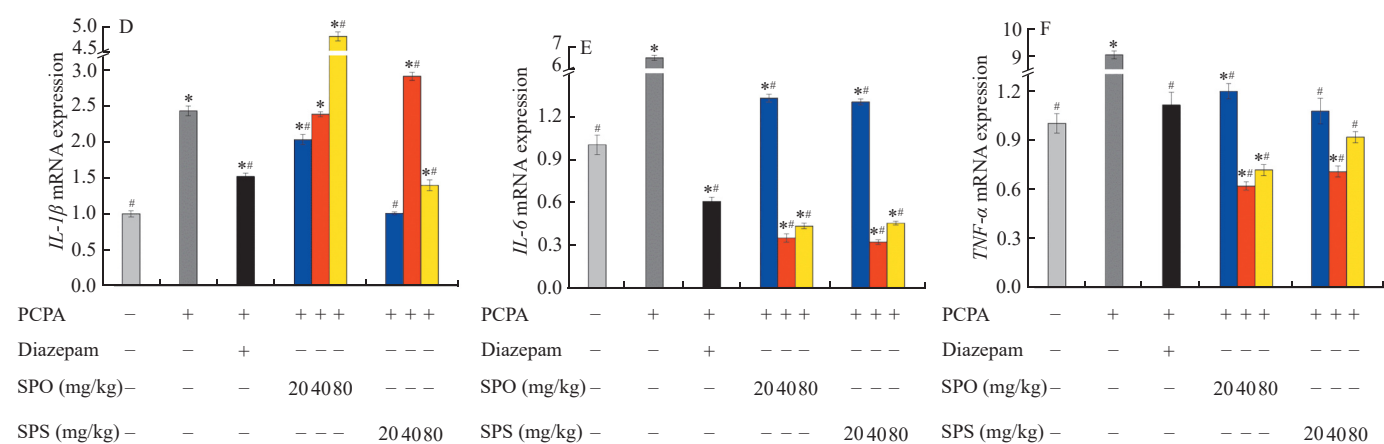


Fig. 4 (Continued)

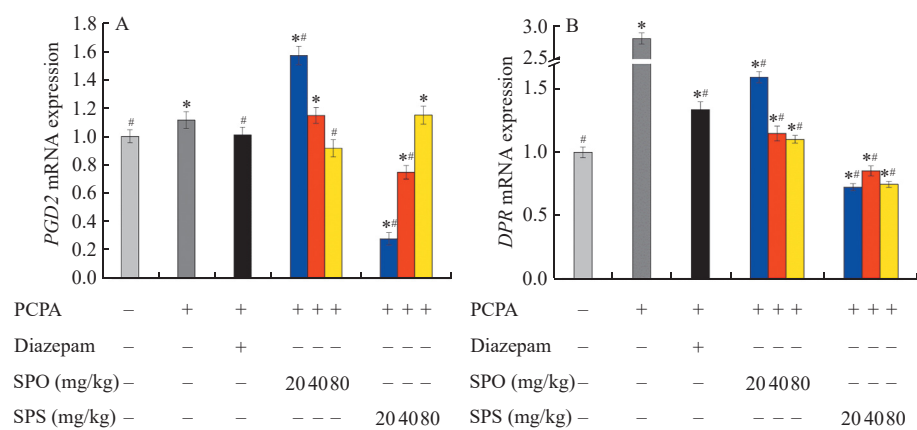


Fig. 5 Effects of SPO and SPS on the mRNA expression of (A) *PGD2* and (B) *DPR* in the hypothalamus. * $P < 0.05$ vs. control group, # $P < 0.05$ vs. PCPA-induced insomnia group.

4. Discussion

As sleep disorders can have deleterious impacts on health and quality of life, a wide variety of pharmacotherapies have been developed to treat insomnia, including hypnotics, antipsychotics, antidepressants, antihistamines, and melatonin receptor agonists^[36]. However, due to the adverse drug effects, the application of TCM is of particular interest. In the present study, the sedative-hypnotic effect of essential oil SPO and saponin SPS from SP were investigated using PCPA-induced insomnia mice.

First, the essential oil and saponin of SP were extracted and named SPO and SPS, respectively. The yield of SPO ($(1.24 \pm 0.04)\%$) was higher than previous study^[37], and also higher than that of SPS ($(0.38 \pm 0.02)\%$). The essential oil content of SPO was $(44.23 \pm 1.37)\%$ and the saponin content of SPS was $(43.72 \pm 1.05)\%$, indicating the main compound of SPO and SPS was essential oil and saponin respectively. PCPA-induced insomnia mice were given SPO and SPS by intragastric administration. The body weight change of mice in each group were tested every 3 days during the feeding, and was summarized in Table 1. SPO and SPS groups had no significant difference in weight compared to the control group, indicating that SPO and SPS had little effect on the health of normal mice after 10 days of administration.

Table 1
Effect of SPO and SPS on body weight (g) of PCPA-induced insomnia mice.

Groups	Day 1	Day 4	Day 7	Day 10	Day 12
1	26.31 ± 1.17	28.22 ± 1.54	29.88 ± 1.28	30.80 ± 1.22	31.12 ± 1.27
2	26.40 ± 1.13	28.44 ± 1.22	30.95 ± 1.46	31.80 ± 1.68	32.04 ± 1.74
3	26.60 ± 1.42	27.22 ± 1.65	30.00 ± 1.92	31.15 ± 2.17	31.25 ± 2.30
4	26.71 ± 0.97	27.36 ± 1.42	30.39 ± 1.28	30.97 ± 0.90	31.81 ± 1.39
5	26.70 ± 1.08	28.02 ± 1.08	29.61 ± 1.24	31.35 ± 1.19	31.73 ± 1.57
6	26.79 ± 1.16	27.39 ± 1.27	30.10 ± 1.62	30.66 ± 2.39	31.46 ± 2.63
7	26.59 ± 1.60	27.04 ± 1.40 [#]	29.30 ± 1.92 [#]	30.58 ± 1.56	30.56 ± 2.02
8	26.51 ± 0.96	27.19 ± 1.60 [#]	29.71 ± 1.80	31.45 ± 2.05	31.97 ± 2.16
9	27.63 ± 1.16 ^{*#}	28.26 ± 1.13	30.40 ± 1.59	30.90 ± 1.97	32.22 ± 1.87

Note: Data expressed as mean ± SEM, $n = 10$; * $P < 0.05$ when compared with Group 1 (normal control group); # $P < 0.05$ when compared with Group 2 (PCPA-induced model group); Groups 4–6 represented low-dose (20 mg/kg), middle dose (40 mg/kg) and high dose (80 mg/kg) of SPO, respectively; Groups 7–9 represented low-dose (20 mg/kg), middle dose (40 mg/kg) and high dose (80 mg/kg) of SPS, respectively.

Then the behavioral tests were conducted to explore the effects of SPO and SPS on behavioral changes in insomnia mice. Epidemiologic studies have suggested that over 20% of insomniacs exhibit depressive symptoms^[38], and depression have been shown to be the largest and most consistent risk factors for insomnia^[39], indicating the

bidirectional link between insomnia and depression^[40]. In this study, tail suspension test was used to evaluate the antidepressant effect of SPO and SPS. During this test, the duration of immobility revealed the duration of depression. The data shown that SPO and SPS were helpful to relieve the depression due to their shorter time of immobility. Similar result was observed in nyctinastic herb decoction^[18], which indicating that SPO and SPS might improve the depression states in insomnia. Subsequently, another behavioral method, sodium pentobarbital-induced sleeping test, was used to evaluate sedative-hypnotic effects^[41]. Previous study reported that insomnia can manifest behavior of long sleep onset and short sleep duration^[42]. In this experiment, the sleep latency time was longest and duration time was shortest in model group, suggesting that the insomnia model was considered successfully established. Both SPO and SPS could shorten the latency and lengthen the duration of sleep induced by sodium pentobarbital in a way. The result suggested that SPO and SPS could not only exhibit sleep-inducing effect but also exhibit sleep-enhancing effect in insomnia mice, possessing centrally acting sleep-promoting properties^[43].

Following, the inner mechanism behind the sleep-promoting effect of SPO and SPS was further explored. There are evidences suggesting that the sleep deprivation causes marked alterations in neurotransmitter receptor expression in brain tissues^[44-45]. Besides, serotonergic neuron was one of the main neurons related to sedative-hypnotic effects^[46]. 5-HT was first believed to be a true neuromodulator of sleep produced by serotonergic neurons^[47]. And the PCPA model with the characteristics of high success rate and typical pathological changes is a recognized insomnia model^[34], which can significantly reduce the content of 5-HT in the brain by inhibiting tryptophan hydroxylation and blocking 5-HT synthesis, thus causing anxiety, insomnia and other symptoms^[47]. In this study, the mRNA expression of 3 main receptors of 5-HT were determined. Theoretically, the mRNA expression of 5-HT receptors should be increase to reverse the lacking 5-HT situation caused by PCPA. It may seem confounding that the mRNA expressions of *5-HT_{1A}* and *5-HT_{2C}* were up-regulated while that of *5-HT_{2A}* was down-regulated after treatment of SPO and SPS. However, previous study reported that activation of *5-HT_{1A}* and *5-HT_{2C}* could lead to anti-depression in different animal models^[48]. Also, some psychotherapeutic agents also downregulate *5-HT_{2A}* receptors with chronic administration including classical antidepressants and antipsychotics^[49-50]. Moreover, *5-HT_{1A}* and *5-HT_{2C}* were more sensitive to SPO, while *5-HT_{2C}* was more sensitive to SPS, indicating that SPO and SPS might act on different focusing targets. These data indicated that SPO and SPS were implicated to modulate the expressions of 5-HT receptors in serotonergic neurons to exert sleep-promoting action.

In addition, GABAergic neuron was another important neuron related to sedative-hypnotic effects, which was postsynaptic neuron of serotonin neuron. It was reported that the release of GABA may be regulated by serotonin receptors, especially the *5-HT_{2C}*^[51]. GABA is the major inhibitory neurotransmitter in the central nervous system (CNS), exerting sedative and calming effect^[52-53]. GABA are released by GABAergic neuron and then combine with *GABA_A* receptors, including *GABA_ARα2*, *GABA_ARα3* and *GABA_ARα5*. Therefore, the levels of GABA receptors were also tested. The results showed that the mRNA expressions of *GABA_ARα2*, *GABA_ARα3* and *GABA_ARα5* were increase in a way compared to insomnia model group. The up-regulated *GABA_A* receptors could increases the outflow of

chloride ions from the end of its axon, forming the primary afferent depolarization (PAD)^[54]. Then PAD reduces or blocks the influx of potassium ions^[52], resulting in a decrease in the amplitude of the action potential at the end of these axons, which lead to presynaptic inhibition and finally achieve the sedative and sleep-promoting effect. Besides, the western blot results showed that both SPO and SPS could increase the content of 5-HT_{1A} in hippocampus and hypothalamus, which was similar to results of mRNA expressions. Additionally, the ability to regulate *GABA_ARα2* expression of SPS was stronger than SPO, which was also consistent with the results of the mRNA expressions. Moreover, GAD, the synthetic rate-limiting enzyme of GABA, was increased with the treatments of SPO and SPS in hypothalamus, while was unchanged in hippocampus, indicating SPO and SPS could enhance the synthesis of GABA in hypothalamus, instead of hippocampus. It can be speculated that SPO and SPS could achieve sedative effect through modulating multi-targets of serotonergic and GABAergic system in insomnia mice, such as the expression of 5-HT receptors, GABA synthetase and *GABA_A* receptors. And they have different regulation ability to different targets, the result also told that SPS showed stronger effect than SPO on a whole.

It was reported that increased proinflammatory cytokines could decreased GABAergic currents, which reducing the release of GABA, thus causing the inhibitory synaptic transmission^[55]. Moreover, SPO and SPS could down-regulate the proinflammatory cytokines to promote the release of GABA, therefore achieving calming effect. On the other hand, sleep homeostasis was related to sleeping hormones. Prostaglandin D2 (PGD2) is currently known as one of the most potential endogenous sleep-promoting substances, which can enhance sleeping by combining with its receptor (DP receptor, DPR)^[56-58]. Unexpectedly, the variation trends of PGD2 and DPR were different. The reason of this result may be speculated that after PCPA modeling, the content of PGD2 and DPR were both up-regulated to reverse the insomnia situation, but the expression of PGD2 was not up-regulated as quickly as the receptor DPR due to their different sensitivity to PCPA, thus resulting the unbalance of hormones secretion. After the treatments of SPO and SPS, the expressions of PGD2 was up-regulated while DPR was down-regulated, attempting to rebalance the secretion of sleep-promoting substances PGD2. Also, the data showed that the ability to hormone rebalance of SPO was stronger than SPS.

Taking all the results into consideration, it is quite rational to conclude that SPO and SPS exerted sleep-promoting in PCPA-induced insomnia mice by regulating the synthesis of neurotransmitter receptors, cytokines and hormone in serotonergic and GABAergic system. The possible molecular mechanism of SPO and SPS was shown in Fig. 6. First, SPO and SPS regulated the expression of 5-HT receptors especially *5-HT_{2C}* receptor to combined more 5-HT released in serotonergic neurons, resulting the increasing action current in GABAergic neurons^[46]. Then they also up-regulated the expression of GAD to produce more GABA, and stimulated the expression of GABA receptors, inhibiting the action current transferred to next neurons and achieving synaptic inhibition in GABAergic neurons. On the other hand, SPO and SPS down-regulated the secretion of proinflammatory cytokines (IL-1β, IL-6 and TNF-α) in insomnia mice, lifting the restrictions of proinflammatory cytokines on action current in GABAergic neurons. Furthermore, SPO and SPS, especially SPS, improved the unbalance expression of PGD2 and its receptor DPR in insomnia mice.

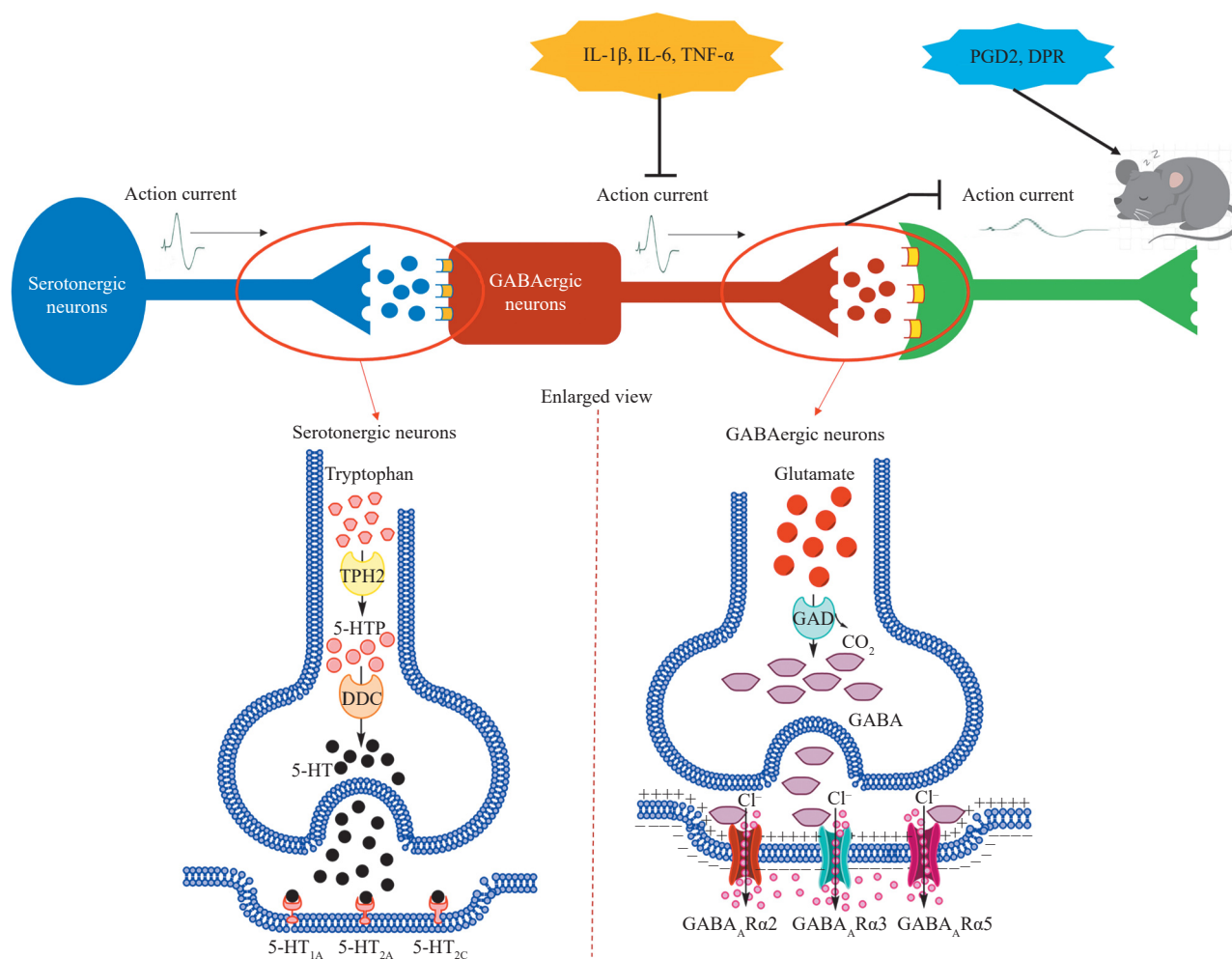


Fig. 6 Possible molecular mechanism of sedative-hypnotic effects of SPO and SPS. TPH2: tryptophan hydroxylase 2; 5-HTP: 5-hydroxytryptophan; DDC: Dopa decarboxylase; $\dashv$: repression effect.

5. Conclusion

In general, SPO and SPS exhibited sedative-hypnotic effect through modulating multiple ways in serotonergic and GABAergic system, including neurotransmitter receptors, cytokines and hormone. Although both SPO and SPS could exert sleep-promoting, they had different focusing targets. SPS had stronger effect on regulation of neurotransmitter receptors while SPO had better rebalance ability to hormone. Further studies are still needed to identify and distinguish the ultimate substances responsible for the sedative-hypnotic potential of SPO and SPS.

Conflict of interest

All authors declare that there is no conflict of interests involved in this study and in this manuscript.

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