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Role of the Volatile Components in the Anti-insomnia Effect of Jiao-Tai-Wan in PCPA-induced Insomnia Rats

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ABSTRACT

Background: Jiao-Tai-Wan (JTW) is widely used for insomnia in traditional Chinese medicine. It has been found that berberine in JTW has a potential anti-insomnia effect, but the anti-insomnia effect of volatile oil, which is another major component in JTW, remains unclear.

Objective: To comprehensively analyze the anti-insomnia effect of volatile oil through chemical analysis, pharmacological research and network pharmacology methods.

Methods: Gas chromatography-mass spectrometry (GC–MS) analysis and network pharmacology platform were used to elucidate the material basis, effect and mechanism of JTW in the treatment of insomnia. A rat model of insomnia induced by p-Chlorophenylalanine (PCPA) was used to verify its anti-insomnia effect. The levels of Melatonin (MT) and Melatonin receptor type 1b (MTNR1B) in the brain and serum of rats were detected by ELISA, Western blot and Real-time PCR methods.

Results: Eighteen volatile oil ingredients of JTW were identified by GC–MS, containing six new components which were found in JTW for the first time. Cinnamaldehyde, Behenic alcohol, Tetradecanal, and Gleenol can promote sleep by targeting MTNR1B according to the predicted results of network-pharmacology. Compared with the model group, JTW can increase the level of MTNR1B in rats' prefrontal cortex and brain stem, while reducing the level of MT in rats' brain stem.

Conclusion: The volatile oil components, such as Cinnamaldehyde, Tetradecanal, and Gleenol, might also be playing a critical role in the anti-insomnia effect of JTW by target MTNR1B.

1. Introduction

Insomnia is a widespread epidemic defined as difficulty falling asleep, repeated awakenings, and poor quality of life (Cao et al., 2016). The importance of good sleep is well known, but the insomnia rate continues to rise due to the increase in life pressure (Si et al., 2020). Many factors are closely related to the occurrence of insomnia, including gender, age, physique, environment and diet, but these factors have not been clearly confirmed. Furthermore, insomnia is also considered to be a key factor leading to multi-system diseases. An epidemiological study pointed out that depression and anxiety were always accompanied by insomnia (La et al., 2020). Meta-analyses revealed that participants with symptoms of insomnia or sleep continuity disorders obviously increased the incidence of hypertension (Ramos et al., 2018). Although the mechanism between insomnia and metabolic syndrome is unclear, there is evidence that insomnia symptoms are related to the compounds of metabolic syndrome.

Current therapeutic approaches for insomnia mainly include cognitive behavioral therapy for insomnia (CBT-I) and drug administration. Drugs approved for the treatment of insomnia include benzodiazepines, histamine, and orexin/hypocretin receptor agonists, whose functions are based on those sleep and waking-related neurotransmitters and hormones (Neubauer et al., 2018). Even though these drugs have been proven to have beneficial effects on patients with insomnia, long-time use still has potential severe risks such as headache, dizziness, and somnolence (Kim et al., 2019; Pinto et al., 2016).

Jiao-Tai-Wan (JTW) is a well-known traditional Chinese formula that has been used to treat insomnia since the Ming Dynasty (SiSi et al., 2022). It is composed of Huang Lian (*Coptidis Rhizoma,*CR) and Rou Gui (*Cortex Cinnamomi,*CIN) at a ratio of 10:1, which can down-regulate neurological inflammation and ameliorate cognitive dysfunction (Ji and Shen, 2011; Kim et al., 2012). A clinical research conducted in China showed that JTW was orally administered for 60 days could improve the Pittsburgh Sleep Quality Index (PSQI) (Zeng et al., 2020). Moreover, an

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experimental study conducted in partially sleep-deprived rats indicated that the administration of JTW could up-regulate the expression levels of circadian proteins cryptochrome1 (Cryl) and cryptochrome2 (Cry2) in the hypothalamus (Huang et al., 2018).

It is reported that CR could significantly reduce the voluntary activities of mice and increase the rate of falling asleep (Ramos et al., 2017). However, a study also showed that after compatibility with CIN, the sedative and hypnotic effects of CR were enhanced (Watanabe et al., 2002). Nevertheless, previous studies rarely mentioned the relationship between the change of volatile oil compounds and the anti-insomnia effect of JTW. Therefore, it is worthwhile to elucidate whether volatile oil ingredients play a critical role in the mechanism of JTW for treating insomnia.

In this study, we acquired the volatile oil ingredients and contents of JTW through GC–MS analysis. The relationship among significant insomnia targets, associated proteins, and volatile oil compounds were established by Network-Pharmacology analysis. A rat model of insomnia induced by p-Chlorophenylalanine (PCPA) was used to verify the effect of JTW on insomnia and its possible mechanisms.

2. Materials and Methods

2.1. Animals

Female Sprague Dawley rats weighing 180–200 g were obtained from the Animal Center of Shanghai Branch, Chinese Academy of Sciences. Six rats were housed per cage under pathogen-free conditions with standard laboratory water and chow, controlled temperature (22 ± 1°C), humidity (50 ± 10%), and 12 h light/dark cycle. Rats were allowed to acclimate for 7 days prior to the experiment. All the animal experiments were performed in consistent with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85–23, revised 1985) and approved by the institutional animal committee of Wenzhou Medical University (wydw2019–0611).

2.2. Drugs and reagents

CR and CIN are the main ingredients of JTW with a ratio of 10:1 (W/W). In this study, CR and CIN samples were purchased from Quzhou Nankong Chinese Medicine Co., Ltd (Zhejiang, China) and identified by Chongliang Lin, a Chinese pharmacist of the First Affiliated Hospital of Wenzhou Medical University, Zhejiang, China. CR and CIN were extracted three times at a ratio of 10:1 by boiling, and solid particles remained. The solid particles were diluted in sterile water to a 0.44 $g{\cdot}mL^{-1}$ final concentration. Zolpidem Tartrate is the most typical drug for treating insomnia and is a positive drug in our research (Rösner et al., 2013). Zolpidem Tartrate Tablet (HangzhouSanofi Pharmaceutical Co., Ltd (Hangzhou, China)) was dissolved in sterile water and achieved a 0.2 $mg{\cdot}mL^{-1}$ concentration. The human dosage was converted to the corresponding dosage in rats according to Chinese Pharmacopoeia (2010) via body surface areas. p-Chlorophenylalanine (PCPA) (Sigma-Aldrich (USA)) was used to induce insomnia animal model in this study. The following antibodies were used in this study for western blotting: vinculin (ab129002, Abcam); Melatonin receptor type 1b (MTNR1B) (ab203346, Abcam); Goat Anti-Rabbit IgG (HRP) (ab205718, Abcam).

2.3. GC–MS analysis

2.3.1. Extraction of volatile oil

CIN (10 g) and CR (100 g) were crushed and placed in a 1 L round bottom flask, and then 600 mL of sterile water was added and soaked for 2 h. The samples were subjected to hydrodistillation for 5 h, according to China Pharmacopoeia (2015). The obtained oils were dried over anhydrous sodium sulfate and stored at 4°C in sealed brown glass vials until tested.

2.3.2. Chromatographic conditions

Quantitative and qualitative analysis of the essential oil was performed using a GC–MS QP2010-Ultra system (Shimadzu Corporation, Kyoto, Japan) equipped with an InertCap 5 MS/Sil column (30 m × 0.25 mm × 0.25 μm). Helium gas was used as the carrier gas at a constant flow rate of 1 ml/min. The shunt ratio was 50:1. Injector and mass transfer line temperatures were set at 280 and 250°C, respectively. Essential oil solution (1 μL) in hexane was injected and then analysed under the following column conditions: initial column temperature at 70°C for 1 min, which then increased to 120°C at a 10°C/min heating ramp, and then increased to 200°C at a 2.5°C/min rate. Finally, it increased to 260°C at a 10°C/min heating ramp.

2.3.3. Mass spectrometric conditions

Electron ionization system with a 70 eV ionization energy. The ionization source temperature was set at 200°C. The mass detector was operated in scan mode with a scanning range of 45–600 m/z.

2.3.4. Results analysis

The major oil components were identified via the National Institute of Standards and Technology (NIST) V11.0 GC–MS library. The relative concentration of each compound in the essential oil was quantified based on the peak area integrated into the analysis program.

2.4. Animal grouping and intervention

Rats were divided into four groups as follows: Control group, Model group, Zolpidem group, and JTW group with 6 rats in each group. Sleep-deprived rodents model induced by intra-peritoneal injection of PCPA was used to this study which is the universal model in investigating the mechanism of insomnia as previously reported (Huang et al., 2018). PCPA was dissolved in weakly alkaline saline and administered intraperitoneally (i.p.300 $mg{\cdot}kg^{-1}$) for once. After injection of 30 h, the circadian sleep rhythm of rats disappeared, revealing that the insomnia model was successfully established. Rats in Zolpidem (1.05 $mg{\cdot}kg^{-1}$) and JTW (dose 2.2 $g{\cdot}kg^{-1}$) groups were administered by gavage after weighing. This dosage was determined to the equivalent dosage in rats according to Chinese Pharmacopoeia, 2010 based on body surface areas. The model group was administered the same amount of sterile water. Drugs and sterile water were administered once a day for one week.

2.5. Serum and tissue collection

Rats were anesthetized by 2% pentobarbital sodium (i.p.0.3 ml/100 g). Then the rats' blood was collected and stranded at room temperature for 30 min, then centrifuged at 3000 rpm and 4°C for 10 min. Finally, the supernatant was aspirated and stored at −80°C. Rats were sacrificed by abdominal aortic hemorrhage, and their brains were quickly removed. Different brain regions, including the prefrontal cortex, hypothalamus, hippocampus, and brain stem were immediately separated on the ice. All the samples were stored in liquid nitrogen for the subsequent experiments.

2.6. Western blot

The prefrontal cortex, hypothalamus, hippocampus, and brain stem, 20 mg of each sample was separately lysed in 250 μL RIPA buffer supplemented with protease (100:1) and phosphatase (50:1) inhibitors on ice for 30 min. Such mixtures were then centrifuged at 12,000 rpm and 4°C for 10 min and collected supernatant. The total protein concentrations were measured by Bicinchoninic Acid Assay Kit (Beyotime Institute of Biotechnology Co., Ltd., Shanghai, China), followed by heat denaturation at 100°C for 10 min. After cooling down, the samples (30 μg protein each) were subjected to polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane. After blocking with 5% skimmed milk for 1 h at room temperature. The

Table 1
Primer sequence.

Gene	Primer
Actin	5′-GGCTGTATTCCCCTCCATCG-3′
	3′-CCAGTTGGTAACAATGCCATGT-5′
MTNR1B	5′-CTGTCGGTGTATCGGAACAAG-3′
	3′-CCAACGGGTACGGATAAATGG-5′

membrane was incubated with a primary antibody at 4°C overnight, then washed, and incubated using a secondary antibody for 1 h, visualized by chemiluminescence (Alegria-Schaffer, 2014). The quantification of target protein was detected by ChemiScope (PowerPac^{TM} Basic, Singapore) analysis program. All bands were normalized to the internal reference vinculin.

2.7. Real-time PCR analysis

The actin gene was used as an internal reference to normalize mRNA levels (Tian et al., 2020). The primers were searched using Primer 3.0 software or Primer bank and their specificity were verified through National centre for Biotechnology Information. The details of gene-specific primers is displayed in Table 1.

Total RNA was extracted with RNA-Quick Purification Kit (Yi Shan Biotechnology Co., LTD., Shanghai, China). Total cDNA was generated by using M-MuLV First Strand cDNA Synthesis Kit (Sangon Biotech) and diluted at 1:15 with sterile water before use. Real-time PCR was performed in a 10 uL system containing diluted cDNA (1 μL), 5 μL of 2X SYBR Green PCR Master Mix (Applied Biosystems, USA), 0.2 μL of each primer (10 μM final concentration) and 3.6 μL of deionized water. PCR was run on ABI 7500 Fast Real-Time PCR instrument. PCR program was set as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The specificity of product amplification was verified by melting curve analysis. The expression level of target gene was normalized to that of actin and then detected by using the comparative Ct ($2^{-\triangle\triangle Ct}$) method (Schmittgen and Livak, 2008).

2.8. ELISA

According to the manufacturer's instructions, ELISA Kits (CUSABIO,China) determined the concentrations of MT in serum and different areas of the brain (Zakharova et al., 2019). The major steps were taken as follows. Firstly, ELISA kits and samples were equilibrated to room temperature for 30 min before measurement. Secondly, 50 μL samples or standards were added to the pre-coated enzymatic standard holes, respectively, setting a blank well without any processing. Then 50 μL HRP-conjugate mixed solution was added to each hole. The enzyme-labeled plates were incubated at 37°C for 60 min. After the incubation, each well was washed three times and fully dried out. Thirdly, 50 μL substrate A and 50 μL substrate B were added to each well, respectively, incubating for 15 min at 37°C. Finally, 50 μL stop solution was added to each hole to terminate the reaction. The optical density at the wavelength of 450 nm was measured with a microplate reader (Varioskan Flash, Thermo Fisher Scientific, USA).

2.9. Drug Targets of volatile oil ingredients

The PubChem database (Kim et al., 2016) (https://pubchem.ncbi.nlm.nih.gov/) is a key chemical information resource. The volatile oil ingredients were imported into the PubChem database, and the 3D molecular structure was exported in SDF files. Swiss Target Prediction (http://old.swisstargetprediction.ch/) and PharmMapper (http://www.lilab-ecust.cn/pharmmapper/) are freely accessed databases designed to identify the potential target of uploaded molecular structure. Finally, a total of 14 volatile oil compounds were removed due to the lack of structural information and sleep-related target. Cinnamaldehyde, Behenic alcohol, Tetradecanal, and Gleenol were kept for further research in this study.

2.10. Insomnia Significant targets

Insomnia's significant targets were extracted from GeneCards (https://www.genecards.org/) database. GeneCards is a comprehensive database that contains genomic, transcriptomic, proteomic, genetic, clinical, and functional information. This study uses "insomnia" and "sleep disorder" as keywords to search for disease-related targets. The targets with probability ≥ 0.5 were used for further research.

2.11. Molecular docking verification

AutoDock is a suite of C programs designed to predict the bound conformations of ligands to macromolecular targets of known structures (Duan et al., 2021). The corresponding proteins were obtained in Protein Data Bank (https://www.rcsb.org/). Then the structure of screened targets was embellished by the PyMOL program. The binding energy < −1.2 kcal/mol was used as the standard for evaluating docking results.

2.12. Statistical analysis

Statistical analyses were performed using GraphPad Prism 8.0 software. All data were analyzed using one-way ANOVA, followed by the least significant difference (LSD) method for comparisons among groups. All data were expressed as the mean ± standard error of the mean (SEM), P values < 0.05 were considered statistically significant.

3. Results

3.1. Analysis of volatile oil components

Volatile oil yield: JTW (0.2 ml); CR (0.05 ml); CIN (0.48 ml). Volatile oil ingredients derived from CR, CIN, and JTW are listed in Table 2. The total ion current chromatogram is shown in Fig. 1. By GC–MS analysis, we found that CR included 7 components, CIN included 10 components, and JTW included 18 components, accounting for 95.06%, 90.58%, and 88.31% of the total volatile oil, respectively. Among them, L-Ascorbic acid 2,6-dihexadecanoate (45.74%), Cinnamaldehyde (66.85%) and n-Hexadecanoic acid (18.03%) have the highest proportion in CR, CIN and JTW, respectively. Interestingly, the proportion of cinnamaldehyde declined from 66.85% in CIN to 6.07% in JTW, while L-Ascorbic acid 2,6-dihexadecanoate was disappeared in JTW. Moreover, six new compounds appeared in JTW, including n-Hexadecanoic acid (18.03%), α-Muurolene (7.61%), Di-epi-α-cedrene-(I) (2.88%), Tetradecanal (1.9%), Cadala-1 (10),3,8-triene (1.59%) and gleenol (1.45%). The ion current chromatographic peak area was used for normalization, the relative percentage of each component in the volatile oil is obtained.

3.2. Molecular structure of volatile oil compounds

The 2D molecular structure of each volatile oil ingredient was drawn by ChemDraw software and shown in Fig. 2, except for ingredients less than 1 percentage.

3.3. Analysis of the ingredient-target network

A total of 18 volatile oil ingredients were separated from JTW, which corresponded with Traditional Chinese Medicine(TCM)'s multi-component characteristics . Among them, 14 ingredients were removed because their associated targets cannot be successfully predicted. The network was composed of 53 nodes (4 volatile oil ingredient nodes and 49 target nodes) and 78 edges in Fig. 3. Each component node was connected to multiple target nodes. The results implied the relationship between ingredients and targets, which provided a foundation for further research of JTW.

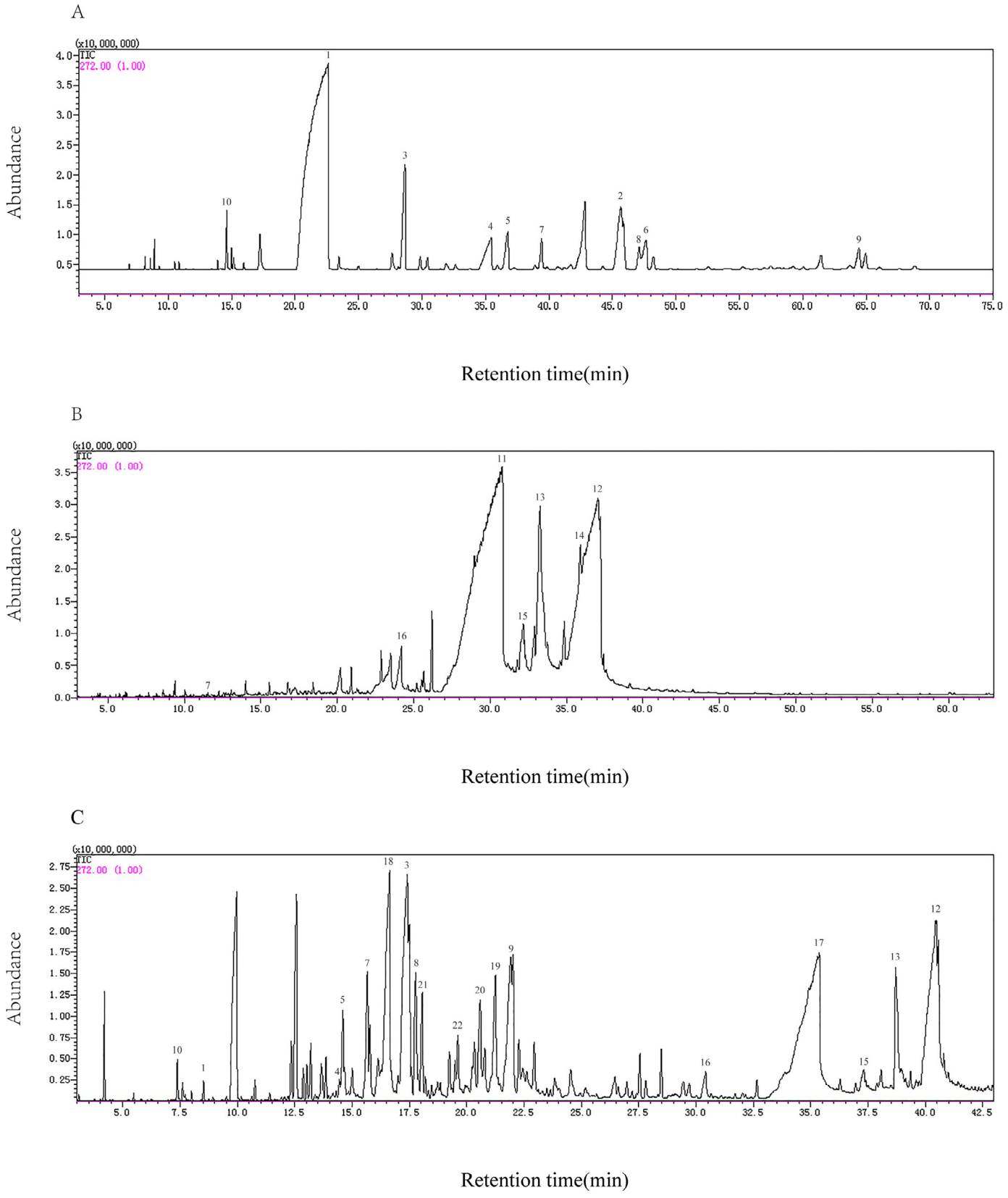

Fig. 1. The total ion current chromatogram. (A) *Cortex Cinnamomi*. (B) *Coptidis Rhizoma*. (C) Jiao-Tai-Wan.

Table 2
Main Volatile oil compounds of CIN, CR, and JTW.

No	Compound name	formula	Retention time (min)			Relative percent (%)		
			CIN	CR	JTW	CIN	CR	JTW
1	Cinnamaldehyde, (E)-	C_9H_8O	22.557		8.534	66.85	—	6.07
2	Isoledene (-)-	$C_{15}H_{24}$	45.678			6.55	—	—
3	α-Copaene	$C_{15}H_{24}$	29.009		17.401	4.74	—	17.19
4	Coumarin	$C_9H_6O_2$	35.116		14.467	3.52	—	0.19
5	Acetic acid, cinnamyl ester	$C_{11}H_{12}O_2$	36.771		14.576	2.02	—	1.09
6	4-Methoxycinnamaldehyde	$C_{10}H_{10}O_2$	47.672			1.89	—	—
7	γ-Muurolene	$C_{15}H_{24}$	39.595	11.433	15.68	1.82	0.01	2.8
8	α-Cubebene (-)-	$C_{15}H_{24}$	47.123		17.767	1.12	—	0.59
9	α-Cadinol	$C_{15}H_{26}O$	64.479		21.896	1.05	—	6.77
10	Benzenepropanal	$C_9H_{10}O$	14.662		7.383	1.02	—	0.25
11	l- (+)-Ascorbic acid 2,6-dihexadecanoate	$C_{38}H_{68}O_8$		30.805		—	45.74	—
12	9,12-Octadecadienoic acid (Z,Z)-	$C_{18}H_{32}O_2$		37.076	40.199	—	26.85	14.53
13	Behenic alcohol	$C_{22}H_{46}O$		33.275	38.734	—	10.26	3.61
14	Butyl 9,12-octadecadienoate	$C_{22}H_{40}O_2$		35.916		—	9.17	—
15	cis-10-Heptadecenoic acid	$C_{17}H_{32}O_2$		32.181	37.314	—	1.86	1.18
16	Pentadecanoic acid	$C_{15}H_{30}O_2$		24.198	30.281	—	1.17	0.58
17	n-Hexadecanoic acid	$C_{16}H_{32}O_2$			35.334	—	—	18.03
18	α-Muurolene	$C_{15}H_{24}$			16.622	—	—	7.61
19	Di-epi-α-cedrene- (I)	$C_{15}H_{24}$			21.233	—	—	2.88
20	Tetradecanal	$C_{14}H_{28}O$			20.58	—	—	1.9
21	Cadala-1 (10),3,8-triene	C15H22			18.041	—	—	1.59
22	Gleenol				19.608	—	—	1.45

CIN: *Cortex Cinnamomi*; CR: *Coptidis Rhizoma*; JTW: Jiao-Tai-Wan.

Table 3
Volatile oil Ingredients and its associated targets.

Ingredient	Target	Uniprot ID	Target	Uniprot ID	Target	Uniprot ID
Cinnamaldehyde						
	MTNR1B	P49286	NOS1	P29475	NOS2	P35228
	MTNR1A	P48039	KAT2B	Q92831	PARP1	P09874
	ACHE	P22303	DNMT1	P26358	MAOB	P27338
	CYP2A6	P11509	DRD2	P14416	SLC6A3	Q01959
	GSK3B	P49841	BCHE	P06276	MAP2K1	Q02750
	TLR4	O00206	MAPK8	P45983	ACE	P12821
					SLC6A4	P31645
Gleenol						
	MTNR1B	P49286	ESR1	P03372	CYP2C19	P33261
	MTNR1A	P48039	ESR2	Q92731	NR3C1	P04150
	ACHE	P22303	SLC6A4	P31645	PPARG	P37231
	CYP19A1	P11511	NR1I3	Q14994	HRH3	Q9Y5N1
	CYP17A1	P05093	BCHE	P06276	DHCR7	Q9UBM7
	SHBG	P04278	SLC6A2	P23975	PER2	O15055
Behenic alcohol						
	MTNR1B	P49286	NR1H4	Q96RI1	HTR2A	P28223
	MTNR1A	P48039	GPBAR1	Q8TDU6	DRD3	P35462
	ACHE	P22303	CYP19A1	P11511	DRD4	P21917
	ESR1	P03372	OPRM1	P35372	GABRA2	P47869
	ESR2	Q92731	ENPP2	Q13822	TACR1	P25103
	SHBG	P04278	PPARG	P37231	CYP3A4	P08684
	NR1I3	Q14994	DRD2	P14416	MAPK8	P45983
Tetradecanal						
	MTNR1B	P49286	MAPK10	P53779	MMP2	P08253
	MTNR1A	P48039	GRM5	P41594	PARP1	P09874
	ACHE	P22303	GSK3B	P49841	NR3C1	P04150
	CYP19A1	P11511	HTR2A	P28223	DRD2	P14416
	NR1I3	Q14994	HTR2C	P28335	DRD3	P35462
	GCK	P35557	TACR1	P25103	OPRM1	P35372
	HTR6	P50406	MMP9	P14780		

3.4. Analysis of common targets

The predicted targets of Cinnamaldehyde, Behenic alcohol, Tetradecanal, and Gleenol were mapped to insomnia significant targets to acquire the common targets, respectively. The common targets are listed in Table 3.

3.5. Analysis of molecular docking

The binding energy of the volatile oil ingredients is listed in Table 4. The results indicated that the volatile oil ingredients had good binding activities with MTNR1B except for Behenic alcohol, as shown in Fig. 4. Furthermore, based on the binding energy, the high to low ingredients were Gleenol, Cinnamaldehyde, Tetradecanal.

Fig. 2. Structural formula of volatile oil constituents. (A) Volatile oil constituents in *Cortex Cinnamomi*. (B) Volatile oil constituents in *Coptidis Rhizoma*. (C) Volatile oil constituents in Jiao-Tai-Wan. (1) Cinnamaldehyde, (E)-. (2) Isoledene (-)-. (3) α-Copaene. (4) Coumarin. (5) Acetic acid, cinnamyl ester. (6) 4-Methoxycinnamaldehyde. (7) γ-Muurolene. (8) α-Cubebene (-)-. (9) α-Cadinol. (10) Benzenepropanal. (11) L- (+)-Ascorbic acid 2,6-dihexadecanoate. (12) 9,12-Octadecadienoic acid (Z,Z)-. (13) Behenic alcohol. (14) Butyl 9,12-octadecadienoate. (15) cis-10-Heptadecenoic acid. (16) Pentadecanoic acid. (17) n-Hexadecanoic acid. (18) α-Muurolene. (19) Di-epi-α-cedrene- (I). (20) Tetradecanal. (21) Cadala-1 (10),3,8-triene. (22) gleenol.

Table 4
The binding energy of MTNR1B to each volatile oil compound.

Compounds	Binding energy (kcal/mol)
Cinnamaldehyde	−3.21 kcal/mol
Behenic alcohol	0.15 kcal/mol
Tetradecanal	−1.45 kcal/mol
Gleenol	−3.9 kcal/mol

3.6. JTW reduced Melatonin levels in the brain stem of rats

Melatonin (MT) disorders or decreased MT levels are characteristic of insomnia (Drake et al., 2018). Thus, it is necessary to evaluate the possible effect of JTW on MT secretion in PCPA-induced insomnia rats. In the model group, we found that MT levels in serum, prefrontal cortex, hypothalamus, hippocampus, and brain stem were lower than those in the control group ($P < 0.01$), as shown in Fig. 5. However, the level of MT in the brain stem was obviously down-regulated compared to the model group ($P < 0.05$) after the treatment of JTW. Moreover, JTW did not affect MT levels in serum and other brain parts.

3.7. JTW increased MTNR1B protein levels in the prefrontal cortex of rats

Based on the previous pharmacological studies, the effect of MT on sleep, particularly such non-rapid eye movement (NREM) sleep was mainly mediated by MTNR1B (Dubocovich, 2007). We determined whether JTW could affect the expression of MTNR1B protein in different areas of the brain. As illustrated in Fig. 6, MTNR1B protein levels in the model group were remarkably reduced compared to the control group ($P < 0.01$). After treatment of JTW for 7 days, the level of MTNR1B protein in the prefrontal cortex increased significantly compared to the model group ($P < 0.05$). Furthermore, JTW did not affect the MTNR1B protein levels in other brain parts such as the hypothalamus, hippocampus, and brain stem.

3.8. JTW increased MTNR1B mRNA levels in rats' brain stem and prefrontal cortex

As shown in Fig. 7, in the model group, the MTNR1B mRNA levels in the prefrontal cortex, hippocampus and brain stem were lower than those in the control group ($P < 0.05$, $P < 0.01$). After treatment of JTW for one week, the MTNR1B mRNA levels in the brain stem and prefrontal cortex were up-regulated compared to the model group

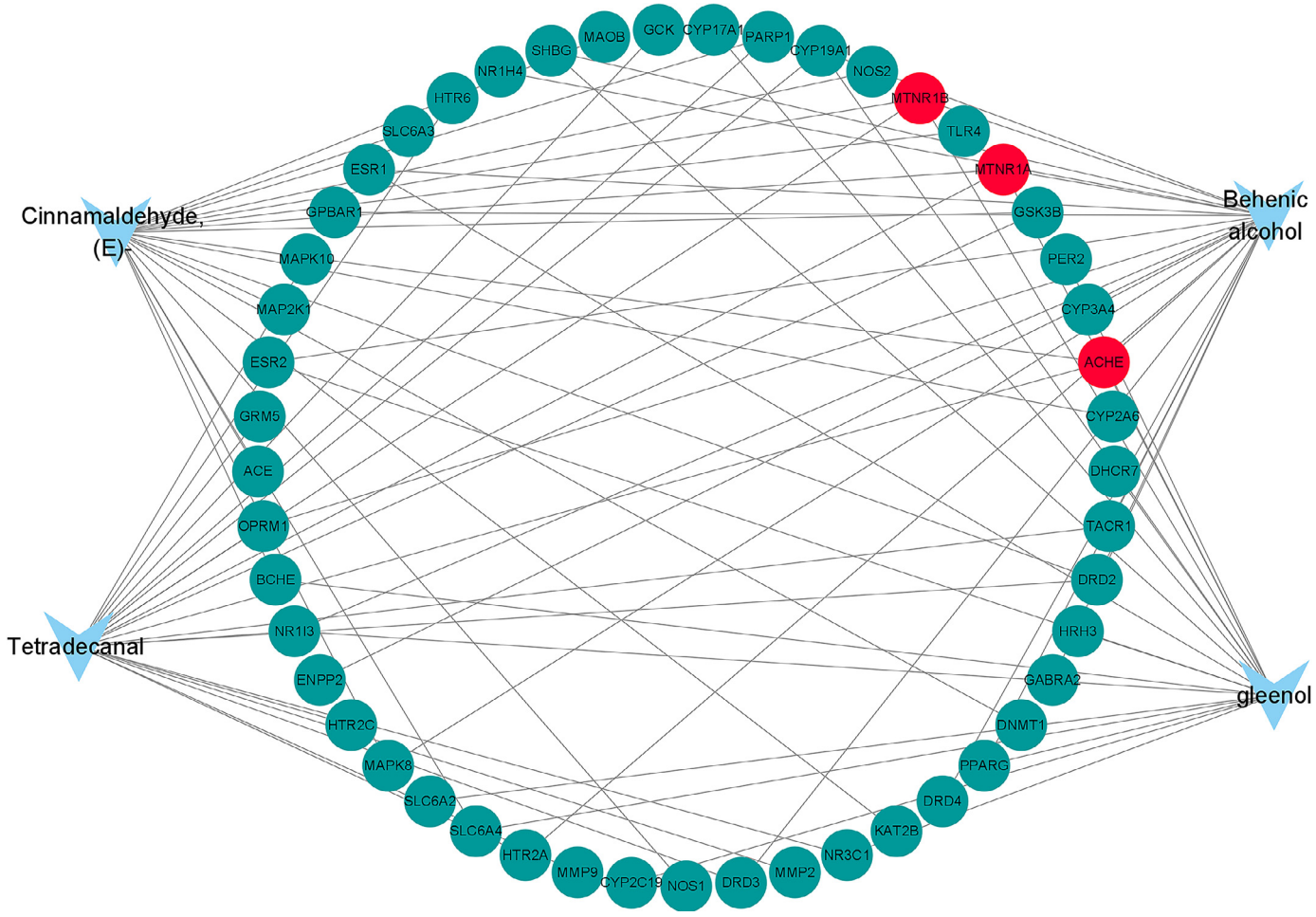

Fig. 3. Ingredient-Target Network. Blue arrows represent volatile oil compounds. Blue circles represent the targets between insomnia and each volatile oil compound. Red circles represent the most important targets. Edges represent the interaction between ingredients and targets.

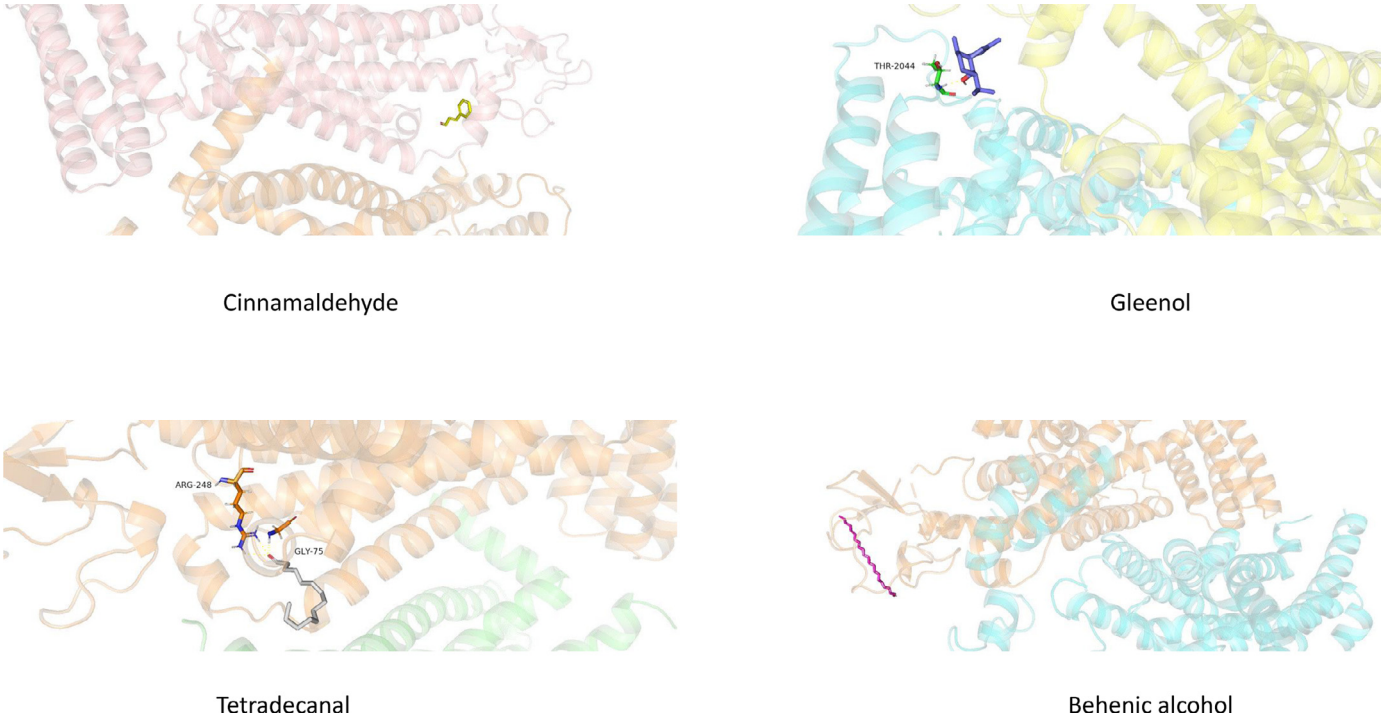

Fig. 4. Molecular docking results.

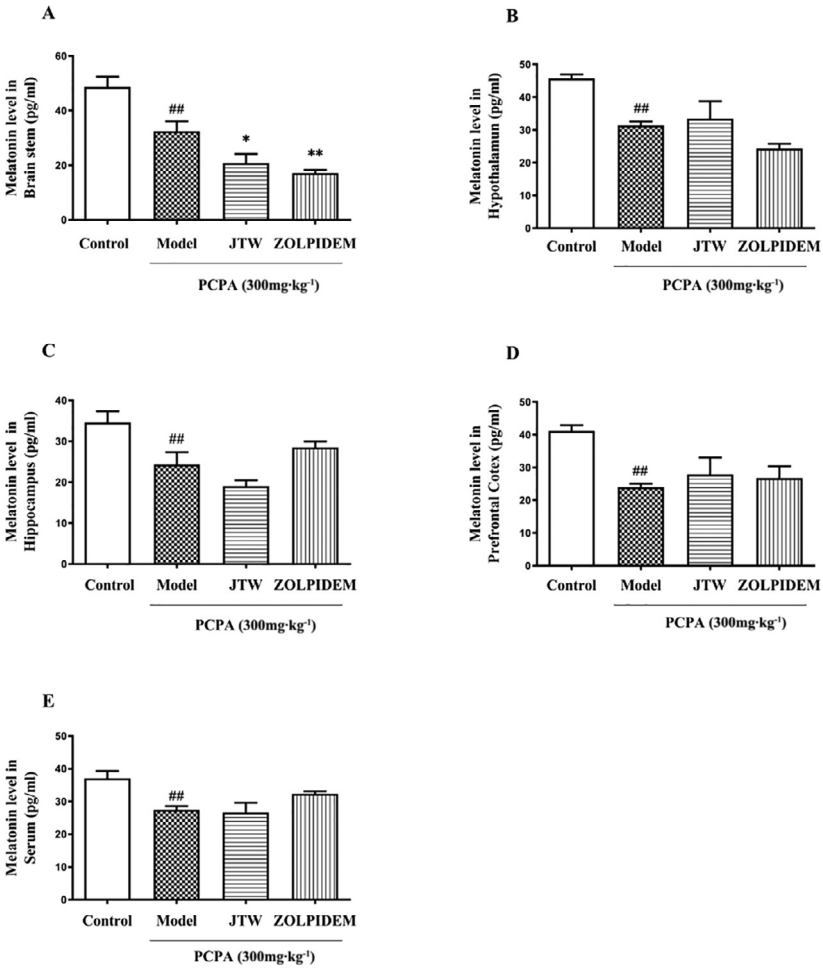

Fig. 5. JTW regulated Melatonin levels in serum and different brain regions of PCPA-induced insomnia rats ($n = 6$). (A) Melatonin content in brain stem. (B) Melatonin content in hypothalamus. (C) Melatonin content in hippocampus. (D) Melatonin content in prefrontal cortex. (E) Melatonin content in serum. Data were expressed as mean ± SEM; $^{\#\#}P < 0.01$ indicates significant differences between the control group and model group; $^{*}P < 0.05$ and $^{**}P < 0.01$ indicates significant differences between the model group and JTW group or Zolpidem group as assessed by ANOVA followed by the LSD test. JTW: Jiao-Tai-Wan; PCPA: p-Chlorophenylalanine.

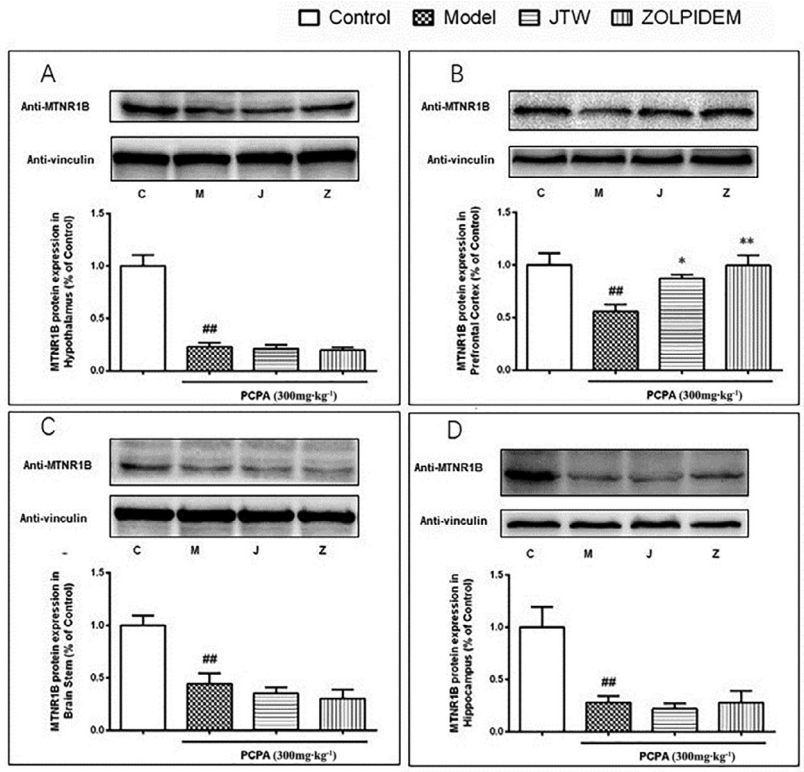

Fig. 6. JTW regulated MTNR1B protein levels in different brain regions of PCPA-induced insomnia rats ($n = 3$). (A) MTNR1B protein level in hypothalamus. (B) MTNR1B protein level in prefrontal cortex. (C) MTNR1B protein level in brain stem. (D) MTNR1B protein level in hippocampus. Data were expressed as mean ± SEM; $^{\#\#}P < 0.01$ indicates significant differences between the control group and model group; $^{*}P < 0.05$ and $^{**}P < 0.01$ indicates significant differences between the model group and JTW group or Zolpidem group as assessed by ANOVA followed by the LSD test. JTW: Jiao-Tai-Wan; PCPA: p-Chlorophenylalanine; MTNR1B: Melatonin receptor type 1b. "C": Control; "M": Model; "J": JTW; "Z": ZOLPIDEM.

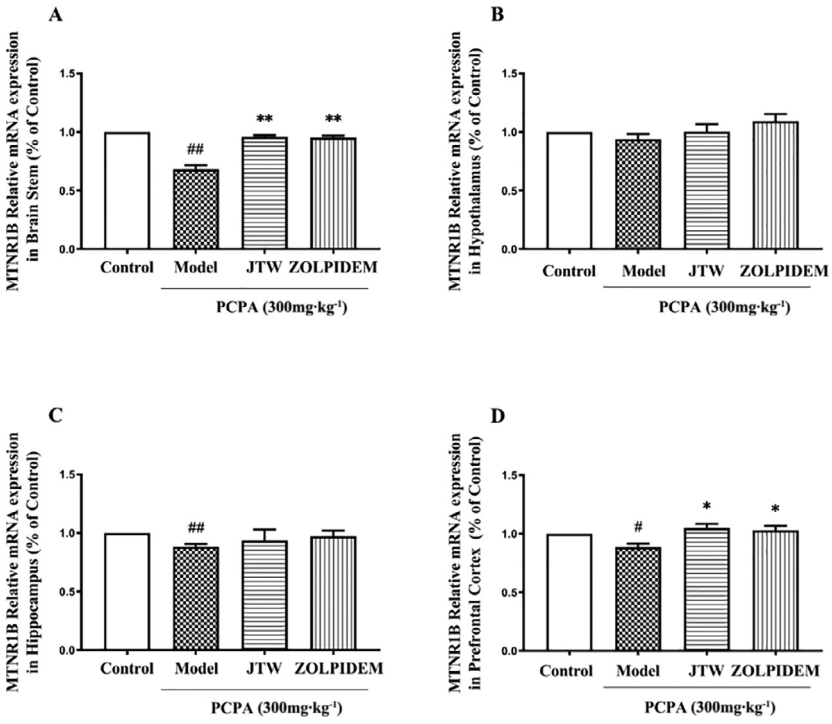

Fig. 7. JTW regulated MTNR1B mRNA levels in different brain regions of PCPA-induced insomnia rats (n = 5). (A) MTNR1B mRNA level in brain stem. (B) MTNR1B mRNA level in hypothalamus. (C) MTNR1B mRNA level in hippocampus. (D) MTNR1B mRNA level in prefrontal cortex. Data were expressed as mean ± SEM; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ indicates significant differences between the control group and model group; $^{*}P < 0.05$ and $^{**}P < 0.01$ indicates significant differences between the model group and JTW group or Zolpidem group as assessed by ANOVA followed by the LSD test. JTW: Jiao-Tai-Wan; PCPA: p-Chlorophenylalanine.; MTNR1B: Melatonin receptor type 1b.

($P < 0.05$, $P < 0.01$). In addition, JTW did not affect the level of MTNR1B mRNA in other parts of the brain, such as the hypothalamus and hippocampus.

4. Discussion

JTW is a traditional prescription composed of CR and CIN and is considered one of the most traditional herbal prescriptions for insomnia (Yeung et al., 2012). Numerous studies have indicated that volatile oil components derived from different Chinese herbal medicines, such as Shi Chang Pu (*Acorus Tatarinowii*), Yi Ye Bai Jiang (*Patrinia Heterophylla*), and Ce Bai (*Cacumen Platycladi Orientalis*), could reduce the frequency of activity, shorten sleeping latency, the number and rate of mice successfully fell asleep increased after treated with these TCM drugs (Shi et al., 2016). Previous studies have shown that the sedative effect of volatile oil components is mainly based on its inhibitory effect on the central nervous system, which increases the levels of NE and 5-HT in the brain of mice and inhibits the release of dopamine (DA) (Shi et al., 2016). However, it is rarely mentioned whether the volatile oil ingredients play a key role in the anti-insomnia effect of JTW. Therefore, this study systematically analyzed the role of volatile oil ingredients in JTW in insomnia from the anti-insomnia action, mechanisms, and material basis.

In this study, GC–MS analysis showed that a total of 18 volatile oil ingredients were separated from JTW with 6 new compounds. Among such new compounds, n-Hexadecanoic acid has the highest proportion. Moreover, the content of cinnamaldehyde in JTW was significantly decreased. It might be due to the reaction between the alkaloids from CR and phenolic acids from CIN during sediment formation, which may adsorb some cinnamaldehyde. Although studies have shown that cinnamaldehyde possesses a certain anti-insomnia effect, it can also increase the absorption and distribution of berberine in the brain and intestine (Kumar et al., 2019; Wang et al., 2020). But it may not be the most important volatile component in JTW in the treatment of insomnia.

Network pharmacology analysis revealed that the predicted targets of Cinnamaldehyde, Behenic alcohol, Tetradecanal, and Gleenol were significantly related to sleep. These targets were mapped to significant insomnia targets to acquire the common targets, including MTNR1B, MTNR1A and ACHE. Melatonin receptor type 1a (MTNR1A) and type 1b (MTNR1B) are two types of G protein-coupled receptors family (GPCR). Both of them are found in the central nervous and numerous peripheral tissues. This distribution pattern of distribution shows a traditional function in neuroendocrine regulation and a role in the arrangement of physiological reflection in both the central nervous system and peripheral tissues (Drew et al., 2001). Molecular docking analysis showed that the volatile oil components had a good binding activity with MTNR1B. In addition, based on the binding energy, the components ranking from high to low were Gleenol (−3.9 kcal/mol), Cinnamaldehyde (−3.21 kcal/mol), Tetradecanal (−1.45 kcal/mol), Behenic alcohol (0.15 kcal/mol). The lower the binding energy, the more stable the structure. It indicates that Cinnamaldehyde, Tetradecanal, and Gleenol are more likely to bind to MTNR1B, which is concerned with the therapeutic effect of JTW.

MT, the neuro-hormone synthesized during the night, is a key factor in the control of circadian rhythm, has a significant impact on sleep disturbances (Tosini et al., 2014). A double-blind, randomised clinical trial revealed that MT supplementation over a four-week period is effective and safe in improving objective sleep quality in middle-aged patients with insomnia (Xu et al., 2020).

It is also reported that the effect of MT on regulating circadian rhythms and the sleep-wake cycle was mainly mediated by its specific receptors (Li et al., 2013; Nishimon et al., 2021).

It is worth noting that the effect of MT on sleep is mediated by MTNR1B (Fisher and Sugden, 2009). This idea was supported by experimental data demonstrating that MT can promote NREM sleep by acting on MTNR1B located in the reticular thalamic nucleus, and infusion of MTNR1B agonist in this nucleus increased the firing rate of the neurons in this area (Ochoa-Sanchez et al., 2011). We found that both MT and MTNR1B levels in different brain regions of PCPA-induced insomnia rats were obviously down-regulated. JTW could regulate MTNR1B and MT levels in different brain areas, but the effects are opposite. The decrease in MT may be due to n-Hexadecanoic acid production in JTW. n-Hexadecanoic acid is a saturated fatty acid that causes apoptosis in many cell types, and MT can inhibit this effect, but the latter will be consumed in large quantities (Cui et al., 2021). The increase in MTNR1B may be based on the direct effect of Cinnamaldehyde, Tetradecanal, Gleenol or due to the negative feedback regulation caused by the decrease in MT.

Usually, protein and mRNA levels show reasonable correlation (Buccitelli and Selbach, 2020), protein biosynthesis is a conserved process and modulated by mRNA (Mauger et al., 2013; Nürenberg-Goloub and Tampé, 2019). The process of mRNA translation can be functionally divided into three phases: initiation, elongation, and termination (Hershey et al., 2012). Initiation of mRNA translation is a major checkpoint for regulating the level and fidelity of protein synthesis (Gualerzi and Pon, 2015). Importantly, this phenomenon is mediated by RNA-binding proteins (RBPs) and microRNAs (miRNAs) (Iadevaia and Gerber, 2015). Recent studies have shown that RBPs and miRNAs control protein levels by regulating mRNA stability and translation (Fukao and Fujiwara, 2017). Notably, miRNAs simultaneously mediates mRNA degradation via independent mechanisms (Fabian and Sonenberg, 2012). In this study, we found that the protein and mRNA expression of MTNR1B were inconsistent. There was a significant difference in the protein expression of MTNR1B and was a non-significant difference in its mRNA expression after the treatment of JTW and zolpidem. Thus, we surmised that JTW and zolpidem inhibit mRNA degradation and promote mRNA translation by regulating mRNA cis-elements and trans-acting factors such as RBPs and miRNAs. Simultaneously, these drugs can increase protein accumulation by blocking the proteolysis or lysis pathway.

5. Conclusion

As an important anti-insomnia traditional Chinese decoction, JTW significantly reduces MT in the brain stem and increases the level of MTNR1B in the prefrontal cortex of PCPA-induced insomnia rats. These effects may be related to its volatile oil components, such as Cinnamaldehyde, Tetradecanal, and Gleenol, which have good binding activities with MTNR1B.

Ethical Approval

All procedures performed in studies involving animals were approved by animal care and use committees where the studies were conducted.

Data Availability

Nil.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Xi Liu: Investigation, Writing – original draft. **Zhengzhong Yuan:** Investigation, Visualization, Funding acquisition. **Congcong Zeng:** Writing – original draft, Writing – review & editing. **Yan Huang:** Investigation. **Xie Xu:** Writing – original draft, Writing – review & editing. **Wenqin Guo:** Writing – original draft, Writing – review & editing. **Hongbin Zheng:** Supervision. **Ruanjuan Zhan:** Supervision, Project administration.

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Supplementary Materials

Nil.

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