

Gan-Dan-Liang-Yi-Tang alleviates p-chlorophenylalanine-induced insomnia through modification of the serotonergic and immune system

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Abstract. Gan-Dan-Liang-Yi-Tang (GDLYT) is a Traditional Chinese Medicine that has been historically used for the treatment of insomnia. However, investigations into its pharmacological ingredients and the mechanism underlying its sedative and hypnotic effects remain limited. The present study reported the detailed mechanisms underlying the sedative and hypnotic effects of GDLYT. Kunming mice were administered GDLYT at various sub-hypnotic doses, which underwent sodium pentobarbital treatment test, pentetrazole induced convulsant studies and p-chlorophenylalanine (PCPA) induced insomnia model. Potentiated hypnotic and sedative effects in mice was studied, and also the changes in related neurotransmitter and immune factors were evaluated. The results suggested that GDLYT possessed weak sedative effects on pentetrazole-induced convulsive activity in normal mice at a dose of 1.3 mg/kg, with an increase in sleep onset in subhypnotic dose of sodium pentobarbital-treated mice. GDLYT was also able to alleviate insomnia induced by PCPA in the rodent models, and increased 5-hydroxytryptamine levels in the prefrontal cortex, hippocampus, hypothalamus and corpus striatum of PCPA-treated rats. Furthermore, the hypnotic effects of GDLYT were modified, which allowed for PCPA-induced immune system changes, including increased interleukin (IL)-1 β , tumor necrosis factor- α and IL-2 expression levels. The results of the present study indicated that GDLYT induced sedative and hypnotic bioactivity by regulating serotonergic activity in the central nervous system and immune system.

Introduction

Gan-Dan-Liang-Yi-Tang (GDLYT) is a prescription drug used for the treatment of insomnia and terrified or sleepless, as described in the Chinese Traditional Medicine book “Bian-Zheng-Lu”. GDLYT is composed of 3 ingredients: Yuan Zhi (*Polygala tenuifolia*), Bai Shao (*Paeonia lactiflora* Pall.) and Chao Zao Ren [*Ziziphus jujuba* Mill. var. *spinosa* (Bunge) Hu ex H. F. Chow]. Yuan Zhi has been used to treat insomnia, anxiety, restlessness and disorientation (1,2). Polygalasaponins, the primary constituents of Yuan Zhi, are able to enhance pentobarbital-induced sleeping behaviors via γ -aminobutyric acid (GABA)-ergic systems in mice (3). Bai Shao, the processed root portion of *Paeonia lactiflora* Pall (Ranunculaceae), is a component of numerous Chinese medicinal formulae prescribed for the treatment of depression-like syndromes; Bai Shao functions by modifying the levels of serotonin [5-hydroxytryptamine (5-HT)] and its metabolite 5-hydroxyindoleacetic acid in the hippocampus (4-6). Chao Zao Ren has been reported to be a component of various Chinese Medicinal Herbs (7). Chao Zao Ren is able to improve sleep quality in patients and inhibit motion sickness effectively by reducing blood hormones levels in the hypothalamic-pituitary-adrenocortical axis (7-9).

Insomnia is a sleep disorder prevalent in women and the elderly (10). Currently, drugs used for the treatment of insomnia predominantly target the γ -aminobutyric acid (GABA) receptor, melatonin receptor, histamine receptor, orexin and serotonin receptor (10). Herbal medicine and Complementary and Alternative Medicine, such as Piper methysticum and the seed of *Ziziphus jujuba* Mill var. *spinosa*, have been widely used in phytotherapy for insomnia (11). GDLYT has been used for thousands of years as a Traditional Chinese Medicine; however, few studies have been conducted on its potential therapeutic effects in animal models. The present study investigated the sedative and hypnotic activities of GDLYT and explored the mechanisms underlying the sedative and hypnotic effects of GDLYT in various mouse models of insomnia. These effects were also investigated by analyzing monoamine neurotransmitters and inflammatory cytokines in the mouse models.

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Materials and methods

Extraction of GDLYT. Plant materials were purchased from Beijing Tong Ren Tang Medicinal Materials Co., Ltd. (Beijing, China) in October 2012. Shade-dried Yuan Zhi, Chao Zao Ren and Bai Shao were extracted 3 times with H₂O for 1 h using a circumfluence extraction method, mixed with 50% EtOH, and the extracts were then filtered prior to the removal of EtOH insoluble parts. The soluble part was concentrated using a rotary vacuum evaporator. The residue was stored for 1 week at room temperature to obtain a dry solid mass.

Animals and ethical approval. A total of 420 Kunming mice weighing 20–24 g and 56 adult male Sprague-Dawley rats weighing 180–200 g were used for the behavioral experiments. All animals were obtained from the Animal Breeding Center of the PLA General Hospital (Beijing, China). The animals were housed in cages (45x60x25 cm) with water and food available *ad libitum* at a constant temperature (22±2°C), under an 12-h light/dark cycle (lights on at 7:00). All animal experiments were carried out in accordance with the Principles of Laboratory Animal Care, the China legislation for the use and care of laboratory animals and the Institutional Animal Care and Use Committee of General Hospital of Chinese PLA.

Sub-hypnotic dosage of sodium pentobarbital treatment. Animals in the control and model groups were administered distilled water, while the drug treatment groups were administered sodium pentobarbital (1.3 mg/kg/day), and the animals in experimental groups were administered GDLYT at doses of 0.65, 1.3, 2.6 and 5.2 g/kg/day. Drugs were administered orally once daily for various durations, sodium pentobarbital i.p. (25 mg/kg) was carried on after 50 min of the last administration, the onset of sleep was observed in each mouse. Mice were considered to be asleep when they lost righting reflex for >1 min. In the sub-hypnotic dosage of sodium pentobarbital treatment test (12), the percentage of sleep onset was calculated as follows: Sleep onset (%) = (No. falling asleep/total no.) x 100.

Anticonvulsant experiments. The modified methods previously outlined by Chindo *et al* (13) and Ngo Bum *et al* (14) were used to estimate the anticonvulsant effect of GDLYT in mice. Briefly, the mice acclimatized to their new environment prior to the start of each experiment, and were kept in individual transparent mouse cages (25x15x15 cm) for 30 min. Pentetrazole (PTZ; 100 mg/kg) was used to induce convulsions (seizures) in the mice. Time latency between the administration of PTZ and myoclonic convulsive behavior was scored as follows: 0, no response; 1, ear and facial twitching; 2, axial convulsive waves observed through the body; 3, body jerks; 4, generalized clonic convulsions with turning over into side position; 5, generalized convulsions with tonic extension episodes and status epilepticus; 6, mortality (15). Following an additional 30 min, the lethality of treatment on the mice was recorded.

Parachlorophenylalanine (PCPA) pretreated model. For the PCPA pretreatment test, rats received intraperitoneal injection

of PCPA (300 mg/kg) between 08:00 and 09:00 once a day for 2 days. At 2 days after PCPA injection, GDLYT at various doses were administered for 7 days, observers were blinded to the treatment. Following PCPA injection, each mouse was observed and total movement distance was recorded, and rats were subsequently anesthetized by chloral hydrate (350 mg/kg) and sacrificed. The hippocampus, piriform cortex, hypothalamus, corpus striatum and brain stem were dissected from the brain on ice and the tissue samples were immediately immersed in liquid nitrogen and stored until further use.

Determination of 5-HT, NE and DA levels by high-performance liquid chromatography (HPLC). The level of 5-HT, NE and DA were determined by reverse-phase HPLC with electrochemical detection, as previously described (16). The 5-HT, DA and NE levels were determined as mg/g wet weight tissue. Identification (by retention times) and content of the compounds (by peak areas) was determined in the tissue samples by comparing 0.2–4 ng/ml 5-HT, NE and DA standard solutions.

Serum cytokine measurements. Serum tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , IL-2, IL-4 and IL-6 concentrations were quantified by specific rat ELISA sandwich assays, performed using antibodies (R&D Systems China Co. Ltd., Shanghai, China) according to the manufacturer's protocol. Absorbance was read at 450 nm on a microtitre plate reader (PerkinElmer, Inc., Waltham, MA, USA) and results were presented as ng/l of serum.

Statistical analysis. The results are presented as means \pm standard error of the mean, indicating the number of animals per group for each experiment. Data were analyzed by one-way analysis of variance followed by Dunnett's post-hoc test for multiple comparisons. In a sub-hypnotic dosage of sodium pentobarbital treatment test, a χ^2 test was used to compare number of mice that fell asleep. $P < 0.05$ was considered to indicate a statistically significant result.

Results

GDLYT induces hypnotic and sedative effects in mice. On the subhypnotic dosage of pentobarbital-treated mice, GDLYT raised the rate of sleep onset in 5 and 7 days with significant effects at 1.3 and 0.65 mg/kg ($P < 0.01$; Table I). Following the PTZ-induced seizures experiment, GDLYT was observed to prolong the latency of convulsions in the treated groups after 5 and 7 days, as compared with the control group at 1.3 mg/kg doses and also protect against the mice mortality in 7 days ($P < 0.05$; Table II).

Effect of GDLYT on PCPA-induced insomnia in pentobarbital-treated mice. Consistent with the results of our previous unpublished study, the results of the present study indicated that treatment with PCPA (300 mg/kg) successfully induced insomnia. GDLYT significantly reversed the insomnia effects in PCPA-treated mice, by decreasing the total movement distance on days 2 and 7 ($P < 0.05$; Table III) compared with the PCPA mice.

Table I. Effect of GDLYT on the sleep onset of mice treated with a sub-hypnotic dose of sodium pentobarbital (n=10-14).

Group	Dosage (g/kg)	3 days		5 days		7 days	
		No. of mice asleep (/10)	Sleep onset (%)	No. of mice asleep (/10)	Sleep onset (%)	No. of mice asleep/total	Sleep onset (%)
Control	-	1	10	1	10	1/12	8
Diazepam	0.0013	9	90 ^a	9	90 ^a	9/13	69 ^a
GDLYT	5.2	3	30	4	40	4/13	31
	2.6	5	50	5	50	5/13	31
	1.3	5	50	6	60 ^b	7/11	39
	0.65	4	40	4	40	4/14	64 ^a

^aP<0.01 and ^bP<0.05, vs. the control group. GDLYT, Gan-Dan-Liang-Yi-Tan.

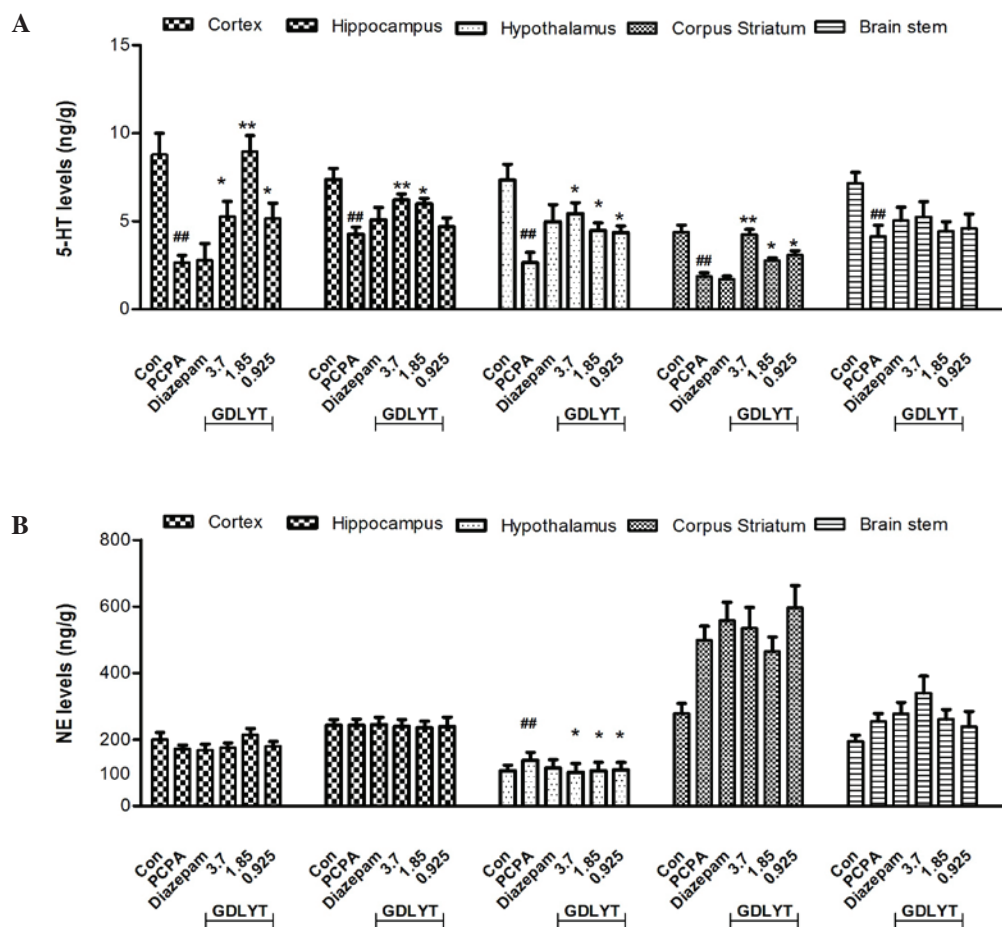


Figure 1. Effect of GDLYT on the concentration of (A) 5-HT and (B) NE in the various brain regions of PCPA-treated mice (n=8). Data are expressed as a mean \pm standard error of the mean. [#]P<0.05, vs. the control group; ^{*}P<0.05 and ^{**}P<0.01, vs. the PCPA group. PCPA, p-chlorophenylalanine; GDLYT, Gan-Dan-Liang-Yi-Tan; 5-HT, 5-hydroxytryptamine.

Effect of GDLYT on monoaminergic neurotransmitter and cytokines in rats. As shown in Fig. 1A, after 7 days treatment, PCPA treatment induced a significant decrease in 5-HT levels (P<0.05) in several areas of the brain, including the prefrontal cortex, hippocampus, hypothalamus, corpus striatum and brain stem, whereas it only induced an increase in NE levels in the hypothalamus (Fig. 1B). PCPA did not have any effect on DA levels in the brain (data has not shown). GDLYT was able

to significantly reverse the effects of PCPA after 7 days administration in the prefrontal cortex, hippocampus, hypothalamus and corpus striatum (P<0.05), as compared with the PCPA group (Fig. 1A). Furthermore, GDLYT decreased NE levels in the hypothalamus (Fig. 1B) and there were no significant changes in DA levels (data not shown). In addition, a decrease in TNF- α and IL-2 levels and an increase in IL-6 levels were observed following treatment with PCPA, although IL-4 levels

Table II. Effect of GDLYT on pentetrazole-induced convulsions and mortality in mice (n=10).

Group	Dosage (g/kg)	3 days (mouse number)		5 days (mouse number)		7 days (mouse number)	
		Latency (sec)	Mortality (%)	Latency (sec)	Mortality (%)	Latency (sec)	Mortality (%)
Control	-	57.2±20.2	90	59.7±17.8	90	54.5±13.3	91
Diazepam	0.0013	128.0±34.7 ^a	30 ^b	129.0±61.3 ^a	10 ^b	80±15.4 ^a	16 ^b
GDLYT	5.2	68.6±22.1	80	66.0±13.6	80	57±13.2	58
	2.6	53.2±9.6	80	131.1±115.8	70	56±6.5	58
	1.3	55.7±11.3	60	164.6±108.9 ^a	50	89±16.0 ^a	36 ^a
	0.65	57.3±13.7	70	144.9±123.9	90	57±10.1	75

The latency represents the latency of myoclonic jerks, expressed as mean ± standard error of the mean. ^aP<0.05 and ^bP<0.01, vs. the control group. The data were analyzed by one-way analysis of variance followed by Dunnett's *post-hoc* test. Mortality was analyzed by χ^2 test. GDLYT, Gan-Dan-Liang-Yi-Tang.

Table III. Effect of GDLYT on movement in PCPA-treated rats (n=8).

Group	Dosage (g/kg)	Total movement distance (m)	
		2-day PCPA treatment	7-day GDLYT treatment
Control	-	16.786±5.981	12.915±2.649
PCPA	0.3	27.348±10.235 ^a	21.791±7.453 ^b
Diazepam	0.001	25.180±7.002 ^a	12.694±1.344 ^a
GDLYT	3.7	24.076±7.262 ^a	9.445±3.296 ^c
	1.85	27.421±9.515 ^a	9.069±2.846 ^c
	0.925	23.481±5.989 ^a	7.648±2.606 ^c

Data expressed as mean ± standard error of the mean. ^bP<0.05, vs. the control group; ^aP<0.05 and ^cP<0.01, vs. the PCPA group. PCPA, p-chlorophenylalanine; GDLYT, Gan-Dan-Liang-Yi-Tang.

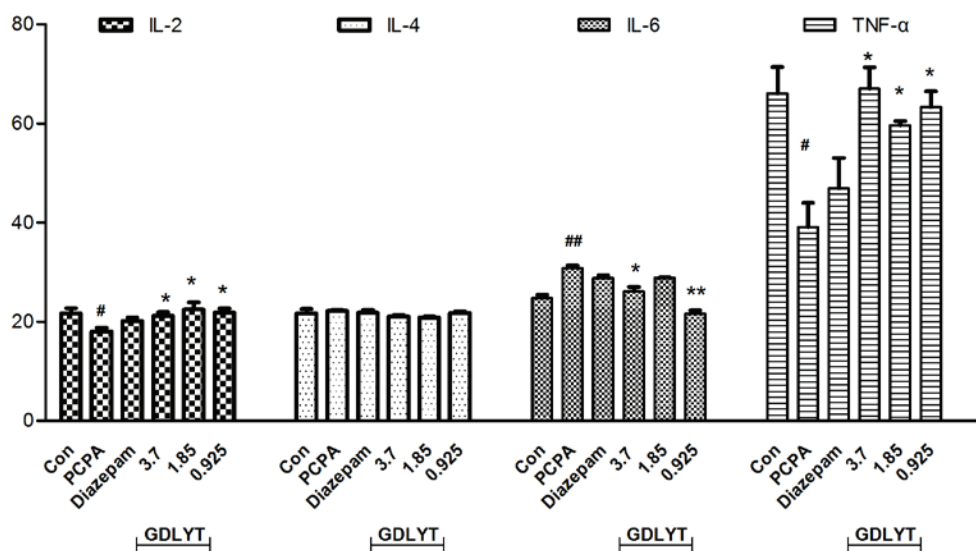


Figure 2. Effect of GDLYT on inflammatory cytokine levels in the PCPA-treated mice. The effect of GDLYT on IL-2, IL-4, IL-6 and TNF-α (n=8). Data are expressed as a mean ± standard error of the mean. [#]P<0.05, vs. the control group; ^{*}P<0.05 and ^{**}P<0.01, vs. the PCPA group. GDLYT, Gan-Dan-Liang-Yi-Tang; 5-HT, 5-hydroxytryptamine; IL, interleukin; TNF, tumor necrosis factor.

were unaffected (Fig. 2). Following treatment with GDLYT at doses of 3.7, 1.85 and 0.925 g/kg, TNF-α and IL-2 levels were

significantly increased, and those of IL-6 were significantly decreased, as compared with the PCPA group (P<0.05; Fig. 2).

Discussion

Insomnia is an common phenomenon causing both physical and mental health impairments (17). However, evidence for treatment options is limited. Insomnia and anxiety are typically treated with adjunctive benzodiazepines, which risk abuse and dependency if used chronically (18). In the current study, the results demonstrated that a traditional herbal medicine, GDLYT, exerts hypnotic-sedative and anticonvulsant effects in mice.

In the brain, endogenous neurotransmitters such as asdopamine, norepinephrine, acetylcholine, serotonin, GABA, histamine and neuropeptides have been suggested to have important roles in sleep mechanisms (19-22). GDLYT decreased the sleep onset in an animal model treated with a subhypnotic dosage of pentobarbital, a classic behavioral pharmacology methods, shown its hypnotic-sedative effect. As PTZ has been reported to interact with the GABA neurotransmitter (11,13,23), the restrain of PTZ-induced seizures suggests that GDLYT possesses anticonvulsant properties through some of bioactive constituents adjusts GABA-ergic neurotransmission.

In addition, chronic administration of PCPA, a serotonin 5-HT synthesis inhibitor, may induce complete insomnia or substantially reduced sleep (24). These insomnia effects were resisted by treatment with GDLYT, which may involve the restoration of serotonin synthesis and thus restored sleep. Therefore, it was hypothesized that the serotonin system was hypnogenic, due to the serotonin-sleep connection. A previous study demonstrated that when serotonin concentration is decreased or following the destruction of the dorsal raphe nuclei in the brainstem, which contain the majority of the serotonergic cell bodies of the brain, sleep is also reduced (25). As reviewed by Dugovic (26), the complex effects of 5-HT in the adjusting on sleep is in part due to the fact that 5-HT may be present in different areas of the brain that are involved in the control of sleep and wakefulness. Consistent with previous reports, the results of the present study demonstrated that different 5-HT levels could be observed in various areas of the brain. GDLYT was able to reverse PCPA-induced 5-HT decrease in several parts of the brain, including the cortex, hippocampus, striatum and hypothalamus. Furthermore, GDLYT also modulated NE levels in the hypothalamus. These results suggested there may be certain activated components of GDLYT that stimulated the serotonergic system.

Cytokines have an important role in immune activation, but are also transported into the central nervous system, where they influence noradrenergic, dopaminergic and serotonergic neurotransmission (27). IL-1 and IL-6 were reported to be associated with psychomotor, sickness behavior and sleep (28,29); IL-2 and TNF- α partly disturb memory and are involved in cognitive impairment. The hyper-secretion of IL-2 has been associated with schizophrenia, and that of IL-6 with depression (30,31). As observed in the present study, the expression levels of IL-2 and TNF- α pro-inflammatory cytokines were decreased, and those of IL-6 anti-inflammatory cytokine were increased following treatment with PCPA inhibitor. Treatment with GDLYT increases the expression levels of IL-2 and TNF- α , and reduced the serum expression levels of IL-6. Therefore, another important mechanism underlying the effect

of GDLYT on sleep disorders in animals is by adjusting cytokine levels so that each can be maintained at normal ranges.

In conclusion, the results of the present study revealed that GDLYT may contain anti-psychoactive components with potential hypnotic, sedative and anticonvulsant properties. The results also suggested that the serotonergic and immune system may participated in the hypnotic-sedative activity of GDLYT. Future studies will be required in order to investigate the mechanism underlying this hypnotic-sedative effect, and to isolate the active ingredient from GDLYT.

Acknowledgements

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