Leptocarpus disjunctus prolongs sleeping time and increases nonrapid eye movement sleep with additional anxiolytic capacity

Abstract

Leptocarpus disjunctus Mast. (Restionaceae) is an edible plant which has indigenous warnings regarding its side effects which can manifest as dizziness. This study investigated hypnotic and anxiolytic properties using several animal models. Anxiolytic activities were evaluated using locomotor determination by elevated plus-maze test, open-field test, and rotarod performance test. Hypnotic activities were performed using pentobarbital sodium-induced sleeping time test. Sleep architecture and quality were obtained from sleep–wake analysis and nonrapid eye movement (NREM) delta activity using electroencephalography. An ethanolic extract of L. disjunctus indicated effective potencies for hypnotic test, locomotor activities, and sleep–wake analysis. Ethanolic extract showed a dose relationship with sleeping time for pentobarbital-induced sleeping time test \( (P < 0.01) \) and also an antagonistic effect on shortening in sleep time induced by flumazenil. The consort significantly decreased locomotor activities among animals undergoing elevated plus-maze test, open-field test, and rotarod performance test, whereas sleep–wake analysis showed that sleeping time and NREM sleep increased. Ethanolic extract of L. disjunctus was shown to be anxiolytic, with the possibly of benzodiazepine-like hypnotic activity.

Key words: Anxiolytic, hypnotic, Leptocarpus disjunctus, nonrapid eye movement delta activity

INTRODUCTION

Herbs have long been used as medicines for treating sleep disorders, and Senna siamea (Lam.) H.S. Irwin and Barneby is still applied as folkloric medicine or as ingredient in herbal remedies for the treatment of insomnia in Thailand. Previous studies reported sedative and anxiolytic effects of S. siamea which reduced locomotor activity and prolonged sleeping time \textit{in vivo}.\cite{1,2} Morinda citrifolia L. fruits are multipurpose, and they are widely used in folkloric medicine as an analgesic and anti-inflammatory drug. Previous research determined central nervous system depressants by evaluating their anxiolytic, sedative, and hypnotic effects.\cite{3} Herbs, therefore, are proven to cause hypnotic and anxiolytic side effects. In Thailand, Leptocarpus disjunctus mast. (Restionaceae) is an edible plant which is consumed in southern districts as a local vegetable, notwithstanding an indigenous warning regarding its side effects that are often exhibited as dizziness. This research aimed to investigate hypnotic and anxiolytic activities of L. disjunctus using several animal models.

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Materials and Methods

Experimental animals
Adult male ICR mice (4–6 weeks old with a body weight range of 35–45 g) from the National Laboratory Animal Center, Mahidol University, Thailand, were used in locomotor coordination, hypnotic and anxiolytic tests. Mice were housed in the Faculty of Science, Rangsit University, Thailand, under standard environmental conditions (24°C ± 1°C, 60%–70% humidity, 12 h light: 12 h dark cycle). Food and water were given ad libitum until 2 h before experiments. All experimental procedures were conducted in accordance with the guidelines of Animal Care and Use Committee, Faculty of Pharmacy, Rangsit University, Thailand (Animal License No. RSEC 01/2013).

Adult male Sprague-Dawley rats (6–8 weeks old with a body weight range of 240–320 g) from Heilongjiang University of Chinese Medicine GLP Animal Center, Harbin, PR China, were used in sleep–wake analysis and delta activity. The rats were housed in the Faculty of Pharmacy, Heilongjiang University of Chinese Medicine, Harbin, PR China, under standard environmental conditions (24°C ± 1°C, 60%–70% humidity, 12 h light: 12 h dark cycle). Food and water were given ad libitum. The procedures were conducted in accordance with the guidelines of Animal Care and Use Committee, Faculty of Pharmacy, Heilongjiang University of Chinese Medicine, PR China (Animal License No. RSEC 02/2015).

Plant material and extraction
*L. disjunctus* plants were collected from Phatthalung and Trang Provinces in Thailand. Plant material was authenticated by Associate Prof. Nijsiri Ruangrungsi. Voucher specimens were deposited at the College of Public Health Sciences, Chulalongkorn University, Thailand. Fresh whole plants were blended in 95% ethanol using an electric blender. The blended plants were macerated with ethanol until exhaustion. Ethanolic extracts were filtered through Whatman number 1 filter paper and then concentrated to dryness in vacuo. Ethanolic fraction was evaporated under rotary evaporator, and extract yields were recorded.

Treatment
Male ICR mice were divided into 19 groups of ten each. Mice in Group I received 5% polysorbate 20 at a similar volume to the treatment groups. Mice in Groups II, III, IV, and V received 10, 50, 100, and 200 mg/kg ethanolic extract. Mice in Groups VI and VII were pretreated with flumazenil (3.5 mg/kg, Wuhan Senwayer Century Chemical, Wuhan, China) in 10% dimethyl sulfoxide and 200 mg/kg ethanolic extract and vehicle control for pentobarbital-induced sleeping time test. Mice in Groups VIII, XII, and XVI received normal saline at the same volume as treatment group and served as the negative control. Groups IX, X, and XI received 10, 50, and 100 mg/kg ethanolic extract for elevated plus-maze test. Groups XIII, XIV, and XV received 10, 50, and 100 mg/kg ethanolic extract for open-field test, and mice in Groups XVII, XVIII, and XIX received 10, 50, and 100 mg/kg ethanolic extract for rotarod performance test.

Male Sprague-Dawley rats were divided into four groups of eight each. Group XX rats in vehicle control received the same volume as the treatment groups (5% polysorbate 20). Rats in Groups XXI, XXII, and XXIII received 50, 100, and 200 mg/kg ethanolic extract.

Pentobarbital sodium-induced sleeping time test
The pentobarbital-induced sleeping time test followed the methodology of previous studies.[4,5] Ethanolic extract was suspended in Tween 20 (5% v/v in saline) and administered orally. Pentobarbital sodium (35 mg/kg of body weight; Merck, Germany) was injected intraperitoneally (IP) to induce animal sleep after 30 min. The sleeping time was recorded and compared to the control group by observing the righting reflex.[6]

Elevated plus-maze test
Following Hosseinzadeh and Noraei, the apparatus consisted of two open arms (15 cm × 5 cm) and two closed arms (15 cm × 5 cm × 12 cm) with a clear acrylic maze located 30 cm above a black floor. Ethanolic extract in sterile saline and vehicle was administered (IP). The mice were placed in the center of the maze and observed for 5 min. The time (in seconds) that the animals spent in the open and closed arms was recorded compared with the control group. An increasing time in the open arms was considered as an anxiolytic effect.[4]

Open-field test
The apparatus consisted of a floor 100 cm × 100 cm divided by red lines into 25 squares of 20 cm × 20 cm with white walls 50 cm high. The test room was illuminated at the same intensity as the colony room.[4,7] Ethanolic extract in sterile saline and vehicle was administered (IP). Mice were placed in the center of the open field, and their behavior was observed for 5 min. The number of leanings (one or two paws in contact with the wall), rearing, and grooming (face cleaning, paw licking, fur licking, head scraping, and rubbing) was recorded. The field was cleaned at the end of each test.

Rotarod performance test
Motor coordination was tested using the RotaRod apparatus (Model 519/EC, Medicraft Electro Medicals (P), Lucknow, India).[4] Ethanolic extract in sterile saline and vehicle was administered (IP). Mice were placed on a horizontal metal rod coated with rubber (3 cm diameter) rotating at a speed of 10 rpm/min. Balancing time on the rod was measured for each animal. The mice were given two trials with a maximum trial time of 300 s and a 30–60 min intertrial rest interval.
Sleep–wake analysis

Surgery

Rats were anesthetized using pentobarbital sodium (60 mg/kg, IP; Merck, Germany) and fixed with stereotaxic apparatus (DW-2000, Taimeng Bio-instruments, Chengdu, China) for chronic stainless steel electrode implantation. Electrodes were implanted for electroencephalogram (EEG) into the right frontal cortex and visual cortex. The electrodes were fixed to the skull with dental cement. An electromyogram (EMG) test was also conducted using stainless steel wire electrodes implanted into dorsal neck muscle. Rats were allowed 7 days to recover from the surgery with postoperative care.

Drug treatment and polysomnography recording

The ethanolic extracts were suspended in 0.5% Tween 20 and administered orally at 9:00 am. Rats were divided into four groups of eight each for sleep–wake cycle testing. EEG and EMG were recorded using an electroencephalograph (MP 150, BIOPAC® Systems, CA, USA) from 09.00 am to 03.00 pm. The signals were amplified, filtered, and digitized at sampling rate of 512 Hz and recorded using AcqKnowledge software version 4.2 (BIOPAC® Systems). Each rat was measured for EEG and EMG in its own acrylic cage in sound-proofed and electrically shielded room.

Nonrapid eye movement delta activity

Delta activity within nonrapid eye movement (NREM) sleep was determined using SleepSign software version 2.0 (Kissei Comtec, Nagano, Japan). The power spectrum densities including delta wave (0.5–4 Hz), theta wave (4–8 Hz), alpha wave (8–13 Hz), and beta wave (13–30 Hz) were integrated and averaged. Delta powers in NREM sleep were expressed as a percentage of the average delta activity.

Statistical analysis

Results from pentobarbital-induced sleeping time test, elevated plus-maze test, open-field test, and rotarod performance test were expressed as means ± standard deviation (SD). Data were compared to control group using Dunnett’s test following ANOVA. Results from sleep–wake analysis and delta activity were expressed as percentage of means ± SD. Data comparisons were performed using Dunnett’s test following ANOVA.

RESULTS

Hypnotic effects

Hypnotic effects of ethanolic extract were determined in pentobarbital-induced sleeping time test. Ethanolic extracts at doses of 50, 100, and 200 mg/kg significantly augmented sleeping time and the dose of 200 mg/kg pretreated with flumazenil reduced sleeping time (P < 0.01) [Figure 1].

Anxiolytic and motor coordination effects

Anxiolytic and motor coordination effects of ethanolic extract were determined using elevated plus-maze test, rotarod performance test, and open-field test. Doses of 10, 50, and 100 mg/kg of ethanolic extract decreased the time that rodents spent in the closed arm of elevated plus maze and also decreased the time which rodents spent on rotarod apparatus. Moreover, rodent behavior in open-field apparatus also decreased [Table 1].

Sleep architecture and delta activity

For sleep–wake analysis, doses of 50, 100, and 200 mg/kg of ethanolic extract increased either sleep time or NREM sleeping time in rats [Figures 2 and 3]. In addition, ethanolic extract did not change EEG activity and also consistently maintained delta activity throughout experimental time [Table 2 and Figure 4].

DISCUSSION

During potentiation of pentobarbital-induced sleeping time test, ethanolic extracts at 50, 100, and 200 mg/kg significantly increased sleeping time compared with vehicle control [Figure 1]. In the mechanistic study, flumazenil which is GABA_A receptor antagonist was orally pretreated for 30 min before treatment with 200 mg/kg ethanolic extract. [10] Sleeping time showed no obvious effect when compared to vehicle control. These findings suggested that ethanolic extracts generated hypnotic activity through GABA_A receptor. Ethanolic extracts also affected average time spent in open arms of elevated plus maze among three doses, indicating an anxiolytic effect. Anxiolytic compounds reduced natural aversion of animals to open arms and promoted exploration. [11] The result was consistent with previous reports that some anxiolytic agents, [12] Treated animals showed habituation behavior after successive open-field test exposures, which was less evident in exploratory behavior [Table 1]. Open-field testing can be used for testing antianxiety agents which
In rotarod performance test, animals treated with ethanolic extract showed decreased locomotor activity, referred to motor coordination effect. This evidence supported that ethanolic extract affected motor coordination. Results obtained from open-field and rotarod performance tests confirmed findings detected in elevated plus-maze and pentobarbital-induced sleeping time tests.

Antianxiety agents affect neurotransmitter production such as GABAergic expression. Ethanolic extract showed effectiveness similar to antianxiety agents by impacting on locomotor activity. From previous studies, Valeriana officinalis L. was also evaluated for its anxiolytic and sedative properties using behavioral paradigms to investigate GABAergic mechanisms, and results demonstrated both sedative and anxiolytic capabilities.

Sleep–wake analysis was performed using an EEG. Waking time significantly decreased after ethanolic treatment [Figure 2]. In addition, ethanolic extract at doses of 50, 100, and 200 mg/kg significantly decreased waking duration, whereas rapid eye movement sleep and NREM sleep duration significantly increased compared to vehicle control group (P<0.01) [Figure 3]. Delta EEG power density was used to describe sleep quality as a measurement of NREM sleep intensity. Results indicated no significant change in EEG activity among four experimental groups [Table 2], and relationship between time and dose responses during NREM sleep showed no statistical difference throughout testing time [Figure 4]. Quality of sleep was not reduced, whereas NREM stage statistically increased. Kava-kava extract also gave a significant decrease in waking stage and increased sleeping stage with significantly heightened delta activity during NREM stage in sleep-disturbed rats. L. disjunctus ethanolic extract may, therefore, contain a component which affects benzodiazepine receptors.
CONCLUSION

*L. disjunctus* displayed effectiveness in both anxiolytic and hypnotic effects and increased duration of NREM sleep. Further studies are required to determine its active compounds and their effects on neurotransmitter expression.

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Conflicts of interest

There are no conflicts of interest.

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