POTENTIAL OF *LEPTOCARPUS DISJUNCTUS* AS HYPNOTIC PLANT: STUDY ON *DROSOPHILA MELANOGASTER* AND MICE MODELS

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ABSTRACT:

Background: *Leptocarpus disjunctus* Mast. is an edible plant which belongs to Restionaceae family. Although, it was reported to cause drowsiness and sleepiness after consumption, it has been continuously consumed as food or food ingredient in Southern region of Thailand. This research aimed to investigate the hypnotic activity of this plant.

Methods: To evaluate the hypnotic activities, pentobarbitone sodium-induced sleeping time using ICR mice was used to examine the sleep prolongation effect, and analysis of sleeping time in *Drosophilla melanogaster* was used as a preliminary model for hypnotic plants.

Results: For sleeping analysis, the ethanolic extract of *L. disjunctus* at the dose of 50, 100 and 200 mg/kg showed statistical significant dose relationship increase in the sleeping time of male ICR mice which was intraperitoneally induced by pentobarbitone sodium. In addition, sleeping time of both male and female *D. melanogaster* which was detected using infrared beam-split monitors, showed marginal increase in *L. disjunctus* (5 mg/ml) treated group when compares to vehicle control group.

Conclusion: *L. disjunctus* has a tendency to be used as a hypnotic plant. *D. melanogaster* can be used as a preliminary animal model for sleeping analysis.

Keywords: Sleeping analysis; *Drosophilla melanogaster*; Hypnotic activity; *Leptocarpus disjunctus*

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INTRODUCTION

Insomnia is a sleep disorder that is characterized by difficulty falling asleep or staying asleep which become more crises in global health. It can be caused by psychiatric, medical conditions and certain biological factors. Sleep is a natural periodic state both body and mind which characterized by consciousness, sensory activity and muscles relaxation. Sedatives are drugs that decrease activity and affects muscle relaxation which serve to relieve anxiety. In addition, the term of hypnotic describes the effect which purpose to initiate, sustain and lengthen sleep [1]. The hypnotic activity has been defined in several animal experiments. Many of the pharmacological tests are based on the potentiation of sleeping time in rodents induced by barbiturates or other sedative agents. Potentiation of hexobarbital sleeping time test is a common *in vivo* method for obtained potentiation of sleeping from suspect agents. The test is used to elucidate CNS-active properties of drugs. Not only hypnotic and sedative but also antidepressants can prolong sleeping time after induced a single dose of hexobarbital [2]. The righting reflex is a measurement for criterion for the duration of

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hexobarbital induced sleeping time. In the past decade, *Drosophila melanogaster* which is a kind of crepuscular animals (active primarily during twilight) has been used as an animal model for studying the genetic components which affect sleep as well as its functions. In circadian behaviour and circadian rhythm research, *D. melanogaster* was used as experimental organism to reveal both genetic base of circadian and rhythmic behaviour by observing its locomotor activity [3, 4]. The sleeping stage of *D. melanogaster* can be conveniently estimated by measuring the locomotor [5]. Their sleeping stage can be satisfied by behavioural characteristics consist of circadian period of immobility consolidation, specific posture or resting place, arousal threshold, the reversibility to wakefulness and a homeostatic regulatory mechanism [6]. Sleep from humans to *D. melanogaster* extends beyond behaviour to pharmacology by detrimental effects similarity. They show behavioural and molecular levels correlation of wakefulness and sleep, thus *D. melanogaster* is an ideal system for studying functions of sleep and its connections to pathological conditions in rodents and humans [7]. Infrared-based monitoring of *D. melanogaster* locomotor activity is an important instrumentation in behavioural characteristics observation. Principles of locomotor detection using infrared beam-split monitor is based on a single *D. melanogaster* which is hosted in a glass tube was attached between food and cotton. A beam of infrared light crosses the midline of the tube and a collector connected to a computer can record and count whenever the fly is breaking the beam by passing through it, and the locomotor activity is measured in terms of detecting movements by number of IR crossings [6]. In Southern of Thailand, *Leptocarpus disjunctus* (Restionaceae family) is the plant which has been consumed as local vegetable; although, there is the indigenous warning about its side effects for sleepiness symptom after consumptions. Hence, this research aimed to investigate its effect on the hypnotic activities using mice and *D. melanogaster* as animal models.

MATERIALS AND METHODS

Experimental animals

Drosophila melanogaster were cultured and employed in Faculty of Pharmacy, Heilongjiang University of Chinese Medicine, Harbin, People Republic of China under standard environmental conditions of temperature, relative humidity and light (25±1 °C, 60-70% humidity, 12 h light: 12 h dark cycle). *D. melanogaster* were cultured in nutrient broth until breeding and spawning. Eggs of *D. melanogaster* were continuously raised until become larva, pupa and adult respectively. Adults were performed gender segregation for preventing undesirable pregnancy within 12 h and sub-cultured in broth by observing their abdomen's tip.

Adult male ICR mice weighing 35-45 g (National Laboratory Animal Centre, Mahidol University, Thailand) were used in pentobarbitone induced sleeping time test. Mice were housed in Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand, under standard environmental conditions of temperature, relative humidity and light (24±1 °C, 60-70% humidity, 12 h light: 12 h dark cycle). Food and water were given *ad libitum* until 2 h before the experimental procedures.

Plant material and extraction

Leptocarpus disjunctus were collected from Patthalung and Trang provinces of Thailand, and the plant material were authenticated by Assoc. Prof. Nijsiri Ruangrungsi. The voucher specimens were deposited at College of Public Health Sciences, Chulalongkorn University, Thailand. Fresh whole plants were blended with 95% ethanol by an electric blender. The blended fresh plants were continuously macerated with ethanol until exhaustion. The ethanolic extracts were filtered through Whatman No.1 filter paper. The ethanolic filtrate was evaporated under rotary evaporator and the extract yields were recorded.

Pentobarbitone induced sleeping time test

Pentobarbitone induced sleeping time in mice was tested according to Carvalho-Freitas & Costa, 2002 [8]. Male ICR mice were divided into 5 groups of ten each. Mice in group I were received 5% polysorbate 20 at same volume of treatment groups. Mice in group II, III, IV and V were respectively received 10, 50, 100 and 200 mg/kg ethanolic extract. Food and water were starved for two hours before the experimental procedures. The various concentrations of extract were suspended in Tween 20 (5% v/v in saline), and were administered orally by gavage (P.O.). The control group was received only vehicle at the same volume as the treated groups. Pentobarbitone sodium (35 mg/kg; Merck, Germany) was injected to induce animal sleep by intra-peritoneally after 30 minutes of administration of the extract. The sleeping time was recorded by observing time of righting reflex [9].

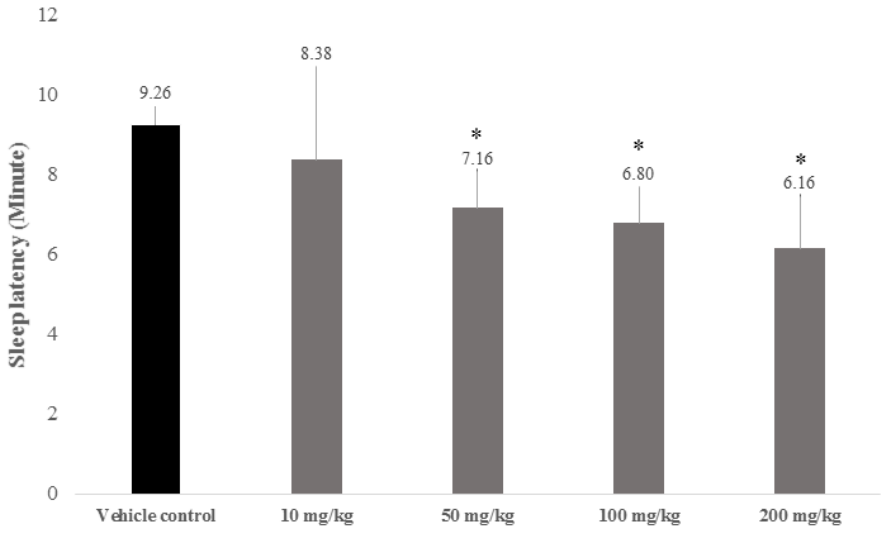

Figure 1 Effects of *Leptocarpus disjunctus* ethanolic extract on sleep latency in pentobarbitone induced sleeping time test. Data are expressed as mean ± SD of time in male mice. **p* < 0.01, compared with vehicle control, Dunnett's tests

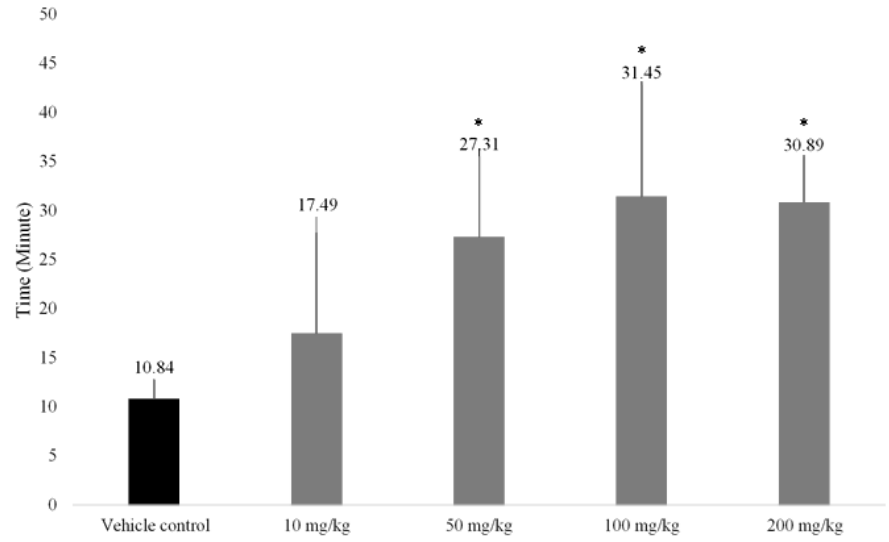

Figure 2 Effects of *Leptocarpus disjunctus* ethanolic extract on righting reflex time in pentobarbitone induced sleeping time test. Data are expressed as mean ± SD of time in male mice. **p* < 0.01, compared with vehicle control, Dunnett's tests

Sleep analysis using *D. melanogaster*

The analysis of sleeping time in *D. melanogaster* was done with slightly modifications according to Gilestro, 2012 [6]. Male *D. melanogaster* were divided into 2 groups of thirty two each, group I and II were respectively received 5% sucrose and 5 mg/ml ethanolic extract suspended in 5% sucrose. In addition, female *D. melanogaster* were also divided into 2 groups of thirty two each. Group III and IV were respectively received 5% sucrose and 5 mg/ml ethanolic extract suspended in 5% sucrose. The cottons were soaked with 20 µl of 5% sucrose in control group and 5 mg/ml ethanolic extract. The soaked cotton were put into the 5 mm diameter tube. The tubes were closed with cap at soaked cotton side. One *D. melanogaster* was put into each tube. The tube's entrance was closed with dry cotton. Each test tube were inserted into the experiment arena and observed the sleeping time under experiment environment by using infrared beam-split monitors (*Drosophila* Activity Monitor, Model DAM2, TriKinetics, Inc., USA). Ambient

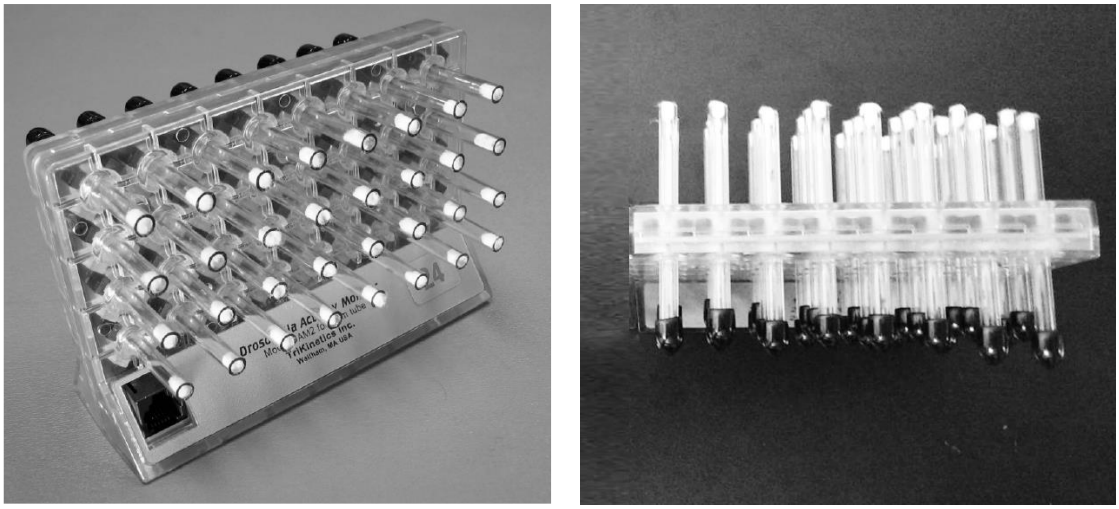

Figure 3 Infrared beam-split movement monitors [11]

Table 1 Effects of *Leptocarpus disjunctus* ethanolic extract on sleeping time in sleep analysis of *Drosophila melanogaster*. Data are expressed as mean ± SD.

	Time (minute)		
	All day	**Day**	**Night**
Female			
5% sucrose	707.031±137.43	238.281±97.41	468.750±78.092
5 mg/ml	802.031±197.59	289.375±104.28	513.438±111.416
Male			
5% sucrose	859.219±168.791	366.875±92.743	492.969±100.032
5 mg/ml	915.156±110.311	401.719±54.855	513.438±73.060

light sensor threshold was 10 lux nominal. Interconnect was 4 wire, 6 position and RJ-11 modular telephone line jack to DAMSystem network for DC power input and data transmission. The sleeping times were recognized by observing the movement of *D. melanogaster* for 24 h. The locomotors rest from sleep were measured by arousal threshold. When *D. melanogaster* fell asleep the arousal thresholds were decreased. Sleep in *D. melanogaster* is a period of inactivity at least five minutes [10].

Ethical consideration

Hypnotic tests were conducted in accordance with the guidelines of the Animal Care and Use Committee, Faculty of Pharmacy, Rangsit University, Thailand and Faculty of Pharmacy, Heilongjiang University of Chinese Medicine, PRC. (Animal License No. RSEC 01/2013)

Statistical analysis

Sleeping times were expressed as means ± SD, treated groups were compared to control group using Dunnett's tests following ANOVA.

RESULTS

Pentobarbitone induced sleeping time test

In this study, the animals were separated into 5 groups. The mice in each group were given a single dose of the vehicle control and, ethanolic extract (10, 50, 100 and 200 mg/kg). During 30 minutes before treatment of pentobarbitone sodium (35 mg/kg) which was injected to induce sleep, the mice in treatment groups obviously appeared drowsiness symptom. The mice were considered asleep by loss of righting reflex observation. The time between onset and righting reflex recovery were recorded. The ethanolic extract (50, 100 and 200 mg/kg) significantly decreased the sleep latency, compared to vehicle control (p<0.01) (Figure 1). Furthermore, the ethanolic extract (50, 100 and 200 mg/kg) significantly prolonged sleeping time, compared to vehicle control (p<0.01) (Figure 2).

Sleep analysis using *D. melanogaster*

In this present study, sixty two male and female *D. melanogaster* were treated with 5 mg/ml ethanolic extract and compared to vehicle control

(5% sucrose). It was found that the sleeping time in both male and female *D. melanogaster* were observed under infrared beam-split monitors (Figure 3). The results were found that the all day, day time and night time sleeping in treatment group of *D. melanogaster* were slightly increased when compared to the vehicle control (Table 1).

DISCUSSION

Pentobarbitone sodium is a short acting barbiturate which was metabolized by microsomal drug enzymes in liver [12]. Pentobarbitone sodium-induced sleeping time test is a popular model for pharmacological and toxicological response. It is an *in vivo* indicator of drug metabolism, which can be carried out on large numbers of animals over a short interval of time. The sleep evaluation was conducted based on the prolongation of pentobarbitone sodium-induced sleeping time basis and observed from the loss of righting reflex. Righting reflex or labyrinthine righting reflex is a reflex that corrects the orientation of the body when the body is taken out of the normal upright position. The reflex can be affected by various types of chemical substance which resulted in the loss of righting reflex after unconsciousness [13]. From the results, the ethanolic extract at the dose of 100 and 200 mg/kg tended to be the most effective doses.

Drosophila melanogaster is a species of fly in the family Drosophilidae. The species is generally known as the common fruit fly. *D. melanogaster* is widely used for genetics, physiology, microbial pathogenesis and life history evolution research. Because of easy care, fast breeding, and laying many eggs [14], it is typically used for preliminary research. *D. melanogaster* was among the first organisms used for genetic analysis because the comprehend processes such as transcription and replication in fruit flies helps in understanding of other eukaryotes, including humans [15]. *D. melanogaster* has emerged as an ideal model organism for studying the genetic components of sleep as well as its regulation and functions. In this present research, the infrared beam-split movement monitors (Figure 3) was used for instrumentation. The individual flies were placed into glass chambers, where their confined motion were detected by detection of the motion as the flies walk back and forth from end to end, counted by infrared light beams and uploaded from the monitors to a host computer for storage and analysis at periodic intervals. It was found that 5 mg/ml of the extract was marginal effective on in both male and female *D. melanogaster* (Table 1). The all-day sleeping time of male and female increased, with non-statistical different compared to vehicle control group (Table 1). Normally, the average sleeping time of male *D. melanogaster* is longer than female [16, 17]. The non-significant difference might be caused by the olfaction and flavour of plant extract which was pungent odour and bitter leading to sleep disturbances, and it was restricted to feed the *D. melanogaster* directly. Moreover, *D. melanogaster* and mice also have cytochrome P450 which is an enzyme inducer for barbiturates [18], pentobarbitone sodium and ethanolic extract may need to activate by xenobiotic metabolizing enzyme. The effect of *L. disjunctus* might be different in mice and *D. melanogaster* because others genetic variation.

CONCLUSIONS

In conclusion, *L. disjunctus* showed the effectiveness in hypnotic effects which conformed to preliminary study of sleep analysis using *D. melanogaster*. Although, there is a limitation in term of drug feeding in *D. melanogaster*, it can be used as preliminary model for screening of hypnotic effect from herbs or chemical substances prior to rodents or other animal models. Furthermore, drug metabolism in *D. melanogaster* should be further studied to clarify the novel perspectives of the physiological effects on hypnotic substances.

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