Behavioral Effects of Acute and Chronic Oral Administration of Barakol in Rats

Chatsri Deachapunya PhD*,
Watchareewan Thongsaard PhD*

** Department of Physiology, Faculty of Medicine, Srinakharinwirot University, Bangkok, Thailand*

Introduction: *Barakol, an active constituent extracted from Cassia siamea, has been shown to have anxiolytic effects similar to diazepam when treated intraperitoneally.*
Objective: *Acute and chronic oral administrations of barakol on anxiolytic, locomotor and exploratory behaviors were examined in rats using an elevated plus maze followed by a holeboard apparatus in comparison with the anxiolytic diazepam.*
Material and Method: *Male Wistar rats were divided into the acute and chronic treatment groups. The acutely-treated rats were given orally with vehicle control (distilled water, p.o.), diazepam (5 mg/kg, p.o.) and barakol (10, 30 and 100 mg/kg, p.o.) while the chronically-treated rats received the same treatment for 30 consecutive days. The anxiolytic behavior was tested on the elevated plus maze for 5 min and immediately followed by the holeboard to test for the directed exploratory behavior for 10 min.*
Results: *Acute and chronic oral administration of barakol (10, 30 and 100 mg/kg, p.o.) produced no significant changes in anxiolytic parameters tested on the elevated plus maze compared to diazepam which significantly increased the percentage of the open/total time and the time spent on the open arms. On the other hand, all parameters tested using the holeboard were not affected by barakol or diazepam when given acutely. When given chronically, all doses of barakol significantly decreased the number of head-dips and the total time spent head-dipping with no changes in the number of grooms or rears per minute.*
Conclusion: *Acute and chronic oral administration of barakol had no anxiolytic and locomotor effects. However, it exerted a sedative effect as shown by a reduction in the directed exploratory behaviors.*

Keywords: *Administration, Oral, Anti-anxiety agents, Barakol, Cassia, Diazepam, Plant extracts, Plants, M edicinal, Rats*

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Barakol is a biological active constituent of *Cassia siamea*, a plant that has been used in Southeast Asia as traditional medicine to treat fever, diabetes, constipation, hypertension and insomnia[1,2]. It was originally extracted by Hassanali-Walji and co-workers in 1969. Its chemical structure was 3a, 4-dihydro-3a, 8-dihydroxy-2, 5-dimethyl-1, 4-dioxaphenalene ($C_{13}H_{12}O_4$)[3] or 2,5-dimethyl-3aH-pyrano-[2,3,4-de]-1-benzopyran-3a,8-diol[4] and a proposed synthetic procedure was described in 1970[5]. Barakol has been found to exert many biological activities such as reduce blood pressure in animal model[6], increase smooth muscle tension[7,8] and stimulate colonic chloride secretion in isolated tissues[9]. In behavioral studies, barakol has been demonstrated to possess dopamine agonist activity, increase nociceptive threshold and suppress serotonergic activity as shown by decreasing 5-hydroxytryptophan-induced head shake behavior[10]. A previous study in our laboratory demonstrated that acute intraperitoneal injection of barakol exerted anxiolytic activity in rats similar to diazepam when tested on an elevated plus maze, a behavioral test for anxiolytic drugs[11]. It also increased exploratory and locomotor behaviors, which were not observed with diazepam. Further *in vitro* studies have shown that barakol inhibits K^+-stimulated endogenous dopamine release from rat striatal slice by acting through a D_2-like autoreceptor[12].

Correspondence to: Deachapunya C, Department of Physiology, Faculty of Medicine, Srinakharinwirot University, Sukhumvit 23, Wattana, Bangkok 10110, Thailand. Phone: 0-2649-5374, Fax: 0-2260-1533, E-mail: chatsri@swu.ac.th

The purpose of this study was to investigate the effects of acute and chronic oral administration of barakol on anxiolytic, exploratory and locomotor behaviors in rats using the elevated plus maze and the holeboard test. In this study, barakol (10, 30 and 100 mg/kg) was fed to the animals in order to avoid infection and complications from daily injections. The acutely-treated rats were given a single doses of barakol orally while the chronically-treated rats received the same dose of barakol for 30 days. On the test day, 30 min after feeding, rats were tested for 5 min in the plus maze and immediately after that, rats were tested for 10 min in the holeboard test. By using these two tests, anxiolytic, exploratory and locomotor behaviors in response to barakol treatment were evaluated. The effects of barakol on the behavioral profiles were compared with the widely used anxiolytic diazepam (5 mg/kg, p.o.).

Material and Method

Animals

Male Wistar rats, weighing 180-200 g, were obtained from the National Laboratory Animal Center, Thailand. They were housed in groups of 4-6 in a room with a 12 hours light: 12 hours dark cycle and allowed free access to laboratory pellets (National Laboratory Animal Center, Mahidol University, Thailand) and water. Animals were naive to elevate plus maze and holeboard apparatus.

Apparatus

Elevated plus maze

The plus maze, considered as a valid model of anxiety in rats, is based on natural aversion for the open arms that can induce fear and anxiety[13-15]. It was made of wood covered with black Formica. It consisted of a maze elevated to a height of 70 cm with two open (45 x 15 cm) and two closed arms (45 x 15 x 10 cm), arranged so that the two pairs of identical arms were opposite each other, connected by an open central area (15 x 15 cm). The closed arms were enclosed by 10 cm high wall. On the test day, each rat was placed in the center of the maze, facing one of the open arms, and its behavior was observed for 5 min. Rats were tested in randomized order with respect to drug treatment as follows: vehicle control, diazepam (5 mg/kg, p.o.) and barakol (10, 30 and 100 mg/kg, p.o.). All treatments were carried out between 8.00-14.00 hours under low-intensity natural light. After each animal was tested, the maze was carefully wiped with a damp cloth. The behaviors were recorded by video camera hanging from the ceiling and the number of open arm and closed arm entries, the total time spent on open arms and closed arms, and the number of rears was scored by two observers. An increase in the number of entries into the open arms and the amount of time spent on the open arms reflects anxiolytic activity whereas a decrease in those parameters reflects anxiogenic activity.

Holeboard

The holeboard is a well-established tool to assay potential sedative effects[16]. It was a wooden box covered with black Formica (60 x 60 x 40 cm), with 16 holes and 4 cm in diameter evenly spaced in the floor. After a 5-min plus maze test, each rat was placed at the center of the floor and its behavior was observed for 10 min. Rats were tested in randomized order with respect to drug treatment between 8.00-14.00 hours under low-intensity natural light. The floor of the holeboard was thoroughly wiped with a damp cloth after each trial. The behavior was recorded by video camera, and the number of grooms, rears, head-dips into the holes and the time spents head-dipping were recorded and evaluated by two observers. The increases in the number of head-dips, the time spent head-dipping, and the number of head-pokes reflects greater directed exploration and is not dependent on locomotor activity[16,17].

Drug and chemicals

Barakol was extracted and purified from *C. siamea* by a method modified in our laboratory[4]. Fresh young leaves of *C. siamea* were obtained from a local Bangkok market and the identification confirmed by comparison with the herbarium specimens in the Botany Section, Technical Division, Department of Agriculture, Ministry of Agriculture and Co-operative, Thailand. The leaves were cut into small pieces and boiled twice with 0.5% sulfuric acid for 30 min. All fractions of the water extract were filtered, combined, and alkalinized with concentrated sodium hydrogen carbonate solution. The mixture was further extracted with chloroform, which was washed with water. The solution was concentrated and shaken with 5% aqueous acetic acid until the extract became colorless. The acidified chloroform extract was neutralized carefully with concentrated ammonia solution and cooled. The crude barakol was crystallized as greenish yellow needles. The yield of barakol was approximately 0.3%. Concentrated hydrochloric acid was added finally to obtain barakol hydrochloride and the mixture was

dried rapidly by vacuum filtration to form crystallized yellow needles of anhydrobarakol hydrochloride. The compound was shown to be a single chemical using thin layer chromatography on silica gel.

The identification of the compound was confirmed using nuclear magnetic resonance (NMR). The physical and spectroscopic characteristics of the compound were evaluated and compared with those in previous reports[4,6,18]. When anhydrobarakol hydrochloride is dissolved in water, the conversion reaction is reversed and so the product used in all the biological experiments is a barakol solution[4]. The purity of the compound was confirmed by high performance liquid chromatography coupled with a photodiode array detector (HPLC-PDA, 190-350 nm, Waters, USA) using Kromasil C8 column (250 mm x 4.6 mm ID, 5 mm particle size) and flow rate of 0.8 ml/min. Barakol was dissolved in distilled water immediately before feeding to the animals.

Diazepam was obtained from tablets (Roche, England) (Welwyn Garden City, UK) to mimic the parenteral use in human. The tablets were grounded and suspended in distilled water immediately prior to feeding to animals. In each treatment group, rats were fed with the drug (5 mg/kg) in a volume of 0.5 ml.

Experimental procedure

Acute treatment

Each rat were randomly fed with vehicle control (distilled water, p.o.), diazepam (5 mg/kg, p.o.) and barakol (10, 30 and 100 mg/kg, p.o.) and returned to its home cage. The lowest dose of barakol (10 mg/kg) was chosen based on the previous study showing that they were behaviorally active when injected intraperitoneally. The two higher doses (30 and 100 mg/kg) were roughly equivalent to the intraperitoneal injection doses (10 and 30 mg/kg). After 30 min, each rat fed with vehicle or drugs was exposed to the elevated plus maze for 5 min. Immediately after the elevated plus maze test, each rat was tested for 10 min in the holeboard apparatus.

Chronic treatment

Groups of 10 rats were fed once daily for 30 consecutive days with vehicle control (distilled water, p.o.), diazepam (5 mg/kg, p.o.) and barakol (10, 30 and 100 mg/kg, p.o.) between 8.00-10.00 hours. Body weight was recorded every day during the treatment. On the 30th day of experiment, after feeding with vehicle or drug for 30 min, each rat were tested in the elevated plus maze and holeboard in the same manner as the acute treatment.

The experimental protocol was approved by the Animal Ethics Committee of Srinakharinwirot University for the use of animal subjects and the procedures are in compliance with the International Guiding Principles for Biomedical Research Involving Animals provided by the National Research Council of Thailand.

Data analyses

All data were expressed as the mean $\pm$ standard error of mean (SEM). The percentages of open/total number of entries and open/total time were calculated for each rat and the mean $\pm$ SEM for each treatment group presented. All data was analyzed using a one-way analysis of variance (ANOVA). The treatment group was compared to the vehicle control group using Dunnett's multiple comparison test. The acute and chronic treatment group was compared using a Student t-test. A $p < 0.05$ was considered significant.

Results

The purity of barakol used in this study was confirmed by HPLC-PDA as shown in Fig. 1. The compound had a characteristic absorbance at 230-260 nm with the highest absorbance at 243.3 nm and the runtime of 15.107 minutes.

Chronic oral administration of diazepam and all doses of barakol for 30 consecutive days had no effect on the body weight compared with the vehicle treated controls (data not shown).

Elevated plus maze

The behavioral effects of acute and chronic administration of diazepam or barakol in rats when exposed to the elevated plus maze are shown in Fig. 2. Rats treated acutely with diazepam (5 mg/kg) showed a significant increase in the percentage of the open/total time (Fig. 2A) [F (4, 38) = 2.59, $p < 0.05$] and time spent on the open arms (Fig. 2B) [F (4, 18) = 3.16, $p < 0.05$], but failed to increase the percentage of the open/total entries (Fig. 2A) when compared with the vehicle-treated rats. The chronic treatment with diazepam for 30 consecutive days increased the percentage of the open/total number of arm entries [F (4, 66) = 2.85, $p < 0.05$], the percentage of the open/total time (Fig. 2A) [F (4, 65) = 3.41, $p < 0.01$], time spent on the open arms (Fig. 2B) [F (4, 68) = 3.41, $p < 0.01$), although the increase in the percentage of the open/total time and the time spent on the open arms were significantly less than that observed with acute

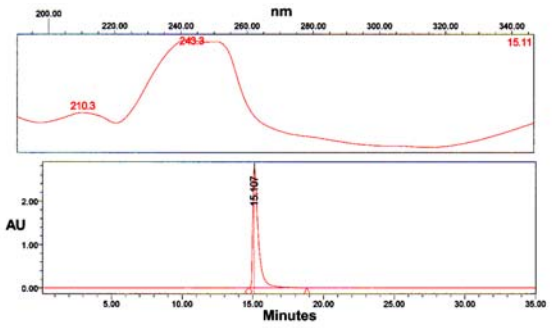

Fig. 1 Chromatogram of barakol (25 mg) dissolved in HPLC water (1mg/ml) measured by HPLC-PDA (190-350 nm, Waters, USA) using Kromasil C8 column (250 mm x 4.6 mm ID, 5 mm particle size) and flow rate of 0.8 ml/min. The compound had a characteristic absorbance at 230 - 260 nm with the highest absorbance at 243.3 nm and the runtime of 15.107 minutes

treatment (Fig. 2A, B). Both acute and chronic treatments with diazepam had no effect on the total number of arm entries or the rear/min. Among animals receiving barakol, only rats acutely treated with barakol (10 mg/kg) showed a tendency, but not significant increase in the percentage of open/total time (Fig. 2A) and time spent on the open arm (Fig. 2B); however, these increases were significant greater than the chronically treated-rats. These behavioral parameters were not affected by higher doses of barakol (30 and 100 mg/kg) treated acutely or chronically. Neither acute nor chronic treatment with all doses of barakol produced significant change in the total arm entries or rears/minute compared with the control group. However, chronic barakol treatment (30 and 100 mg/kg) showed a significant reduction in the total arm entries (Fig. 2C) and rears/min (Fig. 2D) compared to acute barakol treatment.

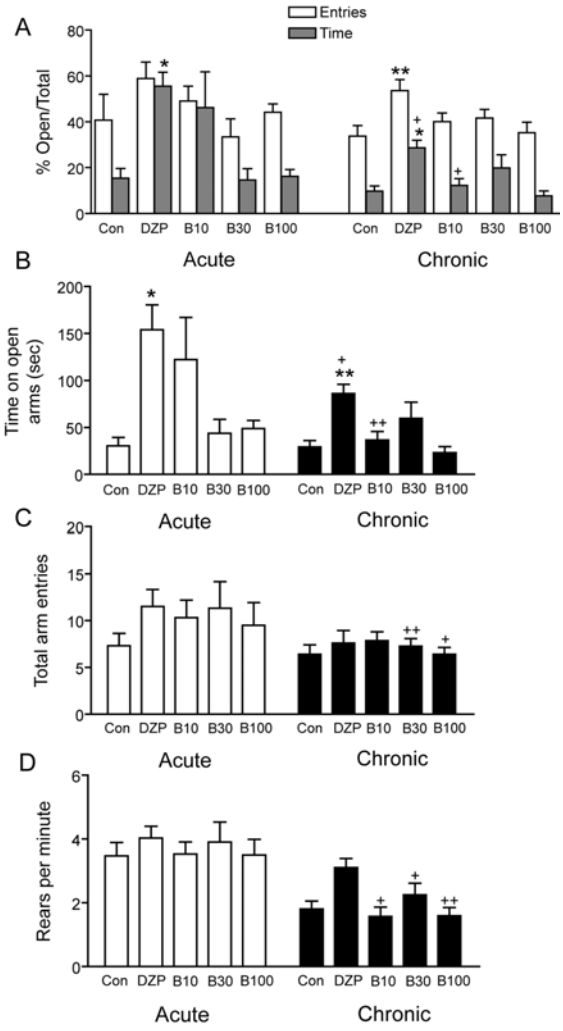

Fig. 2 Effects of acute and chronic oral administration of diazepam (DZP, 5 mg/kg) and barakol (B,10, 30, and 100 mg/kg) on (A) the percentage of the open/total entries and time, (B) the time spent on the open arms, (C) the total number of arm entries, and (D) the number of rears per minute. Behavior was monitored in the elevated plus maze for a 5 min period, 30 min after drug administration. Data represent mean $\pm$ SEM (n = 10-15 per group). * $p < 0.05$ and ** $p < 0.01$ were significantly different from the control group (Con) using Dunnett's test after ANOVA. $^{+}$ $p < 0.05$ and $^{++}$ $p < 0.01$ were significantly different from the corresponding acute treatment using a Student t-test

Holeboard

After a 5-min plus maze test, rats were immediately tested in the holeboard apparatus for 10 min and the data are shown in Fig. 3. Acute and chronic treatments with diazepam or all doses of barakol had no effect on the number of grooms (Fig. 3A) or rears/min (Fig. 3B), although chronic treatment with high dose of barakol (100 mg/kg) showed a significant reduction in the number of grooms and rears/minute when compared with acute treatment. It was noted that chronically vehicle-treated rats showed a marked and significant greater in the numbers of head-dips and time spent head-dipping than acutely vehicle-treatment animals. However, these parameters were not affected by barakol or diazepam treatment when given acutely (Fig. 3C, D). In addition, no significant increases

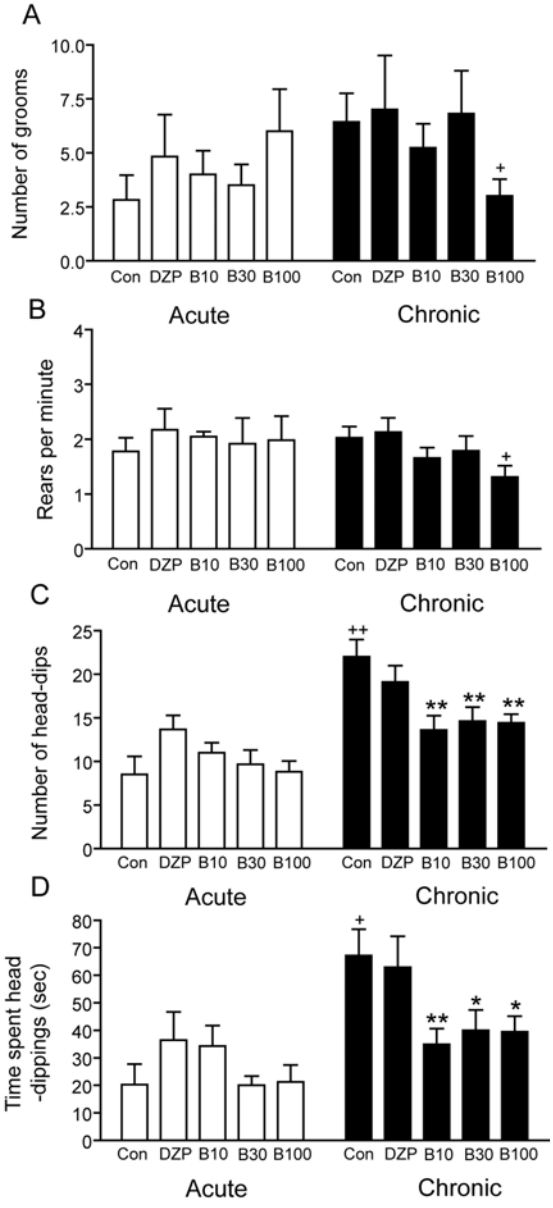

Fig. 3 Effects of acute and chronic oral administration of diazepam (DZP, 5 mg/kg) and barakol (B, 10, 30, and 100 mg/kg) on (A) the number of grooms, (B) the number of rears per minute, (C) the number of head-dips, and (D) the time spent head-dippings. Behavior was monitored in the holeboard for a 10 min period, immediately after the plus maze test. Data represent mean ± SEM (n = 10-15 per group). * $p < 0.05$ and ** $p < 0.01$ were significantly different from the control group (Con) using Dunnett's t-test after ANOVA. $^{+}$ $p < 0.05$ and $^{++}$ $p < 0.01$ were significantly different from the corresponding acute treatment using a Student t-test

in the number of head-dips and time spent head-dipping were observed between acute and chronic treatment of diazepam. In chronic treatment groups, all three doses of barakol significantly decreased the number of head-dips [$F(4, 69) = 4.12$, $p < 0.01$; $F(4, 69) = 3.27$, $p < 0.01$; $F(4, 69) = 3.35$, $p < 0.01$ for barakol 10, 30 and 100 mg/kg, respectively], and the time spent head-dipping [$F(4, 68) = 3.19$, $p < 0.01$; $F(4, 68) = 2.62$, $p < 0.05$; $F(4, 68) = 2.60$, $p < 0.05$ for barakol 10, 30 and 100 mg/kg, respectively]; however, those parameters were not difference from the corresponding acute barakol treatment.

Discussion

The present results demonstrated that acute and chronic oral administrations of barakol (10, 30 and 100 mg/kg for 30 days) had no anxiolytic effect when compared to those observed with diazepam. This indicates that the anxiolytic property of barakol given intraperitoneally found in the previous study[11] was lost when given orally. Moreover, oral administration of barakol had no effect on exploratory and locomotor behavior, but, on the other hand, exerted a sedative effect as shown by a reduction in the directed exploratory behaviors (rears per min in the elevated plus maze and number of head-dips in the holeboard) following long-term treatment.

Previous studies by Thongsaard and co-workers[11] had shown that acute intraperitoneal injection of barakol at the lowest dose (10 mg/kg, i.p.) produced an anxiolytic profile on the plus maze while the higher dose (25 and 50 mg/kg, i.p.) significantly increased only the % open/total number of arm entries and time. In contrast, barakol at the highest dose (75 mg/kg, i.p.) had no anxiolytic action but showed sedative activity. These findings indicate a dose-dependent behavioral effect of barakol with the lower dose producing an anxiolytic activity whereas the higher dose producing a sedative activity. The present study was performed to investigate the effect of acute and chronic administration of barakol orally in order to obtain results for applying to normal usage in human. The results demonstrated that both acute and chronic barakol administration (orally) shown no anxiolytic profiles when compared to the previous results reported by the same laboratory given barakol intraperitoneally. However, as shown insignificantly, acute oral treatment of low dose of barakol had a tendency of anxiolytic effect. The lack of anxiolytic activity of long-term treatment of barakol may be due to several possibilities. Firstly, long-term treatment may result in

accumulation of barakol to a close level that prevents the anxiolytic effect due to other pharmacological actions involving unknown mechanisms. Secondly, long-term treatment may induce tolerance. Most of drugs affecting the central nervous system including well established anxiolytic drugs (*e.g.* benzodiazepines) produce tolerance and withdrawal effects after long-term treatment[19]. In the present study, although oral treatment of diazepam was found to exert anxiolytic activities after chronic treatment, but the response was less compared to acute intraperitoneal treatment. More studies are required to investigate the pharmacokinetics of barakol when administered via different routes. In addition, in a study in which rats received crude water extraction of dry leaf of *C. siamea* (10 mg/kg, p.o.) for 30 consecutive days, an anxiogenic withdrawal effect was observed at 48 and 72 hours after the last dose[20]. Lastly, the route of barakol administration may account for the different results in the present study from the previous acute studies. In this study, barakol was orally given to animals to avoid infection and other complications that might occur from chronic daily intraperitoneal injection, but a single intraperitoneal injection was given in the previous study[11]. More information is required on the precise mechanism of action and pharmacokinetics and pharmacodynamics of barakol in order to clarify the behavioral effects of barakol.

Long-term treatment with either barakol or diazepam failed to change the total arm entries and the number of rears per minute, which are used as an index of locomotor and exploratory behaviors in the plus maze test. These results indicate that in addition to no anxiolytic activity, long-term oral treatment with barakol has no effect on locomotor and exploratory behaviors on the plus maze.

In order to confirm the purity of barakol used in this study, the acute intraperitoneal injection of barakol (10 mg/kg) was tested in the elevated plus maze. The result demonstrated that barakol at the dose of 10 mg/kg shows anxiolytic property in rats as indicated by the significant increase in the percent open/total entries and time (data not shown). It is indicated that the difference in the present study from the previous study[11] is likely due to the route of administration, not the purity of the barakol.

The results in this study demonstrated that acute oral treatment of barakol at all doses did not alter any parameters tested with the holeboard. However, the chronic oral treatment of barakol at all doses decreased exploratory activity as shown by decreases in the number of head-dips and the time spent head-dipping. All of these parameters were unaffected by diazepam treatment. These findings suggest that chronic oral administration of barakol may have a sedative effect, which is correlated with a study showing the ability of barakol (100 mg/kg, i.p.) to prolong the thiopental-induced sleeping time in mice[21]. In addition, barakol treatment had no effect on the number of rears in either the plus maze or holeboard tests. Since the total number of rears is an indicative of motor activity through displacement and vertical activity[22], the results indicate that long-term oral treatment with barakol does not alter locomotor behavior. However, barakol (50 and 100 mg/kg, i.p.) was shown to reduce locomotor activity and rearing in mice and attenuated the hyper-locomotion produced by 1 mg/kg, i.p. metamphetamine[21].

In the holeboard test, both barakol and diazepam, when given orally, had no effect on grooming, which is considered an indicator of displacement behavior associated with serotonergic stimulation[16,23] indicating that long-term oral treatment with barakol may not involve serotonergic function. However, barakol has been shown to suppress 5-hydroxytryptophan-induced head shakes in mice[10,24], indicating the possible serotonergic antagonist activity of barakol. More study is required to confirm the possible effect of barakol on serotonergic function.

The underlying mechanisms for the behavioral effects of barakol remain to be identified because little has been known about the pharmacological and neurochemical changes following acute or chronic treatment of barakol. Previous studies using rat striatal slices demonstrated that barakol decreased K^+-stimulated endogenous dopamine release *in vitro*[12], in a manner similar to the dopamine D_2/D_3 receptor agonist, quinelorane dihydrochloride. The inhibitory effect of barakol on *in vitro* endogenous dopamine release was prevented by the dopamine D_2/D_3 receptor antagonist, S(-)-eticlopride hydrochloride, suggesting an action of barakol at the presynaptic dopamine autoreceptor[12]. As reported previously, barakol may act at the post-synaptic nerve terminal in two different manners. The low dose of barakol (10 mg/kg, i.p.) may act on post-synaptic D_2 receptor to exhibit anxiolytic, exploratory and hyperlocomotor behaviors while the higher dose (100 mg/kg, i.p.) may act on postsynaptic D_3 receptor responsible for the hypolocomotion and sedation[25]. The hypolocomotor and sedative activities of high dose of barakol (50 and 100 mg/kg, i.p.) has been confirmed in mice[21]. However, the anxiolytic activity

of barakol (10, 50 and 100 mg/kg, i.p.) on the elevated plus maze was not observed in that study using mice. The difference in behavioral responses to the same drug treatment was not surprisingly found between rats and mice or even between the different strains of rats[26-28]. In addition, the species differences in behavioral tests may probably due to variations in the absorption or metabolism of compounds among species or strains[29]. Further studies are required to investigate a suitable route of administration, an appropriate dose, and a suitable time course of barakol treatment for either anxiolytic or sedative activities as well as the involved dopaminergic and serotonergic activities.

In conclusion, the present study demonstrated that acute and chronic oral treatment of barakol had no an anxiolytic effect compared to diazepam. Unlike diazepam, barakol reduced directed exploratory activity and exerted a sedative effect. The results from the present and the previous studies[11,12,30] suggest that the acute barakol treatment intraperitoneally exerts an anxiolytic effect while the long-term treatment orally causes sedation. Therefore, it is essential to consider carefully when assessing the value of barakol as anxiolytic or sedative drugs.

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ผลของการให้สารสกัดบาราคอลทางปากอย่างเฉียบพลันและต่อเนื่องต่อพฤติกรรมของหนูแรท

ฉัตรศรี เดชะปัญญา, วัชรีวรรณ ทองสะอาด

*บาราคอลเป็นสารสกัดบริสุทธิ์จากใบขี้เหล็ก (*Cassis siamea*) ออกฤทธิ์ในการคลายความเครียดคล้ายยาไดอาซีแพม (diazepam) เมื่อทดสอบในหนูแรทโดยการให้ทางช่องท้อง การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อทดสอบฤทธิ์เฉียบพลันและต่อเนื่องของการให้บาราคอลทางปากต่อพฤติกรรมการคลายเครียด การเคลื่อนไหว และการตื่นตัวต่อสิ่งเร้าของหนูแรท โดยใช้เครื่องมือ Elevated plus maze เพื่อทดสอบพฤติกรรมคลายเครียดในสัตว์เล็ก และเครื่องมือ Holeboard เพื่อทดสอบพฤติกรรมการตื่นตัวต่อสิ่งเร้าและการเคลื่อนไหวไปพร้อม ๆ กัน โดยแบ่งหนูแรทออกเป็นกลุ่มเฉียบพลันที่ได้รับการป้อนน้ำกลั่น ไดอาซีแพม (5 มิลลิกรัม/กิโลกรัม) หรือ บาราคอล (10, 30 และ 100 มิลลิกรัม/กิโลกรัม) เพียงครั้งเดียว และกลุ่มต่อเนื่องที่ได้รับสารดังกล่าววันละครั้งเป็นเวลา 30 วัน ผลการทดลองพบว่า การให้บาราคอลทางปากอย่างเฉียบพลันหรือต่อเนื่องไม่มีผลในการลดความเครียดเมื่อเปรียบเทียบกับการให้ยาไดอาซีแพมอย่างเฉียบพลันหรือต่อเนื่อง แต่เมื่อให้บาราคอลอย่างต่อเนื่องกลับแสดงผลให้เกิดการง่วงซึมโดยลดพฤติกรรมการเคลื่อนไหวและการตื่นตัวต่อสิ่งเร้า ในขณะที่ไม่พบผลดังกล่าวเมื่อให้ไดอาซีแพมอย่างเฉียบพลันหรือต่อเนื่อง*