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Effects of extracts and neferine from the embryo of *Nelumbo nucifera* seeds on the central nervous system

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Abstract

The effects of embryos of the seeds of *Nelumbo nucifera* on the central nervous system were studied in mice. MeOH extracts of embryos of *Nelumbo nucifera* seeds significantly inhibited locomotor activity in mice. The MeOH extract was successively partitioned between H_2O and *n*-hexane, between H_2O and $CHCl_3$, and between H_2O and *n*-BuOH. $CHCl_3$ extracts strongly inhibited locomotor activity in mice, although other extracts had no effect on locomotor activity. The main alkaloid of $CHCl_3$ extracts, neferine, dose-dependently inhibited locomotor activity in mice. Neferine induced hypothermia in mice and apparently potentiated thiopental-induced sleeping time. An anxiolytic, diazepam, decreased locomotor activity, rectal temperature and enhanced sleep elicited by thiopental, similar to neferine. In addition, neferine and diazepam showed anti-anxiety effects in the elevated plus maze test. Neferine did not affect muscle coordination by the rota-rod test. Neferine did not affect strychnine- nor picrotoxin-induced seizure. In contrast, diazepam had apparent muscle relaxant and anti-convulsant effects. These results suggest that neferine has several central effects and that neferine may participate in the efficacy of the sedative effects of embryos of the seeds of *Nelumbo nucifera*. The mechanisms of the sedative effects of neferine are not similar to those of diazepam.

Keywords: *Nelumbo nucifera*; Bisbenzylisoquinoline alkaloids; Neferine; Sedation; Behavior; Central nervous system

Introduction

"Lian zi xin", embryo loti (embryo of the seeds of *Nelumbo nucifera* Gaertner, Nymphaeaceae), has been used in Chinese traditional medicine as a sedative, antipyretic and hemostat agent (Chiang Su New Medical College, 1978), indicating that it may possess central effects. A previous study reported that bisbenzylisoquinoline alkaloids, such as neferine and liensinine, were isolated from this plant (Furukawa, 1966); however, the central effects of the embryo of the seeds of *Nelumbo nucifera* have not been characterized. Furthermore, it has not been clarified whether the alkaloid involved in this plant contributes to the central effects.

Out of interest in the sedative activity of this herbal medicine, we reexamined the chemical constituents of the embryo of the seeds of *Nelumbo nucifera* Gaertner

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and examined the locomotor activity effects of various extracts of embryo loti in mice. Furthermore, to clarify the central effects, we examined the effects of neferine, which is the main alkaloid of $CHCl_3$ extracts, on locomotor activity, rectal temperature, thiopental-induced sleeping, muscle coordination and drug-induced seizures. Furthermore, we examined the effects of nerefine in the elevated-plus maze test to evaluate anti-anxiety effects.

Material and methods

General experimental procedures

Optical rotations were measured on a JASCO DIP-370 digital polarimeter (Japan). UV spectra were recorded on a Shimadzu UV-2500PC spectrophotometer (Shimadzu, Japan) and IR spectra on a Shimadzu FTIR-8200 spectrophotometer (Japan). ^1H (500 MHz) and ^{13}C (125 MHz) NMR spectra were recorded on a Varian VXR-500 spectrometer (USA) with TMS as an internal standard. Mass spectrometry was obtained with a Hitachi M-4100 mass spectrometer (Japan). Glycerol was used as the matrix for SIMS. TLC was performed on precoated Kieselgel $60F_{254}$ plates (Merck, Germany).

Plant materials

Embryos of the seeds of *Nelumbo nucifera* Gaertner were purchased from Longhua Hospital Shanghai University of Traditional Chinese Medicine in Shanghai, China. A voucher specimen was deposited in the laboratory of Kobe Pharmaceutical University.

Extraction and isolation

Embryos of the seeds of *Nelumbo nucifera* (1200 g) were extracted with hot MeOH. The extracts were evaporated *in vacuo* and the resulting residue (232.6 g) was resuspended in H_2O and extracted successively with *n*-hexane, $CHCl_3$ and *n*-BuOH. The extraction diagram is shown in Fig. 1.

The residue from the $CHCl_3$ layers (8.0 g) was purified by a combination of silica gel CC ($CHCl_3$–MeOH) and preparative TLC ($CHCl_3$–MeOH, 19:1, 9:1; $CHCl_3$–MeOH–NH_4OH, 95:4.5:0.5, 90:9:1; C_6H_6–AcOEt–Et_2NH, 7:2:1) to afford pronuciferine (33.7 mg), thalifoline (6.7 mg), neferine (2.99 g, Fig. 2), liensinine (57.4 mg) and isoliensinine (20.3 mg). The isolated compounds were identified by comparison of their spectral data with those described in the literature.

Animals

Male ICR mice weighing 25–30 g were purchased from SLC Japan (Japan). Mice had free access to food and water, and were maintained on a 12 h dark/light cycle in a room with controlled temperature (23 ± 1 °C) and humidity (55 ± 5%). Experiments were performed in accordance with the Guiding Principles for Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society.

Treatment of extracts and neferine

Extracts of embryos of the seeds of *Nelumbo nucifera* were suspended in 1% carboxylmethyl cellulose-Na and administered p.o. Neferine HCl was dissolved in saline and given i.p. or p.o.

Drugs and treatment

Diazepam and thiopental Na (Wako, Japan) were dissolved in saline and injected i.p. Strychnine nitrate (Wako, Japan) or picrotoxin (Wako, Japan) was dissolved in saline and given s.c.

Locomotor activity

The locomotor activity of animals during the testing period was counted by an activity sensor (NS-AS01, Neuroscience Inc., Japan). Experiments were performed 60 min after administration of extracts or drugs.

Rectal temperature

Rectal temperature was measured by a thermometer (BAT-12, Sensortek, USA). The thermister probe was inserted 2 cm into the rectum.

Thiopental Na-induced sleep

Thiopental Na at 60 mg/kg was injected i.p. 15 min after the administration of neferine or diazepam. The onset time and duration of loss of the righting reflex were recorded.

Elevated plus maze test

The elevated plus maze (method of according to Pellow et al., 1985; Lister, 1987) consisted of two closed arms ($10 \times 50 \times 40$ cm) and two open arms (10×50 cm) emanating from a common central platform (10×10 cm). The closed and open arms were arranged opposite to each other. Thirty minutes after the injection of neferine

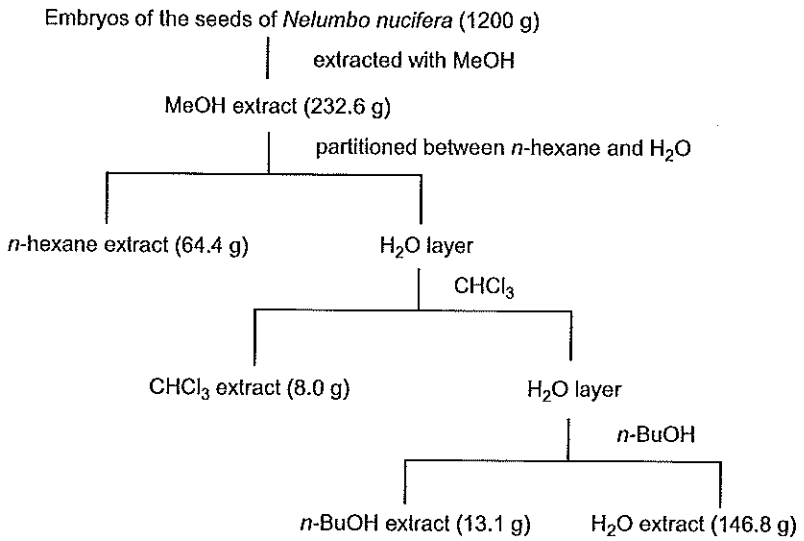

Fig. 1. Extraction procedure from the embryo of *Nelumbo nucifera* seeds.

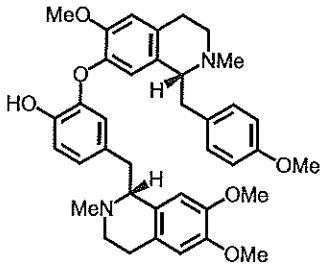

Fig. 2. Chemical structure of neferine.

or diazepam, the mouse was placed at the central platform of the maze with its head facing an open arm and allowed to explore the maze for 5 min. Entry into each arm was defined as placement of four paws into an arm. Number of entries into each type of arm, the percentage of time spent and the percent of arm entries in open arms were recorded.

Rota-rod test

The integrity of motor coordination was assessed with a rota-rod apparatus (Ugo Basile, Italy) at a rotating speed of 8 rpm, by counting the number of falls from the rod in 3 min.

Strychnine- and picrotoxin-induced convulsion

Mice were treated with neferine or diazepam before the administration of strychnine (2 mg/kg, s.c.) or picrotoxin (5 mg/kg, s.c.). The death time and latency of first episode of clonic seizure were recorded. The cut-off time was set as 60 min after the convulsant administration.

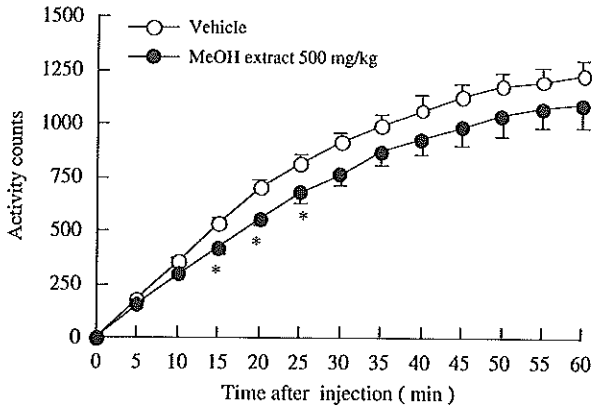

Fig. 3. Effects of MeOH extract on locomotor activity in mice. Results are shown as mean±S.E.M. ($N = 9$–10). MeOH extract was given p.o. *$p < 0.05$.

Statistics

Dose-related effects were evaluated by one-way analysis of variance (ANOVA) followed by Dunnett's test. Significance between two groups was analyzed by Student's *t*-test. Analysis of data on rota-rod test was evaluated by χ^2 test.

Results

Locomotor activity

As shown in Fig. 3, MeOH extracts of embryos of *Nelumbo nucifera* seeds significantly inhibited locomotor activity. Fig. 4 shows the effects of $CHCl_3$, *n*- hexane, *n*-BuOH and water extracts on locomotor activity in mice. Only $CHCl_3$ extracts showed apparent inhibition of locomotor activity.

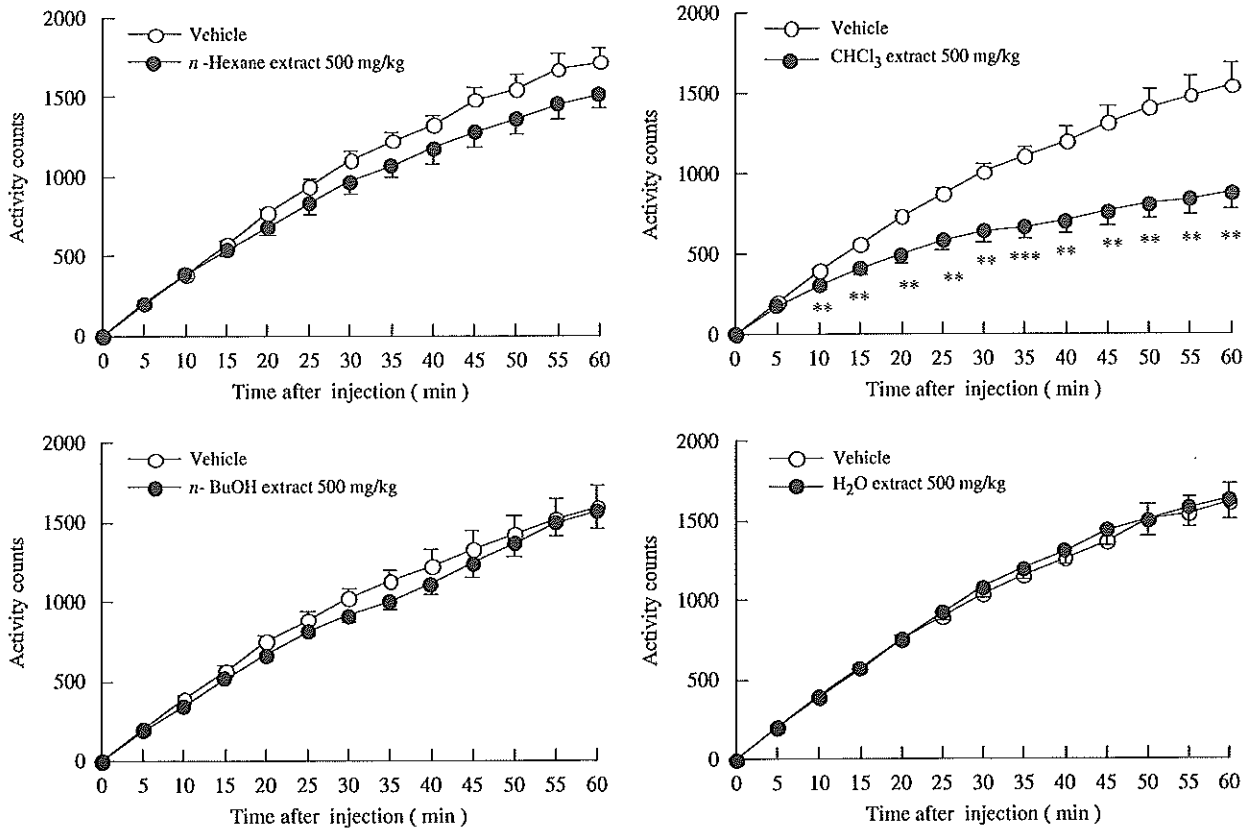

Fig. 4. Effects of *n*-hexane, $CHCl_3$, *n*-BuOH and water extracts on locomotor activity in mice. Results are shown as mean ± S.E.M. ($N = 6$–9). $^{**}p < 0.01$ and $^{***}p < 0.001$. Each extract was given p.o.

Fig. 5 demonstrates the effects of neferine and diazepam on locomotor activity in mice. Neferine above the dosage of 50 mg/kg inhibited locomotor activity. Diazepam at 5 mg/kg decreased locomotor activity.

Rectal temperature

As shown in Fig. 6, neferine significantly decreased rectal temperature in mice. Diazepam also induced significant hypothermia in mice (Fig. 6).

Thiopental-induced sleeping

As shown in Fig. 7, neferine shortened the onset time of thiopental-elicited sleeping dose-dependently. Pretreatment with neferine remarkably prolonged the duration of sleeping induced by thiopental. Diazepam at 1 mg/kg also enhanced thiopental-induced sleeping.

Elevated plus maze test

As shown in Fig. 8, neferine and diazepam increased both the percent of open arm entry or time spent in open arms.

Motor coordination

In the rota-rod test, neferine (25–100 mg/kg) did not affect motor coordination, whereas diazepam impaired motor coordination in mice (Table 1).

Strychnine- and picrotoxin-induced convulsion in mice

Diazepam prolonged the latency of convulsion induced by strychnine and picrotoxin. Diazepam protected against death induced by picrotoxin and prolonged duration until death induced by strychnine (Table 2). Neferine at 50 and 100 mg/kg did not modify strychnine- and picrotoxin-induced convulsions (Table 2).

Discussion

In Chinese traditional medicine, embryos of the seeds of *Nelumbo nucifera* have been recognized to have depressant effects on the central nervous system, such as sedative activity, or to be effective against fever in humans; however, until now, detailed pharmacological

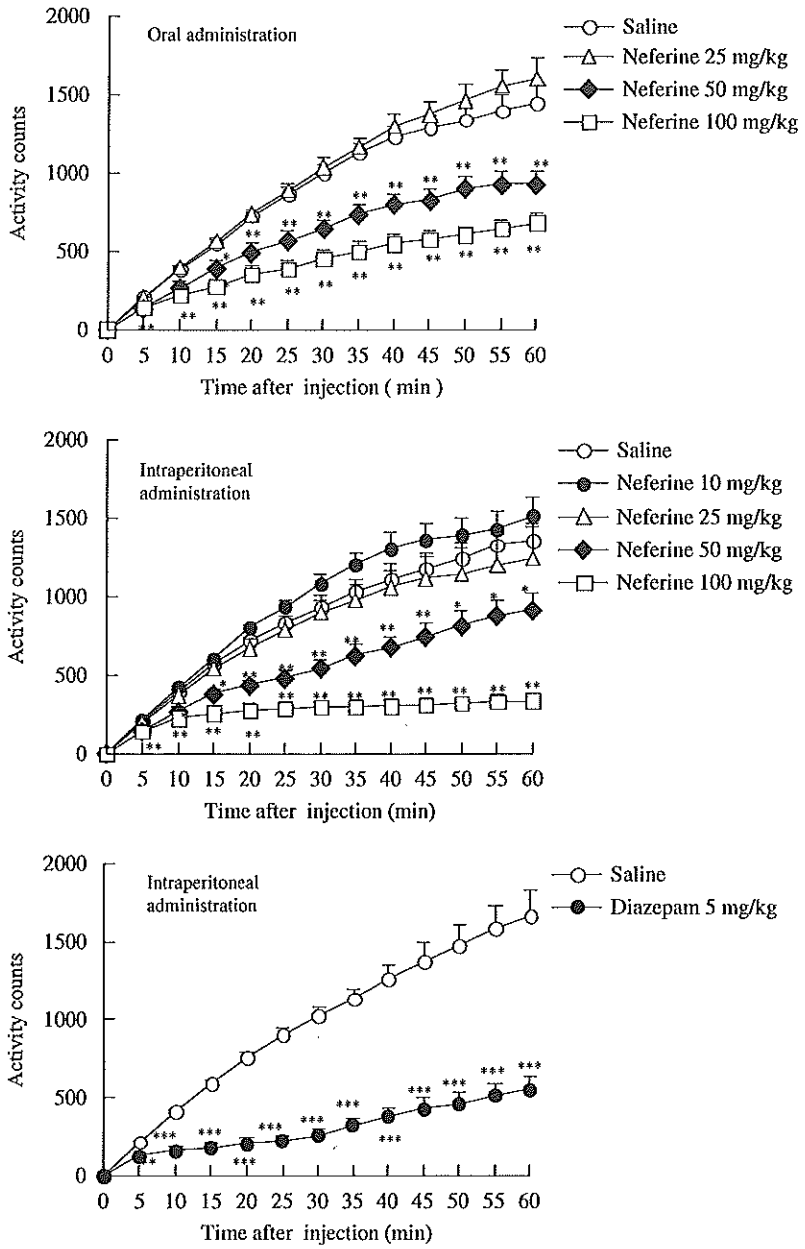

Fig. 5. Effects of neferine and diazepam on locomotor activity in mice. Results are shown as mean±S.E.M. ($N = 6$–8). Neferine was given p.o or i.p. Diazepam was given i.p. $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$.

examination of this plant and its effects on the central nervous system have not been performed. The present study demonstrates that plant extracts and neferine, the major alkaloid in the embryos of the seeds of *Nelumbo nucifera*, have apparent central effects.

MeOH extracts of embryos of the seeds of *Nelumbo nucifera* significantly inhibited locomotor activity. Furthermore, the effects of the partitioned extracts of MeOH, that is $CHCl_3$, *n*-hexane, *n*-BuOH and H_2O, were examined. As shown in the results, $CHCl_3$ extracts apparently reduced locomotor activity, whereas other extracts did not affect locomotion. This indicates that sedative elements may be present in $CHCl_3$ extracts.

As a result of investigating alkaloids in $CHCl_3$ extracts, several alkaloids were identified, including pronuciferine (Bernauer, 1963), thalifoline (Krane and Shamma, 1982), neferine (Furukawa, 1965), liensinine (Pan et al., 1962) and isoliensinine (Tomota et al., 1965) in this study. Thalifoline was isolated for the first time from this plant species. A previous report demonstrated that bisbenzylisoquinoline alkaloids, such as neferine and liensinine, were isolated from this plant (Furukawa, 1966), which is in agreement with the results of this study. Since neferine is a major alkaloid, the effects of neferine on locomotor activity in mice were studied.

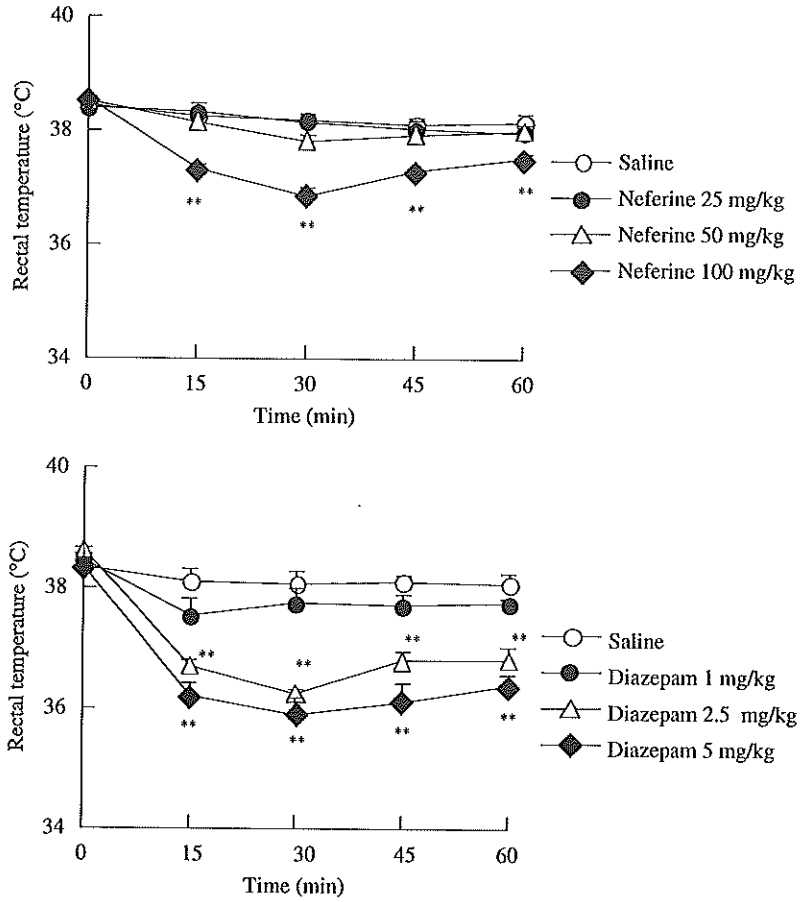

Fig. 6. Effects of neferine and diazepam on rectal temperature in mice. Results are shown as mean ± S.E.M. ($N = 6$–9). ** $p < 0.01$. Neferine and diazepam were given i.p.

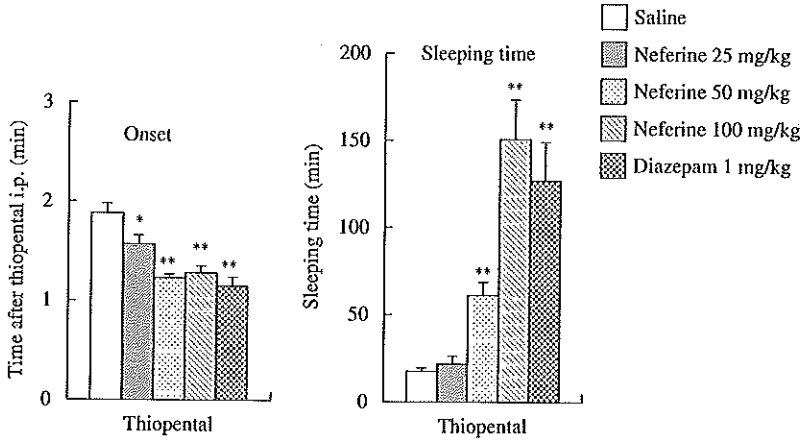

Fig. 7. Effects of neferine and diazepam on thiopental-induced sleep in mice. Results are shown as mean ± S.E.M. ($N = 6$–10). Neferine was given i.p. Thiopental at 60 mg/kg was injected i.p. 15 min after neferine. *$p < 0.05$, **$p < 0.01$ vs. saline + thiopental.

Administration of neferine dose-dependently inhibited locomotor activity in mice. Neferine above 50 mg/kg induced significant effects and administration of 100 mg/kg elicited potent effects. Since embryos of the seeds of *Nelumbo nucifera* may be effective against fever, the effects of neferine on rectal temperature were studied in mice. Neferine induced significant hypothermia in mice. These results demonstrate for the first time that neferine actually induces sedation and hypothermia in mice, suggesting that neferine may contribute to the sedative and anti-fever effects of embryos of the seeds of *Nelumbo nucifera*.

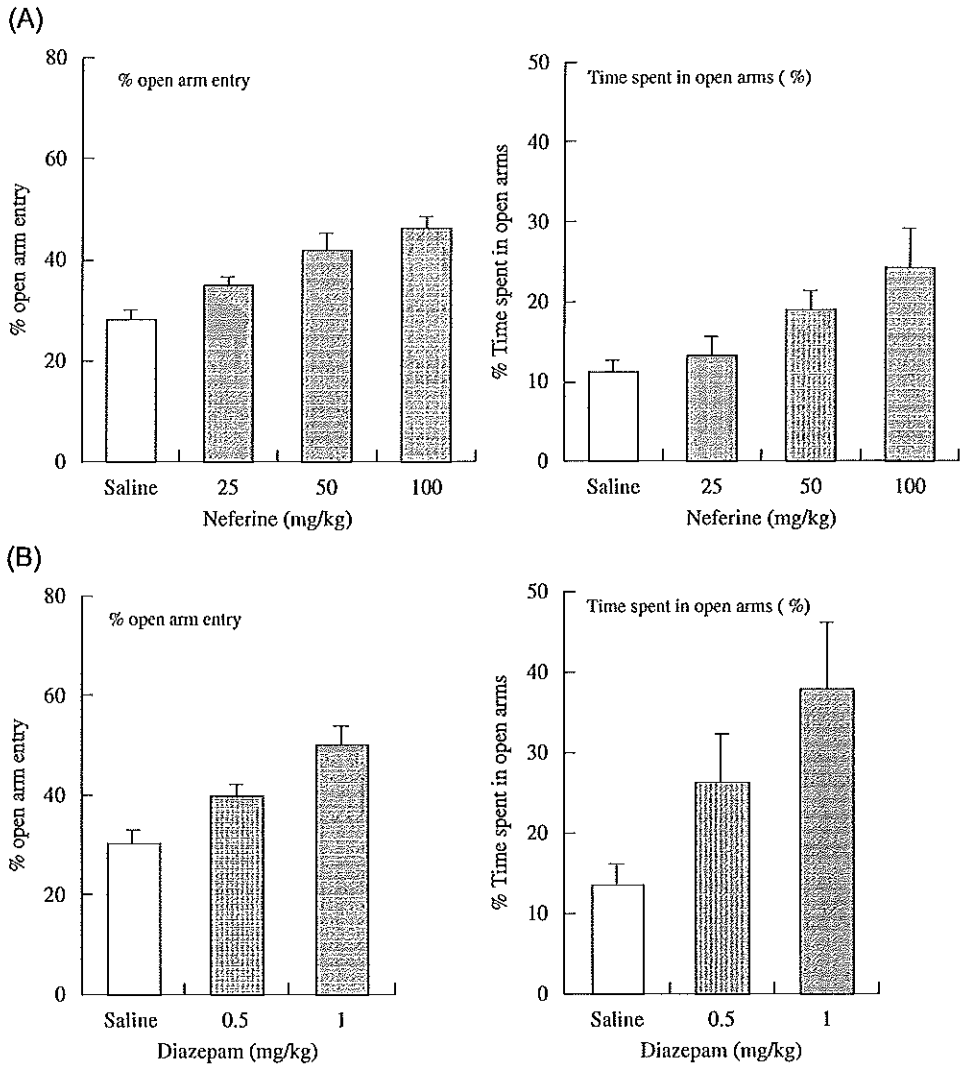

Fig. 8. Effects of neferine and diazepam on the percentage of entries into and time spent in open arms for 5 min in the elevated plus maze. Results are shown as mean±S.E.M. ($N = 6$–10). Neferine and diazepam were given i.p 30 min before experiments. $^{*}p<0.05$ and $^{**}p<0.01$.

Table 1. Effects of neferine and diazepam on motor coordination evaluated by rota-rod test

Group	Motor incoordination
Saline	0/6
Neferine 25 mg/kg	0/6
Neferine 50 mg/kg	0/6
Neferine 100 mg/kg	0/6
Diazepam 5 mg/kg	5/6**

Rota-rod test was performed 15 min after the injection of neferine or diazepam. $^{**}p<0.01$.

An anxiolytic, diazepam, the most widely used benzodizaepine derivative, causes sedation and anti-anxiety effects by activation of benzodiazepine receptors. In the present study, we also found that diazepam inhibited locomotor activity and induced hypothermia,

Table 2. Effects of neferine and diazepam on strychnine- and picrotoxin-induced convulsion in mice

Group	Latency (min)	Death (min)
Strychnine		
Saline	2.2±0.13	2.8±0.18
Neferine 50 mg/kg	2.6±0.20	3.0±0.21
Neferine 100 mg/kg	2.8±0.22	3.3±0.23
Diazepam 1 mg/kg	3.2±0.24*	5.4±0.33**
Picrotoxin		
Saline	9.3±0.42	22.8±1.41
Neferine 50 mg/kg	9.6±0.59	18.4±2.09
Neferine 100 mg/kg	10.5±0.67	29.8±3.26
Diazepam 1 mg/kg	18.4±2.02**	>60**

Results are shown as mean±S.E.M. ($N = 5$–9). Neferine and diazepam were given i.p. 15 min before strychnine or picrotoxin Strychnine at 2 mg/kg and picrotoxin at 5 mg/kg were given s.c. $^{*}p<0.05$ and $^{**}p<0.01$.

similar to neferine. Neferine may therefore show pharmacological activity in the central nervous system, similar to diazepam; therefore, we further studied the pharmacological effects of neferine on the central nervous system compared to those of diazepam.

Both neferine and diazepam prolonged thiopental-induced sleep in mice. The potentiation of neferine on thiopental-induced sleep further supports that neferine has sedative effects, like diazepam. Both neferine and diazepam showed anti-anxiety effects evaluated by the elevated plus maze test. Although diazepam displays sedative effects mediated by the benzodiazepine receptor, it is well known to cause typical side effects and impairment of muscle coordination. Our results also demonstrate that in the rota-rod test, diazepam impairs motor coordination in mice; however, neferine did not affect motor coordination in this test. In addition, diazepam antagonized strychnine- and picrotoxin-induced convulsion, whereas neferine had no effect; therefore, the pharmacological profile of neferine is not the same as that of diazepam. The mechanism of neferine may not relate to benzodiazepine receptors but to other neurotransmitter systems.

In summary, we demonstrated that $CHCl_3$ extracts of the embryos of the seeds of *Nelumbo nucifera* show sedative effects. The major alkaloid neferine induced apparent sedation and hypothermia or anxiolytic effects in mice. The effects of neferine are therefore different from those of diazepam. The anti-hypertensive activity of neferine was characterized (Nishibe et al., 1986), and recently the inhibitory effects of isoliensinine on pulmonary fibrosis were reported (Xiao et al., 2005). Further pharmacological studies of alkaloids, including neferine, in the embryos of the seeds of *Nelumbo nucifera* are now under investigation.

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