Neurotoxicity of *Coscinium fenestratum* stem, a medicinal plant used in traditional medicine

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Abstract

*Coscinium fenestratum* is a common medicinal plant widely used in the Indochina region, but scientific data on its safety is very limited. This study aimed to observe the effect of this plant on neurotoxicity and neurobehavior. Oral administration of plant alcoholic extract at dosages of 5, 10 and 20 mg/kg BW for 14 days increased the rats body weight and decreased the neuron density in the cerebral cortex, hippocampus and striatum. The plant extract significantly increased stereotyped behavior in licking but did not cause anxiolytic activity, anti-depression, sensory motor co-ordination impairment and ataxia. It is concluded that the plant possesses neurotoxicity and is able to induce neurobehavioral changes in rats. Therefore, the application of this plant as either drug or supplementary food should be reconsidered.

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1. Introduction

*Coscinium fenestratum* (Gaertn) Colebr, commonly known as “Ham” or “Ka-min-kreu”, is a common medicinal plant in the family of Menispermaceae and is widely used in the Indochina region. In Thailand, this plant is distributed mainly in the northeast of Thailand especially in Nong Khai and Nakorn Panom provinces. Decoction of the stem of *C. fenestratum* has been used in Thai traditional medicine for rural people in the northeast of Thailand for a long time. Its stem has been claimed to possess hypoglycaemic hypotensive laxative and anti-diabetic activities. In addition, the product of this plant in Sri Lanka is also claimed to be a therapeutic agent for various conditions including ophthalmopathy, inflammation, ulcers, skin disease, abdominal disorders, jaundice, fever and general debility.

Previous studies have reported that the alcoholic extract of this plant possesses anti-oxidant activity. It could protect against hepatotoxicity induced by carbon tetrachloride (Venukumar and Latha, 2002). The extract also exhibited strong anti-feeding (Javasinghe et al., 2003) and hypotensive activities (Singh et al., 1990). In addition, it has been reported that the main components in the stem of *C. fenestratum* are protoberberine alkaloids (Pinho et al., 1992). Berberine and its derivative have been reported to exert profound influences on the nervous system including the
anti-amnesic effect against memory defect induced by scopolamine [Peng et al., 1997].

Recently, this plant has been used as one of the active ingredients in functional food and claimed to contain various health promotion effects. Despite long term use, however, we have little scientific data to support the safety of this plant. Therefore, it is important that medicinal plant which have folklore reputation for medicinal effects should be investigated in order to establish their safety and efficacy.

Based on the unpublished data concerning the same comment of consumers about headache and dizziness after consumption in some cases, the present study was carried out to determine the effect of *C. fenestratum* on the neurobehavioral changes and neurotoxicity in various brain areas of rats.

2. Methods

2.1. Plant materials

The stem of *C. fenestratum* was purchased from Nakorn Panom province during the month of August, 2001. The tree specimen was identified and a voucher specimen from this plant was deposited at the Center of Research and Development of Herbal Health Products, Khon Kaen University under the number HHP-2-462.

2.2. Preparation of the extract

Stems of *C. fenestratum* were washed, dried at room temperature and minced into small pieces. Plant materials are then extracted with 50% ethanol by reflux method. Then the extract was centrifuged at 2500 rpm for 10 min to remove residual debris. The clear supernatant was evaporated under reduced pressure and dried by lyophilizer. The percent yield of extract was 8.53%. The extract was stored at −20°C in a dark bottle until used.

2.3. Animals

Adult male Sprague-Dawley rats (180-200 g, 8 weeks old) were obtained from National Animal Center, Salaya, Nakorn Pathom, and were housed in groups of five per cage in standard metal cages at 22 ± 2°C on 10:14 h light–dark cycle. All animals were given access to food and water ad libitum. The experiments were performed after the approval of the protocol by the Ethical Committee of the Institution, and every effort was made to minimize animal suffering in accordance with the internationally accepted principles for laboratory use and care of the European Community (EEC directive of 1986; 86/609/EEC).

2.4. Drugs

Diazepam (1 mg/tablet), and fluoxetine (20 mg/tablet) (Government Pharmaceutical Organization) were used as standard drugs in this study. They were dissolved in normal and administered via oral route.

2.5. Experimental protocol

All rats were randomly divided into four groups. Each group contained eight rats. The first group was treated with normal saline which used as vehicle. The second to fourth groups were treated with the extract of *C. fenestratum* at doses of 5, 10 and 20 mg/kg BW, respectively via oral route once daily. The doses used in this study are based on the recommendation of traditional herbalists in northeast region of Thailand. In the determination of anxiolytic and anti-depression activities, the animals were divided into five groups. The first to fourth groups were treated as mentioned above and the fifth group was treated with diazepam in the determination of anxiolytic activity whereas fluoxetine was treated in the determination of anti-depression.

2.5.1. Determination of the neurobehavioral effects

The rats were divided into various groups as mentioned earlier. The behavioral profiles were assessed on day 14th after the last dose of treatment. All animals were submitted to the following behavior tasks: (a) elevated plus maze; (b) rotarod test; (c) stereotyped behavior; (d) forced swimming test; (e) walking pattern and ataxia. Diazepam (2 mg/kg BW) and fluoxetine were used as reference drugs for administration to rats belonging to positive control group for the evaluation of anxiolytic and depression activities, respectively.

2.5.1.1. Elevated plus maze test. The elevated plus maze for rat consisted of open arms (50 × 10 cm) and two enclosed arms (50 × 10 cm) with 40 cm high walls, extending from a central platform (10 × 10 cm). The arms were connected with a central square, 10 × 10 cm, to give the apparatus a plus sign appearance. The maze was raised to a height of 50 cm above floor. The maze floor and walls were constructed from dark opaque wood. Each rat was placed on the center of the platform facing an enclosed arm. Animals were tested individually and only once for 5 min according to the following parameters: number of entries in the open and closed arms, and time of performance in each of them. The time of performance measures the time spent by the animal in the opened and closed arms. The maze was cleaned following each trial to remove any residue or odors. Each rat was assessed individually 30 min after the last dose of treatment.

2.5.1.2. Forced swimming test. In order to assess the anti-depressant activity of plant extract, the modified Porsolt test (Porsolt et al., 1977) was conducted. In the first trial, the rats not yet treated were forced to swim in a glass aquarium (22 cm in diameter, 40 cm in height) containing 20 cm high fresh water at 25°C for 15 min. The second exposure, anti-depressant activity of repetitive doses of extract was assessed after 14 days of treatment within 75 min after the last dose of administration. During the test session, the immobility time was recorded by blind observer who has been trained for the observation. The rats were considered immobile when neither hind leg was moving, the rats were slightly hunched forward. The total duration of immobility was measured during the 5-min test. Upon removal from the water, rats were towel-dried and finally returned to their home cage.

2.5.1.3. Rotarod motor co-ordination test. Since the elevated plus maze test was affected by motor defect, the motor co-ordination test was also conducted in order to rule out the motor defect. Motor co-ordination was assessed within 300 s (s) using rotarod test. A rotarod apparatus was used to evaluate motor co-ordination according to the method of Dunham and Miya (1957). Briefly, the animals were placed on a rotating cylinder, 10 cm in diameter covered with texture rubber, suspended 15 cm above an automated stop/start platform. Each rat was subjected to an initial habituation trial at the speed of four rotations per minute (rpm), followed by three test trials at accelerating speed, for three consecutive days. Each trial started at 4 rpm and the speed was accelerated continuously until the animal fell down or up to a maximum of 40 rpm in 300 s. The latency for the animals to fall down onto the platform and the corresponding speed were recorded. When the duration of riding exceeded 300 s, the rat was removed from the rod.

2.5.1.4. Stereotyped behavior. The test was performed in group of eight rat each. The first group received saline as vehicle while the animals in the second to fourth groups received ethanolic extract of *C. fenestratum* at doses of 5, 10 and 20 mg/kg BW for 14 days. Forty five minutes after the last dose of treatment, all animals were observed stereotyped behavior including grooming, rearing and licking for 5 min.

2.5.1.5. Walking pattern and ataxia. After 14 days of treatment, rats were placed in the center of a horizontal 1 m length wooden rod. Ataxia and gait analysis were determined within 90 min after treatment via the ability...
of the rats to walk and balance and the appearance of wide based gait. Rats falling over within 30 steps were regarded as ataxia. The ataxic scores were regarded as following: normal (+4), mild abnormal with ataxia and wide based gait (+3), moderate abnormal with ataxia and unstable gait (+2) and severe abnormal with truncal ataxia and inability to walk (+1).

2.5.2. Acute toxicity test
The alcoholic extracts were administered orally at doses of 5, 10 and 20 mg/kg BW (n = 8). The percentage mortality was noted within 24 h.

2.5.3. Determination of the neurotoxicity
The animals were divided into four groups as mentioned above. The animals were treated with the C. fenestratum extract for 14 days and then they were determined the neurotoxicity using the density of survival neurons in various areas and brain weight as indices. The neurotoxicity was performed after 14 days of treatment in order to mimic the situation of repeated doses toxicity in human according to the guideline for repeated dose toxicity testing (Directive of 75/318/EEC as amended from International Conference on Harmonization of Technical Requirement for Registration of Pharmaceuticals for Human Use).

2.5.3.1. Tissue preparation. Following anesthesia with sodium pentobarbital (60 mg/kg BW), fixation of the brain was carried out by transcardiac perfusion with fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.3. The brains were removed after perfusion and stored over night in a fixative solution that used for perfusion. Then, they were infiltrated with 30% sucrose solution for approximately 4 °C. The specimens were frozen rapidly and 30 μm thick sections were cut on cryostat. They were rinsed in the phosphate buffer and picked up on slides coated with 0.01% of aqueous solution of a high molecular weight poly L-lysine. The alcoholic extracts were administered orally at doses of 5, 10 and 20 mg/kg BW for 14 days and then they were determined the neurotoxicity using the density of survival neurons in various areas and brain weight as indices. The neurotoxicity was performed after 14 days of treatment in order to mimic the situation of repeated doses toxicity in human according to the guideline for repeated dose toxicity testing (Directive of 75/318/EEC as amended from International Conference on Harmonization of Technical Requirement for Registration of Pharmaceuticals for Human Use).

2.5.3.2. Nissl staining. Duplicate coronal sections of the brains were stained with 0.75% cresyl violet, dehydrated through graded alcohols (70, 95, 100% 2x), placed in xylene and coverslipped using DPX mountant.

2.5.3.3. Morphological analysis. Five coronal sections from each rat in each group were studied quantitatively. Neuronal counts were performed by eye using a 40x objective with final field 255 μm² and bregma co-ordination according to the following stereotaxic co-ordinates: (a) frontal cortex: AP 0.2 mm, lateral ± 1–4 mm, depth 1–3 mm; (b) parietal cortex: AP 0.2 mm, lateral ± 5–7 mm, depth 1–4 mm; (c) occipital cortex: AP -4.8 mm, lateral ± 3–5 mm, depth 1–3 mm; (d) temporal cortex: AP -4.8 mm, lateral ± 6–7 mm, depth 3–6 mm; (e) striatum: AP 0.2 mm, lateral ± 1.2–5 mm, depth 3.6–7 mm; (f) hippocampus: AP -4.8 mm, lateral ± 2.4–6 mm, depth 3–8 mm. The observer was blinded to the treatment at the time of analysis. Viable stained neurons were identified on the basis of a stained soma with at least two visible processes. Counts were made in five adjacent fields and the mean number extrapolated to give total number of neurons per 255 μm². All data are represented as number of neurons per 255 μm².

2.6. Statistical analysis
Data are presented as mean ± standard error of mean (S.E.M.). One-way analysis of variance (ANOVA), followed by Newman–Keuls post hoc test. A probability level less than 0.05 was accepted as significance.

3. Results

3.1. Acute toxicity
The rats treated with the plant extract at the dosage range used in this study exhibited some stereotyped behavior. They were alert, with normal grooming and rearing behavior but showed significant increase in licking behavior. There were no signs of passivity, vocalization, motor and secretory activities. The rats also showed no sign of depression. Gait of the animals were normal. Therefore, the extracts were found to produce no acute toxicity except increased licking behavior.

3.2. Body weight and neurotoxicity in various brain areas
The present study demonstrated that oral administration of ethanolic extract of the stem of C. fenestratum at dosage ranges used in this study (5, 10, and 20 mg/kg BW) once daily for 14 days significantly increased body weight of rats while decreased the density of neurons in all areas of cerebral cortex as shown in Figs. 1 and 2. Our results showed no specific area susceptibility in cerebral cortex. Fig. 3 also showed that the plant extract significantly decreased the density of survival neurons in the striatum after the administration of the plant extract for 14 days.

Various areas of hippocampus including CA1, CA2, CA3 and the dentate gyrus were also determined. The significant reduction in density of neurons in all areas just mentioned were first observed at dose of 5 mg/kg BW (Fig. 4). The increasing doses did not produce significant reduction in density of survival neurons in all areas assessed in this study. Among the various areas mentioned above, the dentate gyrus was the most vulnerable area to the plant extract.

3.3. Neurobehavioral study
The standard drug, diazepam increased both the number of open arm entries and time spent in the open arm. However, the significant difference was found only in time spent in open arm. The plant extract used in this study did not show any anxiolytic activity (Fig. 5A and B).
Fig. 6 shows that fluoxetine produced a significant decrease in immobility time in the forced swimming test which confirmed the sensitivity of the test. The plant extract failed to modify the immobility time in this test, and the plant extract administration at dosage used in this study also did not show significant changes in endurance time, and ataxic score as shown in Figs. 7 and 8.

The psychostimulant effect particularly the effect on stereotype behaviors was also observed as shown in Fig. 9. The only significant change was observed in licking behavior while this extract failed to produce significant changes in both grooming and rearing.
4. Discussion

This study represents the first step towards the understanding of the effects of crude extract obtained from stem of *C. fenestratum* on the central nervous system. To date *C. fenestratum* is commonly eaten. However, the results of this study showed that although lack of severe acute toxicity, the repetitive administration even at low dose concentration (5 mg/kg BW) of the plant extract induced neurotoxicity in cerebral cortex, striatum and hippocampus. The neurotoxicity of *C. fenestratum* extract in this study did not show a dose-dependent response manner. This could be partly due to the difference in concentration of various constituents in the crude extract. It was possibly that each chemical constituents of medicinal plants exhibited the biological activity influencing on the survival of neurons in different aspects. In addition, it was also possible that the doses used in this study has already produced saturation in changes already.

Previous studies have shown that stem of *C. fenestratum* contained berberine and its derivative as main components (Tran et al., 2003). These substances were reported to exert cytotoxic effect via the disturbance of enzymes topoisomerase I and II (Sanders et al., 1998). It also complexes with DNA and induces apoptosis (Kuo et al., 1995). Previous studies have reported that flavonoids existing in the plants could exert both anti-oxidant and prooxidant activities depending on the doses (Galati et al., 2002). In addition, the brain has also been reported to be particularly sensitive to oxidative damage due to the high level of lipid content and metabolic rate (Evans, 1993). Therefore, it possibly that the neurotoxicity of an ethanolic extract of *C. fenestratum* may due to the toxic alkaloids, berberine and its derivatives or the flavonoids existing in the extract.

Although the precise mechanism underlying the differential vulnerability of various areas in hippocampus to the extract is beyond our study, we postulated the possibly
underlying mechanism using previous studies data that the specific selectivity area in hippocampus observed in dentate gyrus may relate to the differential distribution of factors contribute important role for the survival of cells such as trophic factors (Mufson et al., 1994), scavenging enzymes (Trépanier et al., 1996) and calcium binding proteins (Goodman et al., 1993; Hof et al., 1996).

Although the plant extract induced marked loss of neuronal density in various brain areas, it failed to induced various changes in behaviors except licking. Our previous unpublished work of our group also demonstrated that the acute effect of plant extract did not show any significant changes in all neurobehaviors assessed in this study. However, our study also had some limitations because we did not measure all behaviors at various periods before the 14th day. Therefore, we still could not cut off the possibility of neuronal adaptation. Previous studies have demonstrated that the key factors which plays crucial role in animal behavior are the interactions between neurotransmitters and receptors, therefore, the lack of significant changes in various behaviors observed in this study may due to the neuroadaptation of various systems to the neuronal activity due to neuronal loss. The repetitive doses of *C. fenestratum* extract may possibly induce the change in density of various receptors and affinity of receptor to neurotransmitter such as in case of ethanol exposure which can induce multiple cellular adaptation that likely contribute to seizure and neuronal toxicity during withdrawal (Mulholland et al., 2003).

It is believed that most behaviors require the integrated activity of many components of the nervous system. However, in this experiment the density of neuronal density was measured in only some areas therefore it may be possible that the areas that we measured lack of positive correlation with of the neurobehaviors.

In addition, it was also reported that changes in neurobehaviors may also due to the learning experiences of animals. Experience has been recognized as major stimulant of brain plasticity in animal species. Previous studies have shown that experience produced multiple, dissociate changes in the brain including increase in synapse formation, increase in glial activity (Kolb and Wishaw, 1998). Therefore, this supported that the changes in neuronal density was not necessary to occur with the alteration in neurobehaviors.

The plant extract also increased stereotyped behavior especially licking. It was known that these behaviors were under the influence of various factors including stressors (Spruyt et al., 1992; Van Erp et al., 1994), and calcium (Satinder, 1968). In addition, stereotypy was also reported to relate to dopaminergic system especially in corpus striatum (Srimon, 1968). Thus, the increase in these parameters in our study may also occur as results of the factors mentioned above. However, the influence of stress was completely resolved using the elevated plus maze which is a well established anxiety model and forced swimming test. The results from our present study showed that the number of entries into opened arms and time spent in the opened arms were not modified after the administration of extract.

Taken together, the alcoholic extract of *C. fenestratum*, medicinal plants reputed for health promoting and various therapeutics effects in the Indochina region, induced neurotoxicity in cerebral cortex, striatum and hippocampus and also increased stereotype behaviors in rats. However, the underlying mechanisms are still required further investigation. In conclusion, our results are surprisingly alleging about the neurotoxicity of reputed and long-term used medicinal plant, thus, serious concerns about long-term used even as drugs or supplementary food should be reconsidered. The application of the plant products for various medical purposes in human should be very careful in order to avoid its toxicity.

**Conflict of interest statement**

There are none.

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