นิพนธ์ต้นฉบับ • Original Article

พิษต่อระบบประสาทของสารสกัดหัวใต้ดินของบอระเพ็ดพุงช้างในหนูแรทเพศผู้

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Neurotoxic Effect of *Stephania venosa* Tubers Extract in Male Rats

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หลักการและวัตถุประสงค์: บอระเพ็ดพุงช้าง โดยเฉพาะหัวใต้ดินของมัน โบราณอ้างว่ามีฤทธิ์ในการรักษามะเร็งและอัลไซเมอร์ อย่างไรก็ตามยังไม่มีรายงานถึงพิษต่อระบบประสาทของสมุนไพรนี้เมื่อบริโภคเป็นเวลานาน การศึกษานี้จึงมีวัตถุประสงค์เพื่อศึกษาพิษต่อระบบประสาทของสารสกัดหัวใต้ดินของบอระเพ็ดพุงช้างในหนูแรทเพศผู้

วิธีการศึกษา: การศึกษานี้ใช้สารสกัดของหัวใต้ดินของบอระเพ็ดพุงช้างด้วยแอลกอฮอล์ โดยป้อนตัวทำละลายหรือสารสกัดที่ขนาด 5 10 และ 20 มิลลิกรัมต่อน้ำหนักตัว (หนูแรท หนัก 180-220 กรัม) วันละ 1 ครั้ง เป็นเวลา 4 สัปดาห์ จากนั้นหนูแรทจะถูกนำสมองส่วนซีรีบรอลคอร์เท็กซ์และฮิปโพแคมปัส มาศึกษาโดยย้อมด้วยเครซิลไวโอเลท

ผลการศึกษา: ผลการศึกษาพบว่าสารสกัดบอระเพ็ดพุงช้างทุกขนาด สามารถลดจำนวนเซลล์ประสาทในสมองส่วนซีรีบรอล คอร์เท็กซ์ (ฟรอนทอล พาไรทอล เทมโพรอล และออกซิพิทอล คอร์เท็กซ์) และฮิปโพแคมปัส (CA1 CA2 CA3 และเดนเทท ไจรัส) อย่างมีนัยสำคัญทางสถิติ

สรุป: การศึกษาครั้งนี้แสดงให้ทราบว่าการบริโภคสารสกัดของหัวใต้ดินของบอระเพ็ดพุงช้างเป็นระยะเวลานานก่อให้เกิดพิษต่อสมองส่วนซีรีบรอลคอร์เท็กซ์และฮิปโพแคมปัสในหนูแรท

Background and Objective: *Stephania venosa* (SV), especially its tuber, is traditionally claimed to be effective treatment of cancer and acted as agent for treatment of Alzheimer's disease. However, the neurotoxicity of this plant in prolonged administration has never been documented. Therefore, this study was aimed to investigate the neurotoxic effects of SV tuber extract in male rats.

Methods: The ethanol extract of tuber SV were used for the experiment. The male rats were orally administered with vehicle or the SV extracts at various doses ranging from 5, 10 and 20 mg kg^{-1} once daily, for 4 weeks. All rats were evaluated the neuronal density of both cerebral cortex and hippocampus by cresyl violet staining.

Results: The results show that all dosages of SV extracts significantly decreased the neuronal density of cerebral cortex (frontal, parietal, temporal, and occipital cortex) and hippocampus (CA1, CA2, CA3, and dentate gyrus) compared to those controls.

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คำสำคัญ: Neurotoxicity, *Stephania venosa*, Cerebral cortex, Hippocampus

Conclusion: The resent study demonstrated that the prolonged administration of alcoholic extract of SV tubers is neurotoxic to cerebral cortex and hippocampus of male rats.

Keywords: Neurotoxicity, *Stephania venosa*, Cerebral cortex, Hippocampus

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Introduction

Since the dawn of history, plants have been most explored and exploited for their bioactive medicinal components, which have always served as “lead compounds” or templates for the rational development of drugs with more specific efficacies and less side effects for human use[1]. Many herbal remedies are frequently used to treat a variety of ailments and symptoms with little or no information about their efficacy or toxicity.

Since a large amount of evidence has accumulated over the years that have implicated oxidative stress as being intimately involved in the neurodegenerative diseases[2], there have been a great number of studies shows that herbal medicine and dietary enrichment with nutritional antioxidants could improve neuronal damage and cognitive function[3-5].

Stephania venosa Spreng (*S. Venosa*; SV) belongs to the Menispermaceae family and is widely distributed in Southeast Asia including Thailand. Accumulating lines of evidence reported that the genus *Stephania* could be a potential source of biologically active compounds which might be used as lead molecules for development of novel drugs[6]. Tubers of this plant have been used in Thai traditional medicine for nerve tonic, aphrodisiac[7], appetizer and for treatment of asthma, hyperglycemia[8] and anti-malarial activities[9]. In addition, the scientific literature strongly supports its in vitro antiproliferative effects on cancer cell lines and has suggested developing this plant as a potential anticancer drug[10]. Moreover, some of the isolated alkaloids were reported to possesses antioxidant activity[11]. In this respect, SV extract could be an attractive candidate as a novel strategy to protect against the neurodegenerative diseases. However, the in vivo neurotoxicity of this plant has never been tested. Therefore, it might be important to examine the impact of this plant on the densities of various neurons in both cerebral cortex and hippocampus in rats.

Materials and Methods

Plant material

The tubers of SV were collected from Ratchaburi Province, Thailand. The herbarium was authenticated by Professor Dr. Pranom Chandranothai, Department of Biology, Faculty of Science, Khon Kaen University, Thailand. A voucher specimen from this plant was deposited at Center of Research and Development of Herbal Products under the number HHP-2-461.

Extraction

The fresh tubers of SV were harvested, chopped into small pieces and dried under the sun light for two days and dried at 50°C until the weight was stable, then grounded into powder. The fine powder of 500 gm of SV was reflux with 50% ethanol for 30 min and filtered with gauze. The filtrate was then centrifuged at 800 g for 10 minutes. The extract procedures were repeated twice. The ethanol of the supernatant fraction was evaporated with lyophilizer and resulted in the final product brown color, kept in dried and dark bottle until used. The percent yield of the final product was 14.29%. The alcoholic extract of SV was prepared as suspending agent with carboxymethyl cellulose to facilitate 0administration via the oral route (gavage).

Animals

Healthy male Wistar rats weighing 180-220 gm and aging eight weeks were obtained from National Laboratory Animal Center, Salaya, Nakorn Pathom. They were housed in group of 5 per cage in standard metal cages at 22 ± 2°C on 12:12 h light-dark cycle. All animals were given access to food and water ad libitum. Experiments were performed to minimize animal suffering in accordance with the internationally accepted principles for laboratory use and care of European Community (EEC directive of 1986; 86/609/EEC) and approved by the Ethical Committee of the Khon Kaen University.

Experimental protocol

All rats were randomly assigned to 5 groups (n=10 in each group). In this study, the doses of the test substances were selected based on our pilot study and earlier reports. Group I: Naive intact control treated rats. Group II: vehicle treated group. Group III-V: SV treated group. The animals were treated with different doses of the alcoholic extract of SV (5, 10 and 20 mg kg^{-1}) via oral route for 4 weeks once daily throughout the experimental period.

Histological procedure

Following anesthesia with sodium pentobarbital (60 mg kg^{-1}), the rats were transcardially perfused with fixative containing 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) in 0.1M phosphate buffer pH 7.3. The brains were removed and stored overnight in the same fixative. They were infiltrated with 30% sucrose solution and kept at 4°C. The specimens were frozen rapidly and 30 μM thick sections were cut on a cryostat (Thermo Scientific Microm HM 525, Germany). The sections were rinsed in phosphate buffer and picked up on slides coated with 0.01% of poly L-lysine.

Cresyl violet staining

Coronal sections of the brains were stained with 0.75% cresyl violet (Sigma-Aldrich, St. Louis, MO, USA), dehydrated through graded alcohols (70, 95, 100% 2x) and xylene and cover-slipped using DPX mountant.

Morphological analysis and neuron counts

Five coronal sections of each rat in each group were studied quantitatively. Neuronal counts in both cerebral cortex and hippocampus were performed by eye using a 40x objective with final field 225 μm2 according to the following stereotaxic coordinates: (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) hippocampus: AP 4.8 mm, lateral ± 2.4-6 mm, depth 3-8 mm. The observer was blind 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 225 μm^2. All data are represented as number of neurons per 225 μm^2

Statistical analysis

Data were presented as mean ± Standard Error of Mean (SEM). One-way Analysis Of Variance (ANOVA), followed by Duncan's test. A probability level less than 0.05 were accepted as significance.

Results

Effect of SV extract on the neuronal density

Representative photomicrographs of the CA1 region in the hippocampal formation are shown in Fig.1A. Marked cell losses in these subfields of the hippocampal formation were observed in the SV treated group.

As shown in Fig.1B and 2, oral administration of vehicle produced no significant changes in the neuronal density in both various areas of hippocampus and cerebral cortex.

Interestingly, SV at all dosages range used in this study significantly decreased the neuronal density in various subregions of the brain as mention previously (p-value <0.05 all; compared to that of control and vehicle treated groups). Unfortunately, our study failed to show the dose-dependent manner in this parameter.

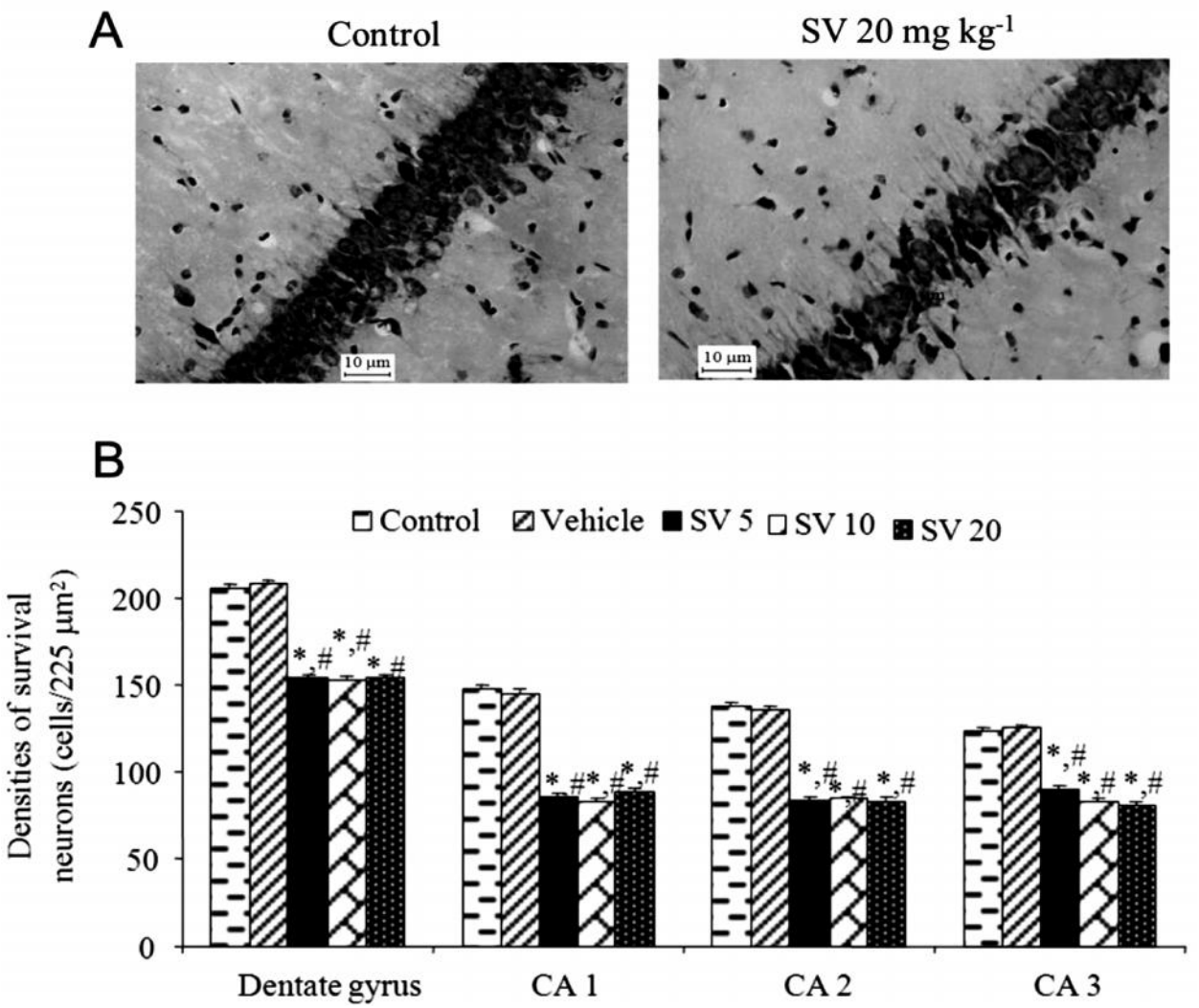

Figure 1 Photomicrographs of Nissl bodies in the CA1 region in the hippocampus at 20X magnification (A). Effect of SV extract (5, 10 and 20 mg kg^{-1}) on neuronal density in various subregions of hippocampus (B). (mean ± S.E.M., *n* = 10) *p<0.05 as compared to naive control group, # p<0.05 as compared to vehicle treated group

Discussion

A great deal of effort has been directed toward searching for the herbal medicines as alternative for drugs or strategies to protect against the neurodegenerative disease. Although *Stephania Venosa* (SV) is extensively used in Thai herbal medicine, it lacks scientific grounds for its neurosafety and to the best of our ability this is the first study to report its possible effects on the neuronal density. Unfortunately, our present study demonstrated that 4 weeks of treatment with alcoholic extract of SV induced neurotoxicity in all areas of cerebral cortex and hippocampus.

It had been reported that the alcoholic extract of SV comprised of various types of alkaloids and flavonoids[12,13]. Previous studies have demonstrated that flavonoids could exert both antioxidant and pro-oxidant activities. The pro-oxidant activity of the phenolic compounds usually occurs in system containing redox-active metal[14]. Thus, this activity of phenolic compounds seems to depend on the metal-reducing properties[15]. It has been known that the imbalance action between pro-oxidant and antioxidant will result in neuronal damage.

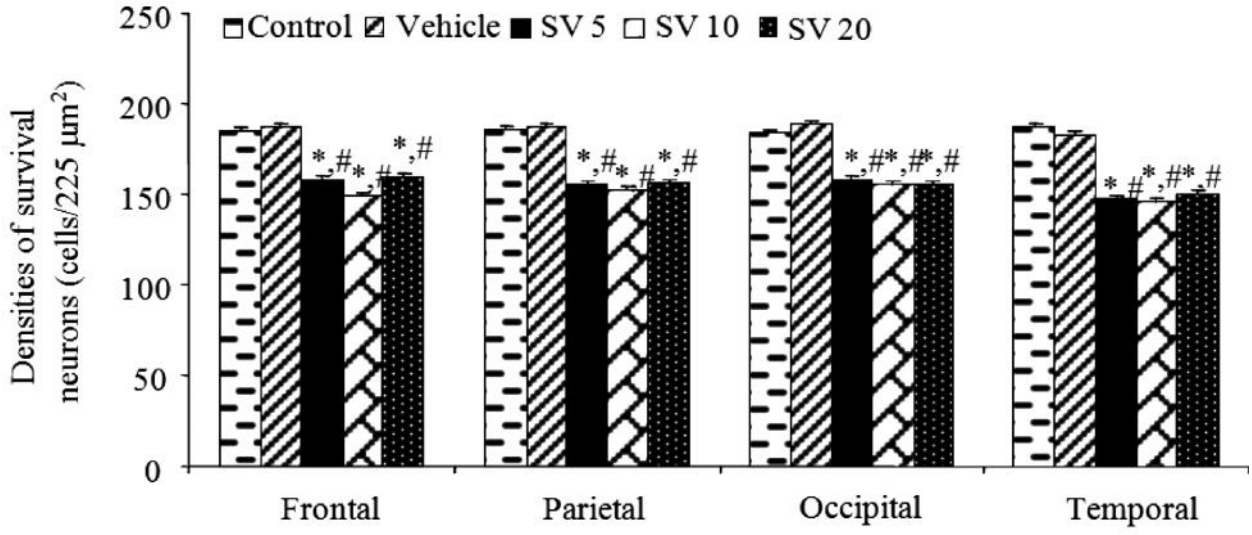

Figure 2 Effect of SV extract (5, 10 and 20 mg kg$^{?1}$) on neuronal density in various subregions of cerebral cortex. (mean ± S.E.M., n = 10) *p<0.05 as compared to naive control group, # p<0.05 as compared to vehicle treated group

In addition, flavonoids may also interact with mitochondria; interfere with pathways of intermediary metabolism leading to the cytotoxicity effect[16]. This effect of SV extract is in agreement with a similar study by Keawpradub and co-worker[17]. On the other hand, these results did not correlate with the activities of the extract previously reported[7]. The disagreement may be due to the different in conditions of study. The previous work had conducted the experiment in vitro while this study determined the effects of SV extract in vivo.

The elevation in intracellular calcium was also postulated to induce neuronal death in the neurodegeneration disorders[18], aging[19] and neurotoxicity of chemical substances[20]. Previous studies showed that the chief constituents of the SV extract were 3 different zones of steroids and terpenes, 8 different zones of alkaloids, 6 different zones of flavonoids and 2 different zones of phenol carboxylic acids[7]. Since this plant extract also contained high phenolic compounds which have been reported to increase the intracellular calcium ion[21]. Thus, we still could not exclude the phenolic compounds in SV extract may possibly by increasing intracellular calcium concentration which in turn induced neurotoxicity in rat's brain. However, this study did not investigate about the possible active ingredients and mechanisms underlying the SV extract induced neurotoxicity; this is planned in future studies.

All results of survival neuron tests in present study failed to demonstrate the neurotoxic effect of SV in the dose-dependent manner (Fig 1-2). These facts still remain unexplained. However, the degrees of Nissl body stained neurons of SV5 and SV10 groups are not different from SV20 group (data not shown) which related with our quantitative results. It is possible that efficacy of neurotoxic molecules in SV crude extract has maximum threshold at 5 mg/kg BW already. In addition, it is possible that our SV extract containing crude substances may have limitation of only substance that can affect nervous tissues. It could be also explained that various factors except neurotoxic factors in SV crude extract may influence on neurotoxic substances. As a result, neurotoxic effect of SV extract was possibly interfered by non-neurotoxic factors. It is not only this study showing failed dose-dependent manner but also many previous studies have been demonstrated[22,23]. Moreover, it was also possible that the relationship between the neurotoxicity in various neurons and SV concentration is not simple. It might be mediated other mediators or other factors, as discussed above, could

modify this phenomenon. However, further researches are still essential to understand the precise mechanism of SV extract induced the neurodegeneration *in vivo* method.

Conclusion

The results of our study provided the evidence to support the neurotoxic effects of SV extract consumption. The results of recent studies should reinforce the need to proceed with caution in using herbal supplements. Whereas more studies at all levels are needed, to characterize both the potential health benefits of individual flavonoids and their potential harmful attributes, it is very possible that the sum of the parts is more important in providing a health benefit to humans than one plant constituent.

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