บทความวิจัย (Research Article)

ฤทธิ์ของสารสกัดกระชายต่อเซลล์ประสาทรับความรู้สึกหลังจากการบาดเจ็บของเส้นประสาทไซแอติกในหนูแรท

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Effect of *Boesenbergia rotunda* L. extract on sensory neuron after sciatic nerve lesion in rat

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บทคัดย่อ

งานวิจัยนี้ต้องการศึกษาฤทธิ์ของสารสกัดจากเหง้ากระชายต่อการเติบโตของเส้นใยประสาทของเซลล์ประสาท และจำนวนของเซลล์ประสาทในปมประสาทหลังจากที่หนูถูกชักนำให้ได้รับบาดเจ็บของเส้นประสาทไซแอติก การเจริญเติบโตของเส้นใยประสาทของเซลล์ประสาทถูกตรวจสอบด้วยวิธีอิมมูโนพยาธิวิทยา โดยอาศัยการย้อมด้วย β-tubulin-III ซึ่งมีความจำเพาะต่อเส้นใยประสาท ผลการศึกษาพบว่าเซลล์ประสาทที่ได้รับสารสกัดกระชายเข้มข้น 1 ไมโครลิตร/มล. มีแนวโน้มส่งเสริมการเจริญเติบโตของเส้นใยประสาทเมื่อเปรียบเทียบกับกลุ่มที่ไม่ได้รับสารสกัด การศึกษาผลของสารสกัดกระชายต่อจำนวนเซลล์ประสาทในปมประสาท หนูได้รับสารสกัดกระชายขนาด 200 มก./กก. เป็นเวลา 28 วัน หลังจากการบาดเจ็บของเส้นประสาทไซแอติก ผลการศึกษาพบว่าจำนวนเซลล์ประสาทรับความรู้สึกด้านที่ถูกชักนำให้ได้รับบาดเจ็บของเส้นประสาทในหนูกลุ่มที่ได้รับสารสกัดกระชายมีจำนวนสูงกว่าอย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับกลุ่มที่ไม่ได้รับสารสกัด อย่างไรก็ตามไม่พบการเปลี่ยนแปลงของจำนวนเซลล์ประสาทรับความรู้สึกในด้านที่ไม่ได้รับการบาดเจ็บของเส้นประสาท นอกจากนี้ยังพบว่าการทำงานของระบบประสาทยนต์ของหนูกลุ่มที่ได้รับสารสกัดกระชายมีแนวโน้มที่ดีขึ้นเมื่อเปรียบเทียบกับกลุ่มที่ไม่ได้รับสารสกัด

คำสำคัญ: กระชาย, การบาดเจ็บของเส้นประสาทไซแอติก, ปมประสาท, การเจริญเติบโตของเส้นใยประสาท

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Abstract

The study was investigated the effect of *B. rotunda* rhizomes extract with respect to neurite outgrowth in vitro and number of sensory neurons after sciatic nerve injury in vivo. Neurite outgrowth from neuron cultures of dorsal root ganglia was evaluated by examination of morphological changes using immunohistochemistry for β-tubulin-III. We observed a statistically nonsignificant trend toward promote neurite outgrowth of sensory neurons of *B. rotunda* treated group at concentration of 1 µl/ml. To study the effect of *B. rotunda* on morphological changes of dorsal root ganglia, adult male Wistar rats were treated with the *B. rotunda* extract for 28 days after sciatic nerve injury at the dose of 200 mg/kg body weight. Overall sensory neuron number in dorsal root ganglia was determined by counting in hematoxylin and eosin sections. The sensory neuron number of *B. rotunda* treated group was significantly higher than in control and vehicle groups at the injury side. However, there were no changes in sensory neuron number of contralateral side. Following sciatic nerve crush, motor coordination was observed on the rotarod treadmill (acceleratingmode). This was a statistically nonsignificant trend toward improved motor coordination.

Keywords: *Boesenbergia rotunda* L., sciatic nerve lesion, dorsal root ganglia, neurite outgrowth

Introduction

Peripheral nerve damage caused by nerve injuries can result in a loss of motor and sensory control of target organ. These functional impairments may be recovered by reinnervation of target organ is achieved by regenerating axons at the lesion site. [1] Therefore, improvement of axonal outgrowth is required for faster regeneration of axons into targets such as skin and muscles, which atrophy in the absence of reinnervation after peripheral nerve injury. Apoptotic mechanisms are related to neuronal death and have been observed after sciatic nerve injury. Macrophages are important players in the progression of neuronal death and promote inflammation through the release of pro-inflammatory cytokines and proteases cause producing of free radicals. [2] Reactive oxygen species (ROS) contribute to tissue damage and remodelling mediated by the inflammatory response after injury. ROS are found in the injured sciatic nerve and dorsal root ganglia and may result in delay nerve regeneration and promote cell death in dorsal root ganglia by retrogradely transported to the cell body. [3]

Plant-derived anti-oxidants have shown great potential for development of nerve injury therapeutics. *Boesenbergia rotunda* L. (Family: Zingiberaceae) or Krachai in Thai as known as finger root is a daily food ingredient and traditional medicinal plant in Southeast Asia and Indo-China. It has been shown to possess anti-inflammatory, antioxidant and antiulcer activities. [4] *B. rotunda* is rich in the active phytochemical substances such as flavonoids including alpinetin, boesenbergin, cardamonin, geraniol, krachaizin, panduratin, pinostrobin, pinocembrin, rotundaflavone, and silybin, [5-7] essential oils including nerol, camphor, cineole, fenchene, hemanthidine, and limonene [8] and olyphenols including caffeic acid, coumaric acid, chlorogenic acid, hesperidin, kaempferol, naringin, and quercetin. [9] Previous study showed that the antioxidant compound composed of β-carotene, α-tocopherol, B complex vitamins improved the process of nerve repair 30 days after its application to the area around nerve injuries. [10]

This study was set out to investigate the antioxidant effect of *B. rotunda* extract on axon outgrowth, in vitro and number of sensory neurons

in rat dorsal root ganglia after sciatic nerve injury, in vivo. The dorsal root ganglia as well as motor coordination functional recovery in response to nerve injury was assessed as an indicator for a possible effect of *B. rotunda* extract during peripheral nerve regeneration.

Material and Method

Plant materials and preparation

Rhizomes of *B. rotunda* were bought from local market in Muang District, Phayao Province from November 2016 - March 2017. The samples were cleaned and cut to small pieces then dried with hot air oven at 60°C for 48 hours. Dried samples (3 kg of *B. rotunda*) were ground and extracted with 95% v/v ethanol by maceration. The ethanol was removed from the extracts under vacuum condition to yield ethanol crude extracts.

Animals and treatment

The study was approved by the Animal Ethics Committee at University of Phayao, Thailand (approval No. 5801040008) on February 23rd, 2017. Young adult male Wistar rats aged about 8 weeks were obtained from Nomura Siam International, Thailand. Rats were divided into four experimental groups (n=5), randomly: a control group, a sham operation group, a control with sciatic nerve injury, and *B. rotunda* treated group with sciatic nerve injury. The cages were housed in a temperature and humidity controlled room with 12/12 h light/dark cycles and animals fed with standard chow and water ad libitum. Rats in *B. rotunda* treated group were received the *B. rotunda* treated extract at dose 200 mg/kg body weight (BW) for 7 days before sciatic nerve crush injury and continually treated for 28 days after injury.

Surgical procedure and behavioral experiment

Rats were anesthetized by intraperitoneal administration of 50 mg/kgBW of pentobarbital. Following surgical preparation in sham group, the left sciatic nerve was exposed through a gluteal muscle incision and after careful hemostasis the muscle and the skin were sutured with nylon suture. In the sciatic nerve crush lesion condition, rats were anesthetized and the left sciatic nerve was exposed through a gluteal muscle. Sciatic nerve was crushed proximal to the tibioperoneal bifurcation with artery forceps (straight 12cm/5") for 1 minute. After that, the muscle and the skin were sutured with nylon suture to close the wound.

The rats were behaviorally analyzed on day 3, 7, 14, 21 and 28 after sciatic nerve crushed lesion. For testing mainly motor function, the time was measured that the rat stays on an accelerating rod (rotarod) as a measure of balance, coordination, and motor-planning.

Primary neuron culture

Rat dorsal root ganglia (DRG) were dissected and collected in RPMI medium with antibiotic-antimycotic. Ganglia were treated with collagenase (5000 U/ml) for 60 min followed by 0.25% trypsin/EDTA for another 15 min. They were then transferred to RPMI medium containing 10% horse serum and 5% fetal bovine serum and dissociated by 5 to 10 passages through a fire-polished Pasteur pipette. DRG neurons were transferred into CO_2-Independent Medium (Gibco Invitrogen) and plated on 35 mm glass dishes coated with 0.1 mg/ml poly-d-lysine (overnight) and 0.2 mg/ml laminin (for 4 h). One hour after plating, DRG neurons were treated with *B. rotunda* extract at concentration of 1 µl/ml. Cultures were maintained in CO_2-Independent Medium with and antibiotic-antimycotic at 37°C for 24 hours.

Immunohistochemistry and histology

After 24 h in culture, neurons were fixed with 4% paraformaldehyde (PFA) for 20 min, permeabilized with 0.01% Triton X-100 (Sigma Aldrich) in Phosphate Buffered Saline (PBS) for 5 min and blocked with blocking buffer (10% goat serum in PBS) for 30 min. Cells were incubated with primary antibodies against neuronspecific β -III Tubulin (ThermoFisher, 1:1000) at 4°C overnight. After that, cells were washed with PBS and incubated with secondary antibodies (Goat anti-Mouse IgG (H+L) Secondary Antibody, Horseradish peroxidase (HRP); ThermoFisher, 1:200). 3, 3′-Diaminobenzidine (DAB); (Sigma-Aldrich) staining was used to visualize the cell using light microscopy. DAB labeled neurons were analyzed using light microscopy. Thirty neurons from each culture were used to study the neurite outgrowth. The longest vector from the center of the cell body to one of the growth cones was measured as maximal distance.

For histology of DRG, the rats were scarified on day 28 after sciatic nerve injury. The lumbar DRG (L3-L5) were collected and fixed in 4% PFA overnight. Following three washes in PBS. DRG then used for paraffin method [11]. Ten μm thickness sections were stained with hematoxylin and eosin (H&E). Sensory neurons of the lumbar DRG were counted in 5,000 μ^2 area of DRG.

Statistical analysis

Statistical analysis was performed using ANOVA followed by Tukey's multiple comparisons test. All data points are presented as mean values±SEM applying Prism software (*=P<0.05, **=P<0.01, ***5=<0.001).

Results

Effect of *B. rotunda* extract on neurite outgrowth

Dissociated neurons were plated on a growth promoting substrate (poly-D-lysine/laminin) and labeled with β -III Tubulin antibody after 24 h in culture. The length of the longest axon per neuron as a measure for the neuronal capacity to induce axon elongation. Obvious, *B. rotunda* extract at concentration of 1 μl/ml showed a statistically nonsignificant trend toward improved axon outgrowth patterns of sensory neurons **[Figure 1].** The maximal distance remained unchanged by *B. rotunda* extract treatments.

Measurement of DRG neurons

Histological study of DRG was applied by H&E staining. After sciatic nerve injury, there were morphological changes in ipsilateral DRG. In contrast, contralateral DRG showed no changed in number of neurons in all experimental groups. However, *B. rotunda* extract at dose of 200 mg/kgBW revealed the effect to protect sensory neuron death after nerve injury in ipsilateral DRG. Number of neurons in ipsilateral DRG of *B. rotunda* treated group was significantly higher than control and vehicle groups (P<0.05) **[Figure 2].**

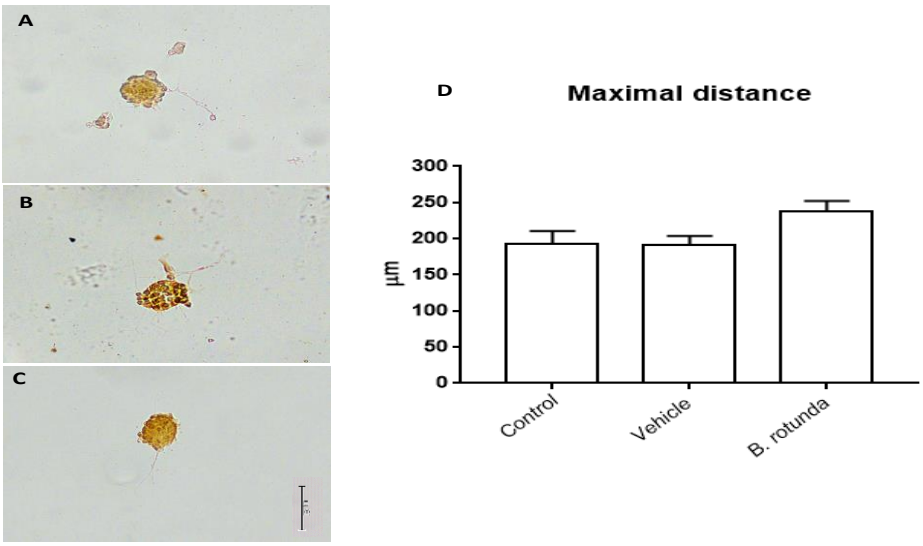

Figure 1 Representative examples of neuronal morphologies of young adult DRG neurons stained with β -III Tubulin antibody. DAB staining images are shown to document the neurite outgrowth of DRG neurons (A= DRG neuron of control, B= DRG neuron of vehicle, C= DRG neuron of *B. rotunda*). In cultures treated with *B. rotunda* extract at concentration of 1 µl/ml, the length of the longest axon (maximal distance) show a statistically nonsignificant trend toward enhanced neurite outgrowth (D). Bar=100 µm.

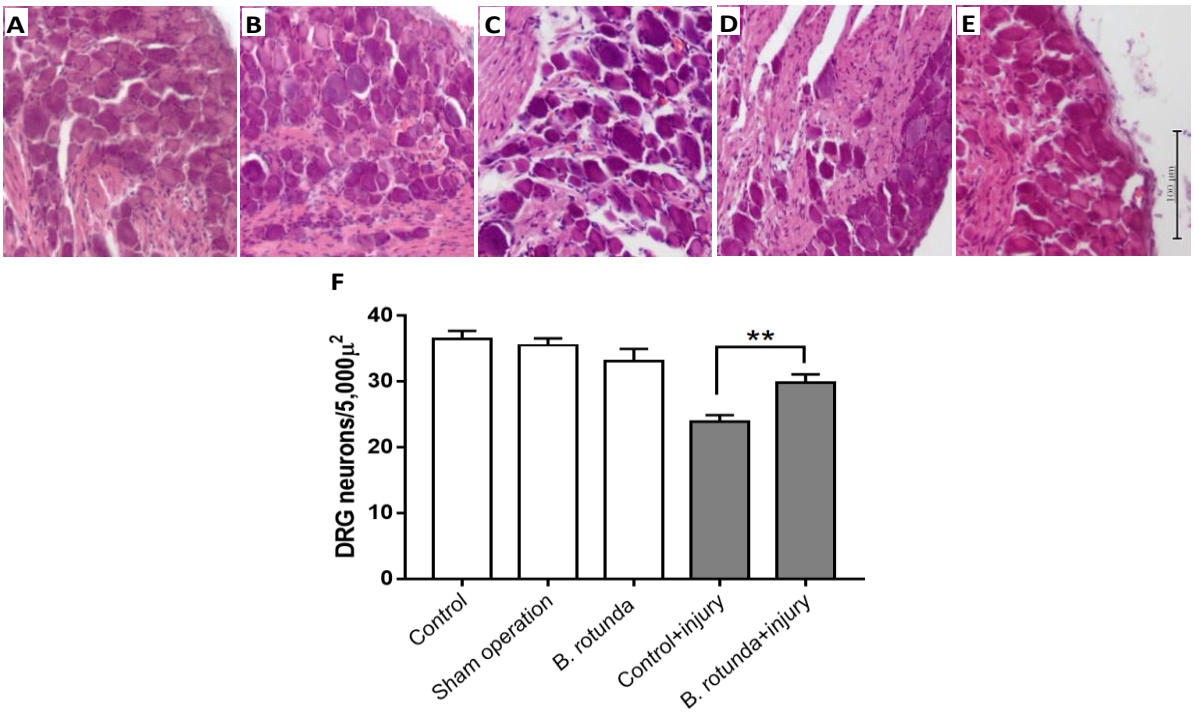

Figure 2 Morphological changes in DRG 28 days after sciatic nerve injury. In contralateral DRG (A= DRG of control, B= DRG of sham operation, C= DRG of *B. rotunda*) there were no changes in DRG structure. In contrast, ipsilateral DRG (injury side) (D= DRG of control, E= DRG of *B. rotunda*) show alternation in histology. Neuron number in *B. rotunda* treated group was significantly higher than control and vehicle groups (F) Bar=100 µm.

Effect of B. rotunda extract on motor coordination recovery

Motor coordination function was accessed by the rotarod treadmill test on day 3, 7, 14, 21 and 28 after operation. Functional recovery for motor coordination and balance did not reveal statistically significant differences between the experimental groups **[Figure 3].**

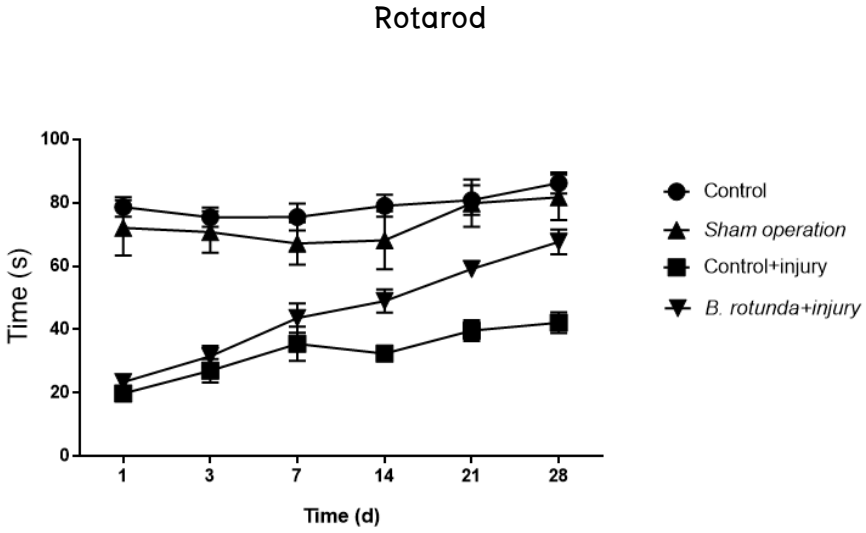

Figure 3 Functional recovery following sciatic nerve crush lesion in rats. Treadmill running (rotarod) reveals a statistically nonsignificant trend toward improved motor coordination in *B. rotunda* treated group during day 14 of training when compared to injured control and injured vehicle groups.

Discussion

Following sciatic nerve crush lesion, motor performance shows a trend to improve faster in rats treated with *B. rotunda* when compare to injured control and vehicle treated group. This observation may be attributed to faster axon outgrowth leads to regenerating myelinated axons reached to the muscle. [12] The results show the effects of *B. rotunda* on neurite outgrowth in DRG neuron culture. Oxidative stress (ROS) has been reported to induce neurite degeneration and inhibited neurite outgrowth. [13] *B. rotunda*, is a plant possessed anti-oxidant such as flavonoid and phenolic compounds. [14] Study has been reported that anti-oxidant has shown to increase the length of the neurite in hippocampal neuron cultures. [15]

The experiment was induced sciatic nerve injury to investigate the retrograde degeneration of DRG neurons. It has been shown that neuroinflammatory processes was occurred in the injured nerve and played a crucial role in neuronal cell death in peripheral nerve. [16] Oxidative stress such as reactive oxygen species (ROS) and nitric oxide (NO) had shown to induce cell death after tissue injury. The chronic increases in ROS levels may trigger cell death by interfering with normal cellular operations. [17] In addition, Macrophage activation in nerve injury leads to the production of cytokines, free radicals and inflammatory mediators which induce the apoptotic cell death of neurons observed in many neurodegenerative diseases. [18] In this study, *B. rotunda* can protect DRG neuron death after the lesion of sciatic nerve. The reduction of dead neurons by apoptosis, which may promote by antioxidants and anti-inflammatory effect of plant extraction. [19]

In conclusion, the study reveals that *B. rotunda* plays a role in stimulation of axon elongation in vitro and can reduce number of DRG neuron loss during sciatic nerve injury. The consumption of antioxidant and anti-inflammatory-rich foods, such as *B. rotunda* can limit neurodegeneration caused by oxidative stress and inflammatory.

Acknowledgement

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