

Lion's Mane, *Hericium erinaceus* and Tiger Milk, *Lignosus rhinocerotis* (Higher Basidiomycetes) Medicinal Mushrooms Stimulate Neurite Outgrowth in Dissociated Cells of Brain, Spinal Cord, and Retina: An *In Vitro* Study

Snehlata Samberkar,^{1,2,*} Sivasangkary Gandhi,^{1,3} Murali Naidu,^{1,2} Kah-Hui Wong,^{1,2} Jegadeesh Raman,^{1,3} & Vikineswary Sabaratnam^{1,3}

¹Mushroom Research Centre, Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia; ²Department of Anatomy, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia; ³Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia

* Address all correspondence to: Snehlata P. Samberkar, Department of Anatomy, Faculty of Medicine, University of Malaya, 50603, Kuala Lumpur, Malaysia; Tel: +60379673792; Fax: +603 79674724; E-mail: snehlata@um.edu.my

ABSTRACT: Neurodegenerative disease is defined as a deterioration of the nervous system in the intellectual and cognitive capabilities. Statistics show that more than 80–90 million individuals age 65 and above in 2050 may be affected by neurodegenerative conditions like Alzheimer's and Parkinson's disease. Studies have shown that out of 2000 different types of edible and/or medicinal mushrooms, only a few countable mushrooms have been selected until now for neurohealth activity. *Hericium erinaceus* is one of the well-established medicinal mushrooms for neuronal health. It has been documented for its regenerative capability in peripheral nerve. Another mushroom used as traditional medicine is *Lignosus rhinocerotis*, which has been used for various illnesses. It has been documented for its neurite outgrowth potential in PC12 cells. Based on the regenerative capabilities of both the mushrooms, priority was given to select them for our study. The aim of this study was to investigate the potential of *H. erinaceus* and *L. rhinocerotis* to stimulate neurite outgrowth in dissociated cells of brain, spinal cord, and retina from chick embryo when compared to brain derived neurotrophic factor (BDNF). Neurite outgrowth activity was confirmed by the immunofluorescence method in all tissue samples. Treatment with different concentrations of extracts resulted in neuronal differentiation and neuronal elongation. *H. erinaceus* extract at 50 µg/mL triggered neurite outgrowth at 20.47%, 22.47%, and 21.70% in brain, spinal cord, and retinal cells. *L. rhinocerotis* sclerotium extract at 50 µg/mL induced maximum neurite outgrowth of 20.77% and 24.73% in brain and spinal cord, whereas 20.77% of neurite outgrowth was observed in retinal cells at 25µg/mL, respectively.

KEY WORDS: medicinal mushroom, *Lignosus rhinocerotis*, *Hericium erinaceus*, neurite outgrowth activity, neurodegenerative disease

ABBREVIATIONS: BDNF, brain-derived neurotrophic factor; DAPI, 4',6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline.

I. INTRODUCTION

Aging is an inevitable process and the fraction of world's population over 65 years of age is expected to increase to 80–90 million by the end of 2050.¹ Out of many diseases that threaten aging humans, neurodegenerative diseases such as Alzheimer's disease, dementia, and Parkinson's disease² and

retinal diseases like diabetic retinopathy, glaucoma, and age-related macular degeneration can be very traumatic.³ Neurohealth is a major concern as one ages. Retrospective studies have proved that natural products like mushrooms are suitable candidates for neurohealth.⁴ Out of 14,000 species of familiar mushrooms,⁴ roughly 2000⁴ are acknowledged to be fit for human consumption. Researchers have reported that

many medicinal mushrooms promote neurotrophic properties such as neurite outgrowth stimulation,^{5,6} nerve regeneration,⁷ neuroprotection,⁸ and anti-oxidation.⁹ Mushrooms may have potential in the prevention or treatment of age-related neurodegenerative complaints.¹⁰

The mushrooms selected for this study were *Hericium erinaceus* (Bull.:Fr.)Pers. (Hericaceae, higher Basidiomycetes) and *Lignosus rhinocerotis* (Cooke) Rivarden (Polyporaceae, higher Basidiomycetes). *H. erinaceus*, a rare mushroom also known as lion's mane, monkey's head, and Yamabu shitake, is scattered throughout North America, Europe, and Asia.^{11,12} It has been used in Chinese and Japanese cuisine and as an herbal remedy to treat various human diseases including gastric ulcers for hundreds of years.¹² A health syrup called "Houtou" is prepared from dried fruit bodies. *H. erinaceus* tablets are used to treat ulcers, inflammation, and tumors of the alimentary canal.¹³ It is also evident that *H. erinaceus* has facilitated functional recovery subsequent to peripheral nerve injury.⁷ Based on this study, our focus is on regeneration of the central nervous system (CNS) using brain, spinal cord, and retinal explants and cells after dissociation, using chick embryo as a model.

L. rhinocerotis is also known as tiger's milk mushroom or "cendawan susu rimau" in the local language. This species is distributed only in the tropical rainforest in regions of South China, Thailand, Indonesia, Malaysia, Philippines, and Papua New Guinea. *L. rhinocerotis* has been used as a general tonic, antipyretic, and antipruritic; it has also been used to treat fever, cancer, food poisoning, swollen breasts, cough, and asthma and to assist in wound healing, among other uses.¹⁴ Its usage is limited in spite of its medicinal properties due to unavailability. Sclerotium of *L. rhinocerotis* documented enhancement of neurite outgrowth activity in PC12 cells.¹⁵ Based on their potential, in this study, both mushrooms with different concentrations (25–100 µg/mL) were treated with tissue samples and compared with negative and positive controls. Neurite extensions were confirmed by immunofluorescence staining.

II. MATERIALS AND METHODS

A. Preparation of Mushroom Aqueous Extracts

Mushrooms require the correct combination of humidity, temperature, substrate (growth medium), and inoculum (spawn) to grow. In Malaysia, *H. erinaceus* is cultivated on a substrate containing rubberwood sawdust, rice bran, and calcium carbonate at a ratio of 100:5:1. After 2 months of spawn run at 27±2 to 32±2°C at a mushroom farm (Ganofarm Ltd., Tanjung Sepat, Selangor, Malaysia), approximately 300 g of fresh fruit body per 800 g of substrate was harvested.¹⁶ Fresh fruiting bodies of *H. erinaceus* were purchased from the mushroom farm. Fresh fruit bodies were sliced, frozen, and freeze-dried. The freeze-dried fruit bodies were then blended in a Waring commercial blender and stored in airtight containers at 4°C prior to assay.¹⁶ *L. rhinocerotis*, a rare species, is found in the forests of Malaysia. In this study, the freeze-dried powder of sclerotia of cultivated *L. rhinocerotis*^{10,17} was purchased from Ligno Biotek Sdn Bhd (batch no. TM02). The freeze-dried powders of both mushrooms were then soaked separately in distilled water (1:20, w/v) and were agitated at 150 rpm for 24 h.¹⁵ The mixture was then double boiled in a water bath at 100°C for 30 min, cooled, and filtered by Whatman filter paper No. 4. The aqueous extract was freeze-dried and kept at –20°C prior to use.^{6,18}

B. Preparation of Explants Culture and Trypsinization

Fertilized chicken eggs were collected from Charoen Pokphand Jaya Farm (M) Sdn Bhd (Negeri Sembilan, Malaysia) and then were incubated at 39°C in a humidified incubator. The brain (day 4), spinal cord (day 6), and retina (day 9) were dissected on their respective days.¹⁹ Based on a modified method of Gibco Life Technologies (Selangor, Malaysia), the tissue samples (brain, spinal cord, and retina) were finely chopped, washed with phosphate-buffered saline (PBS) twice, and centrifuged at 3000 rpm for 3 min after adding trypsin. The supernatant

was discarded and the cell pellet was resuspended twice with 2–5 mL of prewarmed (37°C) complete media. The samples were centrifuged at 3000 rpm for 3–5 min. The supernatant was discarded and the cell pellet was added to fresh complete media and incubated at 37±2°C in a 5% CO₂ humidified incubator for 24 h.

C. Neurite Outgrowth Assay

Two-day-old cultured cells were seeded into 12-well plates at a cell density of 5×10⁴ cells per well. The mushroom aqueous extracts in Dulbecco's modified Eagle's medium (DMEM) at concentrations of 25, 50, 75, and 100 µg/mL (w/v), and brain-derived neurotrophic factor (BDNF) at 10 ng/mL (w/v) were tested for neurite outgrowth stimulation activity. Cells in complete DMEM without treatment served as the negative control. Plates were incubated at 37±2°C in a 5% CO₂ incubator for 2 days.¹⁵

D. Scoring of Neurites

Neurite extensions were scored under an inverted microscope (Nikon Eclipse TS100) with the aid of a handheld counter. A cell was scored positive for bearing neurites if it had at least one thin extension longer than the diameter of its cell body.²⁰ In a well, 10 fields with an average of randomly chosen 250–300 cells per well were examined and photographed using a Nikon DS-Fi1 camera and were processed with Nikon's NIS-Elements D imaging software.⁶

E. Neurofilament Staining

A neurofilament was used as an indicator for neurite outgrowth and immunofluorescence staining was used for confirming neuronal extension, which is an increase in axonal length. Based on the standard method,²¹ primary neuronal cells were seeded in 12-well plates and exposed to treatment for 2 days. The cells were fixed with 4% paraformaldehyde for 20 min at room temperature. After two washes with PBS, the cells were incubated with primary antibody, antineurofilament 200 antibody produced in rabbits (Sigma, St. Louis, MO, USA) (1:80

dilution in blocking buffer) for 1 h. The cells were washed and then incubated with secondary antibody, anti-rabbit IgG-fluorescein isothiocyanate (FITC) antibody produced in sheep (Sigma, St. Louis, MO, USA) (1:80 dilution in blocking buffer) for 1 h in the dark at room temperature. The cells were then washed thrice. The coverslips were then mounted with 4',6-diamidino-2-phenylindole (DAPI), which stained the nucleus. Images were observed and captured with a fluorescent microscope (Nikon Eclipse 80i microscope using FITC and DAPI filters).

F. Statistical Analysis

All experiments were carried out in three replicates. Results were expressed as the means ± SD. All data were subjected to analysis of variance using GraphPad Prism Statistical Software version 7 (GraphPad Software Inc., La Jolla, CA, USA). The differences among samples were evaluated using Duncan's multiple range test, where *p*<0.05 was considered significant.

III. RESULTS AND DISCUSSION

Neurons in the mature CNS are unable to regenerate injured axons and the neurons that remain uninjured are unable to form novel connections that might compensate for ones that have been lost.²² Subsequently, due to a break in the communication between healthy neurons, a cascade of events takes place that leads to neuronal degeneration and cell death. The factors responsible for failure of regeneration are several and include poor regenerative ability of CNS neurons, inhibitory properties of astrocytes,²³ and inhibitory molecules produced by oligodendrocytes and myelin.²⁴ Overpowering these issues will facilitate the nerve regeneration for restoration of function following damage, through accident, injury, or neurodegenerative disease.

Natural products have been traditionally accepted as remedies due to the popular belief that they present minor side effects.²⁵ In traditional Chinese medicine, mushrooms have always been prepared for medicinal use by hot water extraction. The number of mushrooms, however, studied for

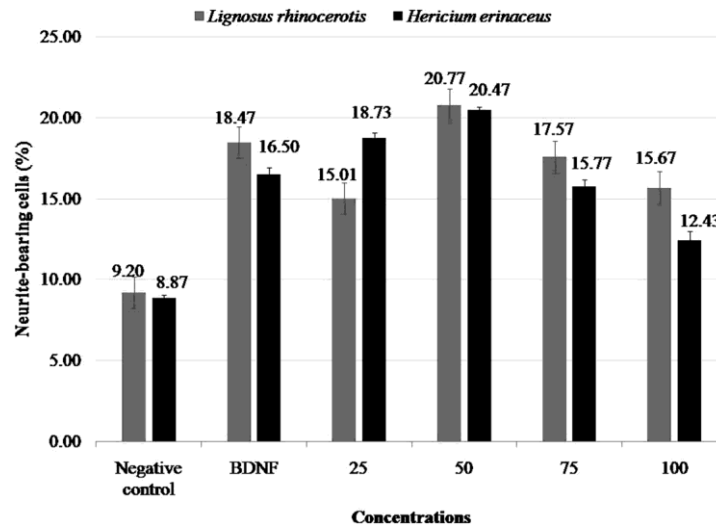


FIG. 1: Effect of varying concentrations of *H. erinaceus* and *Lignosus rhinocerotis* aqueous extracts on neurite outgrowth stimulation on chick embryo's brain cells.

neurohealth activity are few and *H. erinaceus*¹⁰ is one of them. Its chemical composition was documented by Kawagishi et al.⁵ Further study on aqueous extracts proved that polysaccharides could induce neuronal differentiation and promote neuronal survival.²⁶ Ongoing research in our laboratory shows that *H. erinaceus*,⁶ *L. rhinocerotis*,^{15,27} and *Pleurotus giganteus* (Berk.) Karunarathna & K.D. Hyde (morning glory mushroom, cow's stomach mushroom)²⁸ exhibit neurite outgrowth stimulatory effects in NG108-15 and PC12 cell lines. Inspired by this, researchers focused their goal on peripheral nerve regeneration following crush injury to the rat peroneal nerve by using aqueous extract of *H. erinaceus*. This study proved that daily administration of aqueous extract of *H. erinaceus* has beneficial effects on recovery of the injured rat peroneal nerve in early stages of regeneration.⁷

Another medicinal mushroom is *L. rhinocerotis*. Retrospective studies have shown that an aqueous extract of *L. rhinocerotis* sclerotium induced neurite outgrowths of 24.4% and 42.1% at 20 $\mu\text{g/mL}$ (w/v) of aqueous extract alone and a combination of 20 $\mu\text{g/mL}$ (w/v) aqueous extract and 30 ng/mL (w/v) of NGF, respectively, in rat pheochromocytoma cells (PC12 cells).¹⁵ Similarly, the present study showed neurite outgrowth of 20.77% and 24.73% at 50 $\mu\text{g/mL}$ in brain and spinal cord cells and 20.77% at 25 $\mu\text{g/mL}$

in retinal cells. Consistent with our previous study, the sclerotia of *L. rhinocerotis* reported neurite outgrowth in N2a.²⁹ It was recently shown that sclerotial extract performs better than mycelial extract.²⁹ Advanced study shows maximum neurite extension for *L. rhinocerotis* and curcumin at 21.1% at 20 $\mu\text{g/mL}$ and 29.47% at 10 $\mu\text{g/mL}$ on PC-12 cells.³⁰ Combining 20 $\mu\text{g/mL}$ of *L. rhinocerotis* with 1 $\mu\text{g/mL}$ curcumin gave 27.2% neurite extension in PC12 cells.³⁰ Taken as a whole, these medicinal mushrooms have shown neurological properties such as neuronal survival and neurite outgrowth activities including improvement in recovery and function in both *in vivo* and *in vitro* mammalian nervous systems.³¹

The aqueous extracts of *H. erinaceus* and *L. rhinocerotis* showed a gradual dose-dependent twofold increase in neurite outgrowth stimulation at 25 and 50 $\mu\text{g/mL}$ concentration compared to the negative control. Figure 1 shows the neurite outgrowth stimulation on brain cells after 48 h incubation decreased the extension in a dose-dependent manner observed at 75 and 100 $\mu\text{g/mL}$ concentrations. The maximal stimulated outgrowth on brain cells treated with aqueous extracts of *H. erinaceus* and *L. rhinocerotis* was 20.47% and 20.77%, respectively, at 50 $\mu\text{g/mL}$, comparable to that of the BDNF-treated cells (positive control), whereas *H. erinaceus* showed significant ($p < 0.05$) neurite outgrowth of 18.73%

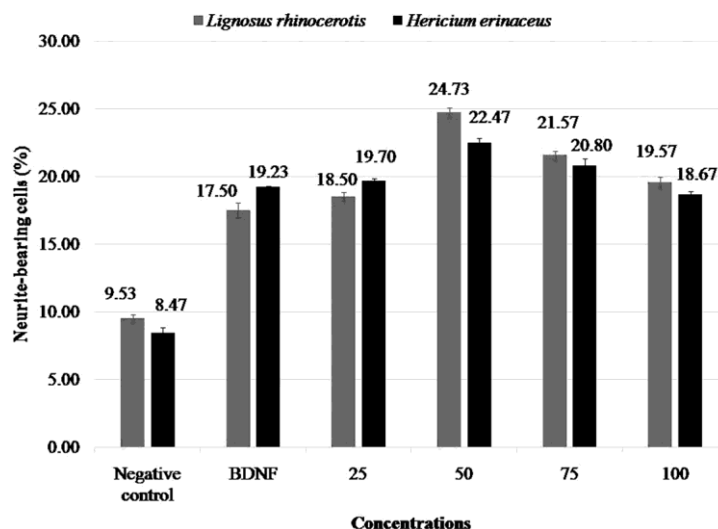


FIG. 2: Effects of varying concentrations of *H. erinaceus* and *Lignosus rhinocerotis* aqueous extract on *in vitro* neurite outgrowth stimulation on chick embryo’s spinal cells.

at a concentration of 25 µg/mL. *H. erinaceus* and *L. rhinocerotis* aqueous extracts showed significant ($p<0.05$) neurite outgrowth of 22.47% and 24.73%, respectively, at 50 µg/mL on the spinal cord (Fig. 2). The aqueous extract of *L. rhinocerotis* showed significant ($p<0.05$) neurite outgrowth (20.77%) at 25 µg/mL on retinal cells; in contrast, the aqueous extract of *H. erinaceus* (21.70%) exerted its maximum neurite growth at 50 µg/mL (Fig. 3). Comparing both of the aqueous extracts,

H. erinaceus exerted a significantly potent neurite outgrowth on retinal cells at a lower concentration compared to *L. rhinocerotis* aqueous extract. Retinal cell stimulation was significant ($p<0.05$) at 50 µg/mL by *H. erinaceus*. Neurite outgrowth is confirmed by neurofilament staining as shown in Fig. 4. Figures 4A, D, and G show BDNF at the concentration of 10 ng/mL used as a positive control in the brain, spinal cord, and retina. Figures 4B, E, and H show the negative control in all three

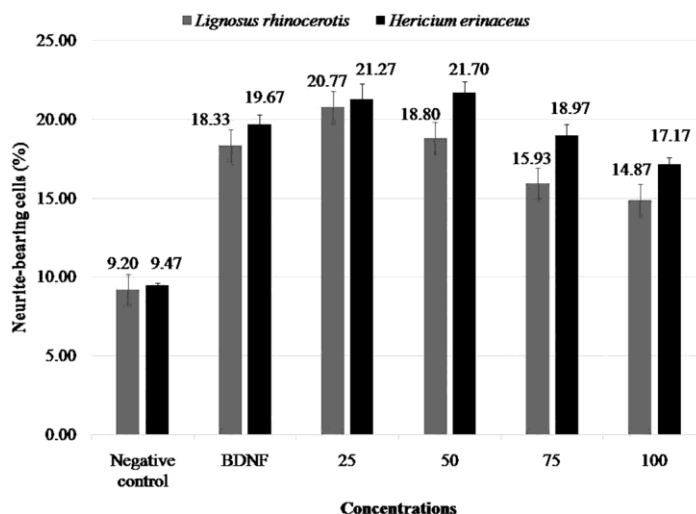


FIG. 3: Effects of varying concentrations of *H. erinaceus* and *Lignosus rhinocerotis* aqueous extract on *in vitro* neurite outgrowth stimulation on chick embryo’s retina cells.

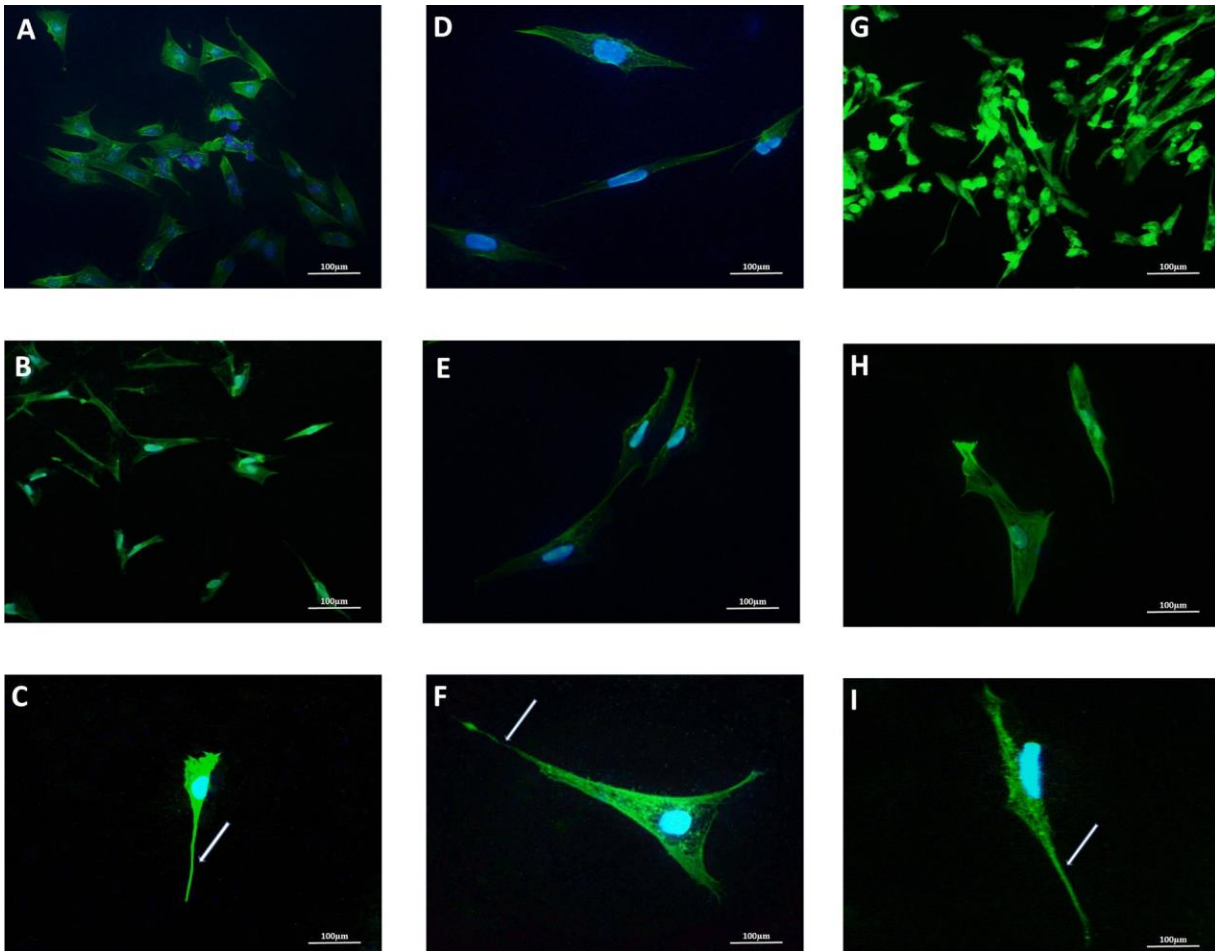


FIG. 4: Fluorescent microscopy image of *in vitro* neurite outgrowth in chicken embryo's brain, spinal cord, and retinal cells by *Lignosus rhinocerotis* aqueous extracts. Brain and spinal cord cells were treated with hot aqueous extract *L. rhinocerotis* at a concentration of 50 and 25 µg/mL for retinal cells after 48 h of incubation at 37±2°C in a 5% CO₂ humidified incubator. (A) Brain positive control: BDNF (10 ng/mL). (B) Negative control of brain cells without extract. (C) Brain cells treated with hot aqueous extract of *L. rhinocerotis* at 50 µg/mL. (D) Spinal cord positive control: BDNF (10 ng/mL). (E) Negative control of spinal cord cells without extract. (F) Spinal cord cells treated with hot extract of *L. rhinocerotis* at 50 µg/mL. (G) Retina positive control: BDNF (10 ng/mL). (H) Negative control of retinal cells without extract. (I) Retinal cells treated with hot aqueous extract of *L. rhinocerotis* at 25 µg/mL. (C), (F), and (I) Cells show an exuberant long neurite outgrowth (arrow) as compared to (A) (D), and (G).

samples, which have either no neurite, or neurite with insufficient length to be scored as positive. Figures 4C, F, and I show neurite outgrowth in the brain, spinal cord, and retinal cells confirmed by neurofilament staining. Neurite extension is marked by an arrow. Recent research suggested that these neurofilaments are closely related to many neurodegenerative diseases, such as amyotrophic lateral sclerosis, Parkinson's disease, and Alzheimer's disease. Using *in vitro* assays, cultures, and transgenic

mice, these studies provided new insights into neurofilament function. The function of each subunit, the relationship of neurofilaments with other cytoskeletal elements and their clinical significance are topics of increasing attention.

IV. CONCLUSIONS

H. erinaceus and *L. rhinocerotis* aqueous extracts were examined for neurite outgrowth activity in

the brain, spinal cord, and retinal cells of chicken embryo. Four different concentrations (25, 50, 75, and 100 µg/mL) were tested. Among the concentrations, *H. erinaceus* aqueous extract, 50 µg/mL, stimulated neurite outgrowth in brain and spinal cord cells. *L. rhinocerotis* sclerotium extract showed similar neurite outgrowth activity in retinal cells at 25 µg/mL after 48 h of incubation. Immunofluorescence staining confirmed neurite outgrowth.

ACKNOWLEDGMENTS

The authors thank the University of Malaya for Bantuan Kecil Penyelidikan (BKP) grant BK003-013, RG 193/11AFR, and UMRG RP 005B-13 AFR, and Mrs. Cheng Poh Guat of Vita Agrotech, a mushroom farm in Tanjung Sepat, Selangor, Malaysia for the continuous supply of fresh *H. erinaceus* mushroom. The technical assistance by the staff of the Department of Anatomy is greatly appreciated.

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