Neuritogenic activity of hot water extract from *Hericium erinaceus*

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ABSTRACT – Hot water soluble extract was prepared from *Hericium erinaceus* and its neuritogenic activity on PC12h cells was analyzed, which is a clone originating from a rat pheochromocytomon. The moisture content of freeze dried hot water extract was 12.08%. The extract was mainly composed of carbohydrate (51.24%) followed by crude protein (24.04%), crude fat (0.26%), dietary fiber (5.09), and ash (12.18%). Fatty acids, glucan and inorganic constituents were found as minor components. The neuritogenic activity of hot water extract was evaluated under microscopic observation of neurite outgrowth in PC12h cells and by measuring the neurite length of individual cell. The extract exhibited strong effect of neurite outgrowth in a dose-dependent manner from 0.01 mg/mL to 1 mg/mL, in which longer neurite outgrowth was observed as the treatment dose increased.

KEYWORDS – *Hericium erinaceus*, Hot water extract, Neuritogenic activity, Proximate composition, PC12h cell

Introduction

Hericium erinaceus growing on old or dead broadleaf trees is a well-known edible and medicinal mushroom belongs to the *Aphyllophorales*, *Hydnaceae* family in oriental countries. The mushroom was called “Yamabushitake” in Japan or “Houtou” in China and it is taken as a food in Japan and China without harmful effects. *H. erinaceus* was successfully cultivated indoors on conifer sawdust in artificial cultivation (Mizuno *et al.*, 1999a).

Various biological functions of *H. erinaceus* have been investigated in recent years which, related to antimicrobial effect (Kim *et al.*, 2000), anti-tumor activities (Mizuno, 1996b; Mizuno, 2002), immunomodulatory effect (Liu *et al.*, 2002), antioxidant properties (Mau *et al.*, 2002), cytotoxic effects (Kuwahara *et al.*, 1992), hypolipidemic effects and promotion of synthesis of nerve growth factor (NGF) (Kawagishi *et al.*, 1994; Lee *et al.*, 2000). The antimicrobial effect of *H. erinaceus* extracts and standard antibiotics was quantitatively assessed by the presence of clear zones indicating strong inhibition, and hazy

(partial) inhibition zones. However, bacteria were found to be more sensitive to the bioactive compounds compared to *H. erinaceus* (Wong *et al.*, 2009).

A number of bioactive molecules, including antitumor substances, have been identified in *H. erinaceus*. Galactoxyloglucan, xylan and glucosylated polysaccharides were antitumor and immunostimulating polysaccharides from *H. erinaceus* (Wasser, 2002). Polysaccharides exert their antitumor effects primarily by activating various immune system responses in the host, such as complement system activation, macrophage-dependent immune system responses, and upregulation of interferon expression. These polysaccharides have various chemical compositions and belong primarily to the β -glucan group (Lee *et al.*, 2009). *H. erinaceus* is reported not only to have a hypoglycemic effect but also to reduce the elevation rates of serum triglyceride and total cholesterol levels when administered. The treatment of diabetes often involves medication to control blood glucose levels; some of these medications have undesirable side-effects. Thus, *H. erinaceus* with hypoglycemic effects can be safely

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used in the management of diabetes (Wang *et al.*, 2005).

Among previously reported biological function of this mushroom, recent research focused on its neuritogenic activity due to the existence of hericenone group which stimulate the synthesis of NGF (Nerve growth factor). Hericenones C, D, E, F, G and H and erinacines A, B, C, D, E, F, G, H and I were isolated from the methanol extract of the fruit body and mycelium of *H. erinaceus*, respectively, all of which promote nerve growth factor(NGF) synthesis in rodent cultured astrocytes. NGF has potent biological activities, such as preventing neuronal death and promoting neurite outgrowth, and is essential to maintain and organize neurons functionally (Obara and Nakahata, 2002). It is assumed that functional deficiency of NGF is related to Alzheimer's disease and plays a role in the etiology of the disease process (Allen and Dawbarn, 2006; Takei *et al.*, 1989). NGFs are proteins which are unable to cross the blood-brain barrier and easily metabolized by peptidases. Therefore, its application as a medicine for treatment of neurodegenerative disorders will be difficult. Alternatively, research has been carried out on low-molecular weight compounds that promote NGF biosynthesis. Recent research has reported neuritogenic activity from edible and medicinal mushroom, such as *Tremella fuciformis* (Park *et al.*, 2007).

In this study, we investigated proximate composition of *H. erinaceus* and its biological function in hot water extract from *H. erinaceus*. The neuritogenic activity of the extract was investigated using PC12h cells and its nerve growth factor (NGF)-like activity was also calculated.

Materials and Methods

Composition analysis

1) Analysis of the proximate composition

Proximate analysis of *H. erinaceus* fruiting body and its hot water extract were analyzed to measure content of the crude protein, crude fat, dietary fiber, ash and moisture according to the official AOAC methods.

Moisture was removed by oven dehydration at 105°C for 72 h from sample (1 g). Ash was determined by weighing the incinerated residue obtained at 550°C after 10 h. The dietary fiber content was calculated by combining enzymatic and gravimetric methods. Each of the samples was analyzed using the AOAC TDF method (Prosky *et al.*, 1988) to provide duplicate samples for nitrogen and ash determination. In brief, aliquots of samples (1 g of dry matter) were treated with 100 μ L thermostable α -amylase (3,000 U/mL, Megazyme, Wicklow, Ireland) for 30 min at 150 rpm in a boiling water bath (95°C) and an purified protease (50 mg/mL, Megazyme, Wicklow, Ireland) for 30 min at 150 rpm at 65°C to solubilize protein and amyloglucosidase (3300 U/mL, Megazyme, Wicklow, Ireland) for 30 min at 150 rpm at 65°C to remove glycogen. After a 90% ethanol precipitation, the ethanol-insoluble residue recovered by filtration was dried and weighed, and the weight was corrected for ash and residual protein content. Crude fat content of the samples was estimated by exhaustive Soxhlet extraction of a known weight of dried sample with ethyl ether (b.p. 40~60°C) for 8 h (A.O.A.C. 7.045, 1975). Finally, crude protein was determined by a micro-Kjeldahl method, and a conversion factor of 6.25 was used to quantify the nitrogen percentage of the crude protein. The percentage of all the fractions (crude protein, crude fat, moisture and ash) were added together and subtracted from 100 to obtain the total carbohydrate percentage.

2) Analysis of glucans

The total glucan contents were quantified following enzymatic hydrolysis with exo-1,3- β -glucanase (200 U/mL, Megazyme, Wicklow, Ireland) and β -glucosidase (4 U/mL, Megazyme) by modified AOAC methodology for mushrooms. The β -glucan contents were determined according to contents of β -glucan. The β -glucans are solubilised in concentrated potassium hydroxide (2 N) from sample (100 mg). And then the samples were treated with 0.2 mL of amyloglucosidase (1630 U/mL, Megazyme, Wicklow, Ireland) plus invertase (500 U/mL, Megazyme, Wicklow, Ireland). The samples were incubated at 40°C

for 30 min at 150 rpm. Samples were read on a UV-visible spectrophotometer (Uvikon 933, Milan, Italy) at 510 nm. The percentage of β -glucans were subtracted from total glucan contents to β -glucan contents.

3) Analysis of fatty acids composition

Sample (1 g) added pyrogallol solution (50 mg/mL) was extracted with diethyl ether/petroleum ether (1:1) on water bath at 80°C at 120 rpm for 1 h. The fatty acids in the extract were simultaneously hydrolyzed and derived to their methyl ester forms with 1 mL of NaOH/methanol at 85°C for 10 min and then a complete derivation was added with 1 mL of BF_3 (14%) at 85°C for 10 min. And then the sample evaporated to dryness under a stream of nitrogen. Individual samples were passed through an anhydrous Na_2SO_4 column and redissolved in 3 mL of iso-octane. The derived free fatty acids were separated in An Agilent Technologies 7890N gas chromatograph equipped with a flame ionization detector and a fused silica capillary column (SP^{TM} -2560, 100 m $\times$ 0.25 mm i.d. $\times$ 0.2 μm film thickness, Supelco, Bellefonte, PA) with a stationary phase of 5% phenyl methyl silicon. Operating conditions were as follows: Helium was the carrier gas (1 mL/min), detector (285°C), injector (225°C), and injected volume (1 μL).

4) Analysis of inorganic constituents

Inorganic constituents were determined by first destroying the organic matter in the dried sample. Calcium, sodium, potassium, iron and phosphorus was determined spectrophotometrically (A.O.A.C. 3.064, 1975). Sample (0.5 g) added H_2O_2 (3 mL) and AgNO_3 (5 mL) was roasted in an Microwave at 200°C to determine K, Ca, Na, Fe, and P. After roasting, samples were placed in a beaker and were diluted distilled water (final weight: 15 g). The Na and Ca contents were determined by flame photometer, and Cu, Zn, Mn, and Fe by Inductively Coupled Plasma Spectrometer (OPTIMA 5300 DV, PerkinElmer, U.S.A)

Neuritogenic activity of *H. erinaceus* hot water extract

1) Preparation of hot water extract from *H. erinaceus*

The hot water extract of *H. erinaceus* fruiting body was obtained as follows: freeze-dried mushroom (50 g) was milled into powder using a food mixer, and the mushroom powder was extracted with water 6 hours at 100°C. The extract was centrifuged at 10000 rpm (using Vision scientific Co. Ltd., VS-24SMTi) for 40 min at 4°C. Supernatant was collected and filtered twice through No.4 Whatman filter paper, and filtrate was evaporated at 40°C. And then the extract was freeze-dried for 48 h.

2) Cell culture

PC12h cells, a subclone of PC12 cells isolated by Dr. Hatanaka (Hatanaka, 1981) and kindly donated by Pulmuone Co., Ltd. (Institute of Food & Culture R&D Center, Pulmuone Co., Ltd., Seoul, Korea), were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 5% (v/v) horse serum and 5% (v/v) fetal bovine serum in a 100 mm petri dish coated with collagen (Type I, Sigma, U.S.A.) under 10% CO_2 at 37°C. PC12h cells undergo certain NGF-responsive cellular events, including neurite outgrowth. PC12h cells are much more sensitive to NGF than PC12 cells (Hatanaka, 1983).

3) Assay for neuritogenic activity in PC12h cells.

Sample preparation: *H. erinaceus* hot water extract was prepared through 0.22 μm syringe driven filter (Millex HV, Millipore, France). The concentration of *H. erinaceus* hot water extract were 0.01 mg/mL, 0.1 mg/mL, 0.5 mg/mL, and 1 mg/mL. Also, the yogurts were centrifugated at 10,000 rpm for 30 min at 4°C into sterile sealed tubes. Supernatant was collected and filtered twice through No.4 Whatman filter paper, and then filtrate was freeze-dried for 48hours.

For morphological studies, cells were plated in 6 well plates coated with collagen at a density of 2×10^5 cells in 2 mL medium per dish. After 48 h of culture, the medium was replaced with serum-free DMEM/Ham'sF12 (1:1) medium (DMEM/F12, Gibco) supplemented with sodium selenite (30 μM /L), transferrin (10 mg/mL), insulin (10 mg/mL), progester-

one (256.1 µg/mL), and hot water extract *H. erinaceus*. On the other hand, Nerve growth factor (sigma-Aldrich, St. Louis, USA) 0.1 µg/mL was used negative control. And positive control was distilled water filtered through 0.22 µm syringe driven filter (Millex HV, Millipore, France). After 48 h, the neuritogenic activity was evaluated by measuring the length of the longest neurite of individual cells using an image processor system (Leica Qwin, Germany) attached to a phase-contrast microscope. One hundred cells in at least 10 random fields in two culture dishes were measured, with the values averaged.

Results and Discussions

Composition analysis of *H. erinaceus* and its hot water extract

The proximate composition of *H. erinaceus* and its hot water extract are shown in Table 1. The freeze dried *H. erinaceus* showed moisture content of 4.80%. The mushroom was mainly composed of dietary fiber (31.35%) followed by crude protein (25.71%), carbohydrate (25.65%), ash (9.01%) and crude fat (3.44%). On the other hand, the *H. erinaceus* hot water extract showed moisture content of 12.09%. Main components of *H. erinaceus* hot water extract were followed by carbohydrate (51.24%), crude protein (24.21%), ash (12.18%), dietary fiber (5.09%) and crude fat (0.26%). Considerable differences between freeze dried *H. erinaceus* and its hot water extract weren't apparent. Ko *et al.* (1999) reported the protein content of freeze-dried shiitake mushroom is 18.12%. The protein content of *H. erinaceus* was high compared to shiitake mushroom and other mushrooms (Kalac, 2009).

The glucan contents of *H. erinaceus* and its hot water extract are shown in Table 2. Glucan contents of this mushroom were 7.62%, β-glucan contents were 7.20%. Also glucan contents of hot water extract were 2.65% and β-glucan contents were 2.19%. Great attention has recently been paid to mushroom β-glucans, due to their health-positive effects. β-glucan research and applications have

Table 1. Proximate composition (g/100g)¹ of freeze-dried *H. erinaceus* and its hot water extract

Proximate composition	Freeze-dried <i>H. erinaceus</i>	<i>H. erinaceus</i> hot water extract
Carbohydrate	25.65 ± 2.075	51.24 ± 1.22
Crude protein	25.71 ± 0.23	24.21 ± 0.23
Crude fat	3.44 ± 0.89	0.26 ± 0.00
Dietary fiber	31.35 ± 1.79	5.09 ± 0.87
Ash	9.01 ± 0.66	12.18 ± 0.79
Moisture	4.80 ± 0.28	12.09 ± 0.19

¹Average ± standard deviation, n=3

Table 2. Glucans composition (g/100g)¹ of freeze-dried fruiting body of *H. erinaceus* and its hot water extract

Glucans	Freeze-dried <i>H. erinaceus</i>	<i>H. erinaceus</i> hot water extract
α-glucan	0.421 ± 0.010	0.459 ± 0.075
β-glucan	7.204 ± 0.702	2.194 ± 0.260
Total glucan	7.625 ± 0.693	2.653 ± 0.189

¹Average ± standard deviation, n=3

Table 3. Fatty acids composition (g/100g)¹ of freeze-dried fruiting body of *H. erinaceus* and its hot water extract

Fatty acids	Freeze-dried <i>H. erinaceus</i>	<i>H. erinaceus</i> hot water extract
Saturates	0.186 ± 0.014	0.095 ± 0.0002
Mono-unsaturates	0.151 ± 0.016	
Linolein Acid (C18:2n-6)	0.469 ± 0.046	0.038 ± 0.0006
Total fatty acids	0.806 ± 0.014	0.133 ± 0.0003

¹Average ± standard deviation, n=3

been successful, mainly in the east-Asian countries and deal mostly with cultivated mushroom species (Wasser, 2002).

The fatty acids of *H. erinaceus* and its hot water extract are shown in Table 3. Fatty acids contents (0.806%) of this mushroom were low. Very high proportions were found Linolein acid (C18:2n-6) in freeze-dried *H. erinaceus* and its hot water extract. The nutritional value of this mushroom lipid is limited, due to low total lipid contents and the absence of desirable n-3 fatty acids.

Major inorganic constituents of *H. erinaceus* and its hot water extract are given in Table 4. The inorganic constituents in freeze dried *H. erinaceus* were on the whole slightly lower than its hot water

Table 4. Inorganic constituents composition (mg/100g)¹ of freeze-dried fruiting body of *Hericium erinaceus* and its hot water extract

Inorganic constituents	Freeze-dried <i>H. erinaceus</i>	<i>H. erinaceus</i> hot water extract
Na	1.273 ± 0.041	
K	1515.000 ± 24.042	1992.500 ± 487.196
Fe	0.669 ± 0.007	0.232 ± 0.004
Ca	2.402 ± 0.566	10.936 ± 0.601
P	463.200 ± 9.617	572.075 ± 18.773

¹Average ± standard deviation, n=3**Table 5.** Usual content (mg/100g) of major inorganic constituents in mushrooms

Inorganic constituents	Usual content
Na	10 - 40
K	2000 - 4000
Ca	10 - 50
P	500 - 1000

(Pavel Kalac, 2009)

extract, except Iron. Kalac (2009) reported content of major inorganic constituents in mushrooms (Table 5). According to Kalac (2009), *H. erinaceus* and its hot water extract contain non-specific levels of inorganic constituents. But, inorganic constituents of this mushroom are somewhat higher or comparable with the most vegetables.

Neuritogenic activity of *H. erinaceus* hot water extract

Hot water soluble extract was prepared from *H. erinaceus* and its neuritogenic activity on PC12h cells was analyzed, which is a clone originating from a rat pheochromocytomon. NGF has been reported to induce morphological changes in PC12h cells by improving neurite outgrowth (Hatanaka, 1983). NGF was shown to improve the differentiation of PC12h cells (Fig. 1; Table 6). The average neurite length of cells treated with 0.1 µg/mL of NGF is 49.01 ± 11.06 µm. After treatment of PC12h cells with the hot water extract of *H. erinaceus* at a concentration range of 0.1 and 1 µg/mL for 48 h, the longest neurite length of each cell was measured (Fig. 1; Table 6). They were significant increase in differentiation com-

Fig. 1. Neuritogenesis induced by NGF in PC12h cells (*200). Scale bar = 100 µm (a) Control (negative vehicle); (b) NGF 0.10 µg/mL. Morphological changes induced hot water extract of *H. erinaceus* in PC12h cells. Scale bar = 100 µm. (*200); (c) Hot water extract of *H. erinaceus* 0.01 mg/mL; (d) Hot water extract of *H. erinaceus* 0.10 mg/mL; (e) Hot water extract of *H. erinaceus* 0.50 mg/mL; (f) Hot water extract of *H. erinaceus* 1.00 mg/mL.**Table 6.** Effect of *H. erinaceus* hot water extract on PC12h cells neuritogenesis

	Neurite length (µm)
Control	12.931 ± 2.281
NGF 0.10 µg/mL	49.013 ± 11.058
Concentration of	
<i>H. erinaceus</i> hot	
water extract	
0.01 mg/mL	24.639 ± 4.728
0.10 mg/mL	44.569 ± 6.891
0.50 mg/mL	62.989 ± 7.783
1.00 mg/mL	76.353 ± 13.853

Average ± standard deviation, n=100

pared to the neurite length of the control. The average the longest neurite length of cells treated with 1 mg/mL of *H. erinaceus* is 76.35 ± 13.85 µm. The hot water extract of *H. erinaceus* exerts neu-

ronal differentiation activity in PC12h cells by inducing neurite outgrowth at low concentration (0.1 mg/mL) compared to nerve growth factor (0.1 µg/mL of NGF).

Park *et al.* (2007) reported that the hot water extract of *Tremella fuciformis* tends to induce neurite outgrowth in PC12h cells. Also Park *et al.* (2006) reported the neuritogenic activity of genipin containing fraction from Korean gardenia fruit previously. When PC12h cells were treated with 5 µg/mL of genipin containing fraction from Korean gardenia fruit, they observed similar neuritogenic activity compared to 1 µg/mL of hot water extract of *T. fuciformis*. The average neurite length of cells treated with 1 µg/mL of hot water extract of *T. fuciformis* is 77.84 ± 0.92 µm.

Conclusion

In this study hot water extract of *H. erinaceus* fruiting body was prepared and analyzed. As a result of hot water extract analysis, the proximate composition, glucans, fatty acids and inorganic constituents were similar to freeze-dried *H. erinaceus*. In neuritogenic activity of hot water extract, hot water extract exhibited neurite outgrowth on PC12h cells. Treatment of PC12 cells with NGF induced neuronal differentiation. Also, treatment of PC12h cells with hot water extract did. These results suggest the possibility using hot water extract *H. erinaceus* derivatives as NGF substitutes. The degree of neurite outgrowth activity differed among the compound concentration. In 1 mg/mL hot water extract exhibited the highest neuritogenic effect and the lowest neuritogenic effect is 0.01 mg/mL. It means neuritogenic effect depends on the concentration of hot water extract.

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