ANTIOXIDANT ACTIVITIES OF EXTRACTS FROM THE GENUS *PHELLINUS* SPECIES

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ABSTRACT

Ethanolic extracts of fruit bodies of five species (eight strains) of the genus *Phellinus*, and five solvent extracted fractions derived from one of these extracts (*Ph. baumii* PB-10), were evaluated for antioxidant activity and the capacity to protect PC12 cells against H_2O_2-induced oxidative damage. Extracts of all eight strains of *Phellinus* spp. exhibited antioxidant activity and protected PC12 cells against oxidative damage at different magnitudes of potency. Strongest antioxidant activity was exhibited by extracts of *Ph. baumii* PB-10, with recorded IC_{50} values for superoxide radical and hydrogen peroxide scavenging activity of 3.76 μg/mL and 4.24 μg/mL, respectively. Radical scavenging activities, reducing potential and anti-lipid peroxidation capability of five different extracts (petroleum ether, chloroform, ethyl acetate, n-BuOH and ethanol-petroleum phase) derived from *Phellinus baumii* were studied. The results showed the ethyl acetate and n-BuOH extracts exhibited stronger antioxidant capacity. Radical-scavenging activity and protection levels against H_2O_2-induced damage to PC12 cells were highly correlated with the flavonoid content of the extracts and isolated fractions.

KEY WORDS: Species of the genus *Phellinus*, Extracts, Antioxidant activities.

INTRODUCTION

Reactive oxygen species (ROS) are among the major sources of primary catalysts that initiate oxidation *in vivo* and *in vitro* [1]. The triplet state oxygen can react with other molecules to yield ROS such as hydrogen peroxide (H_2O_2), superoxide (O^{2-}), and hydroxyl radicals (OH) [2]. ROS have been associated with the beginning of many diseases and degenerative processes in ageing [3]. Other free radicals, such as DPPH and ABTS, can also lead to the oxidative damage. Natural products with antioxidant activity may be used to help the human to reduce oxidative damage. Many natural antioxidants have already been isolated from different kinds of plant materials [4].

Species of the genus *Phellinus* Quél. (Hymenochaetaceae, Aphyllophoromycetideae, higher Basidiomycetes) have been shown to contain various bioactive substances including triterpenoids, polysaccharides and flavones [5]. *Phellinus* species are believed by traditional Chinese herbalists to be effective in treating many gynecopathic ailments [6]. Much of the research carried out on *Phellinus* in recent years has focused on the chemical composition, structure and pharmacology of polysaccharides and proteoglycans [7-9] derived from mushroom fruit bodies, while the flavone components have received relatively little attention. We have now prepared ethanolic extracts and other solvent extracted fractions rich in flavonoids from the fruit bodies of different *Phellinus* species and measured their antioxidant activities. Our aim is to provide a scientific basis for earlier empirical observations, and to promote the future development of *Phellinus* species as important medicinal mushrooms.

MATERIALS AND METHODS

Materials. Fruit bodies of eight different strains of five species of the genus *Phellinus* were cultivated at the Institute of Edible Fungi (IEF), Shanghai Academy of Agricultural Sciences (SAAS). Strains were obtained from the Herbarium of the Edible Fungi Culture Collection Center, Branch of China Culture Collection Center of Agricultural Microorganisms, and maintained at the Research Center of Medicinal Resources, IEF. Designation and origin of the strains were as follows: *Phellinus baumii* PB-10, (Korea); *P. gilvus* KSH, (Korea); *P. linteus* PLSH, (Korea); *Ph. igniarius* JSH, (Japan); *P. badius* NFSH, (South Africa); *P. igniarius* PI-12, (China); *P. linteus* MYSH, (China); and *Phellinus* sp. CSH, SA06 (China). The designation of CSH was based on our ITS1-ITS4 sequencing data and matching with an identical sequence in GenBank.

Rutin, luminal and nerve growth factor (NGF) were purchased from Sigma-Aldrich. Superoxide dismutase was purchased from LanJi Science and Technology Development Co. Ltd (Shanghai, China), while all other chemicals were from local suppliers and of AR grade.

Preparation of Ethanol extracts and other Solvent Fractions. Fruit bodies of all the studied *Phellinus* strains were cut into small pieces and dried at 50-55 °C for 48 h. Dried material (2000 g) was immersed twice in 10 vols 60% (v/v) ethanol and refluxed at room temperature (~24 ºC) for 24 h. Combined aqueous ethanolic extracts were centrifuged (3,500 g, 10 min, 20 °C) to removed suspended impurities, concentrated by vacuum evaporation and then freeze-dried.

The dried ethanol extracts 60 g was resuspended with 500 ml 20 % ethanol. 1 L of petroleum ether was used to extract the sample for three times. The collected extracts were combined. And then the samples were fractionated successively with 3 L of chloroform, 3 L of ethyl acetate (EtOAc), and 3 L of n-BuOH in the same ways. All the solvent fractions were removed under reduced pressure to be dried. Finally, five different extracts (petroleum ether, chloroform, ethyl acetate, n-BuOH and ethanol-petroleum phase) were collected.

Determination of Flavone Content. Total flavone content of the crude ethanolic extracts was determined by the $NaNO_2$-$Al(NO_3)_3$ colorimetric assay using rutin as the standard [10].

Superoxide Radical Scavenging Activity. Superoxide radical scavenging activity of the crude ethanolic extracts was measured by the chemiluminescence-based method according to Guo and Wang [11]. Test samples (2 μL) dissolved in 70% ethanol to various concentrations (5, 10, 20, 40 and 60 μg/mL) were dispensed into each well of a 96-well plate. K_2HPO_4-KH_2PO_4 buffer (0.05 mol/L) served as the control. Pyrogallol (8 μL, 6.25×10^{-4} mol/L), luminal (50 μL, 1 mM) and sodium carbonate buffer (100 μL 0.05 mol/L) were then added to each well and the intensity of luminescence determined at 0.6 s intervals for a total of 30 s using a Clarity Microplate Luminometer (Bio-Tek, USA). Superoxide radical scavenging activity was calculated using the following equation:

Scavenging ratio (%) = (luminescence value of control - luminescence value of test sample) / luminescence value of control × 100%

IC_{50} refers to the sample concentration when the scavenging ratio was 50%.

Hydroxyl Radical Scavenging Activity. The radical scavenging activity was determined through the CuCl-phenanthroline-H_2O_2 system according to the method established by HongFei

Fu *et al* [12] with some modifications. The Clarity Microplate Luminometer was used in the assay. The mixt composed of 10 μL of sample solution and positive control, 10 μL of a 1.0 mmol/L CuCl solution, 10 μL 1 mmol/L 1,10-phenanthroline solution were added in the 96 microwell plate, and then 10 μL 0.6 % H_2O_2 was added in every cell from pump one and 150 μL Na_2CO_3-$NaHCO_3$ (pH=8.5, 0.05 mol/L) were added from pump two to process the reaction. The chemiluminescence intensity was recorded with a program in the processor once every 6 s and was kept 30 s. 70 % ethanol was used as a control. Hydroxyl radical scavenging activity was calculated using the following equation:

Scavenging ratio (%) = (luminescence value of control - luminescence value of test sample) / luminescence value of control × 100%

IC_{50} refers to the sample concentration when the scavenging ratio was 50%.

Hydrogen Peroxide Scavenging Activity. Hydrogen peroxide scavenging activity of the crude ethanolic extracts was measured by the chemiluminescence-based method according to Qin *et al* [13]. Aliquots of H_2O_2 (10 μL) were first dispensed into each well of a 96-well plate. Test samples (10 μL) dissolved in 70% ethanol to various concentrations (2, 5, 10, 20, 40, 60 and 80 μg/mL), luminol and sodium carbonate buffer solution (150 μL) were then added to each well. Double distilled water served as the control. The intensity of luminescence was read every 0.6 s for 30 s and the scavenging ratio value for each sample was calculated as follows:

Scavenging ratio (%) = (luminescence value of control - luminescence value of test sample) / luminescence value of control × 100%

IC_{50} refers to the sample concentration when the scavenging ratio was 50%.

DPPH Radical Scavenging Activity. The scavenging activity of the DPPH free radical was assayed according to the method of Brand-Williams *et al* [14] with slight modification. 100 μL of various concentrations of five different phases samples were added to 100 μL of 6×10^{-4} mol/L DPPH· radical solution mixed with 900 μL of 70 % ethanol (final concentration of DPPH· was 6×10^{-5} mol/L). The mixture was shaken and stood at 25 °C for 20 min; the absorbance of the solution was measured at 517 nm. 100 μL of 70 % ethanol was used as a control. The radical scavenging capacity of the tested samples was measured as a decrease in the absorbance of DPPH· radical and was calculated by using the following equation [15]:

Inhibition (%) = $[(A_{control}-A_{sample})/ A_{control}]\times100$

Reducing Potential. The reducing power was described by a modified method of Neeraj Mishra *et al* [16] and use of ascorbic acid as a standard. 50 μL different concentration of samples were mixed with 500 μL of 0.2 mol/L sodium phosphate buffer (pH=6.6) and 500 μL of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. After 500 μL of 10% trichloroacetic acid were added, the mixture was centrifuged at 4000 g for 10 min. The upper layer (500 μL) was mixed with 400 μL of distilled water and 100 μL of 0.1% of ferric chloride and the absorbance of the solutions at 700 nm was then measured. The absorbance is proportional to reducing power.

Anti-Lipid Peroxidation Capability. Anti-lipid peroxidation capability was assayed as described previously (Yu Cao and Isao Ikeda [17]) with minor modifications. The fresh rats' hepatic tissue were dissected and homogenized in 4 °C PBS (20mM, pH 7.4) to produce a 10% homogenate (v/v). The homogenate was mixed with 20 μL $FeSO_4$ (6 mmol) and 20 μL H_2O_2 (60 mmol). The mixture was incubated with the test samples in the presence of sample at 37 ◦C for 1.5 h. The reaction was stopped by addition of 200 μL trichloroacetic acid (TCA, 20%, w/v). The

mixture was centrifuged at 4000 g for 10 min to remove precipitation. 500 µL thiobarbituric acid (TBA, 1%, w/v) added to 300 µL supernatant were heated at 100 °C for 15 min. After centrifugation at 4000 g for 10 min, the absorbance (A_1) was measured at the wavelength of 532 nm. A_0 was measured by using 70 % ethanol as the control. The inhibition of lipid peroxidation was determined by quantification of MDA. The inhibitory rate was obtained according to the formula [18]:

Rate of inhibition of lipid peroxidation (%) = $(1-A_1/A_0)\times100$

Protection of PC12 Cells against Oxidative Damage. PC12 cells were grown in DMEM culture medium and harvested during the logarithmic phase by centrifugation after culturing at 37 °C under 5% CO_2. Cell pellets were resuspended in sterile culture medium to a concentration of 2×10^4 cells/mL and 190 µL of this suspension were added to each well of a 96-well plate. Ten µL H_2O_2 (0.75%) were then added to each well and, after standing under the test conditions for 4 h, 199 µL DMEM culture medium were added to each well followed by 1 µL sample of various concentrations (10 µg/L, 50 µg/L, and 150 µg/L). Controls consisted of a normal control group (without H_2O_2), a H_2O_2 group (without sample) and a positive control group (1 µL 100 µg/L NGF). Well plates were incubated at 37 ºC in a 5% CO_2 atmosphere for 48 h after which aliquots (20 µL) of Alamar Blue reagent (Biosource Nivelles, Belgium) were added to each well and the incubation continued for a further 12 h. Changes in extinction values at 570nm and 600nm were measured using a micro ELISA autoreader **(**Bio-Tek Instruments, Winooski, VT, USA).

Cell viabilities were calculated according to the Biosource protocol:

Cell viability (%) = $[117216 \times A_{\lambda570}\,(\text{sample}) - 80856 \times A_{\lambda600}\,(\text{sample})]/$
$[117216 \times A_{\lambda570}\,(\text{control}) - 80856 \times A_{\lambda600}\,(\text{control})] \times100$

Statistical Analysis. All data were given as means±standard deviation (SD). Comparisons between the means of various treatment groups were analyzed using SPSS 13.0 followed by analysis of variance (ANOVA). $P < 0.05$ was considered to be significant.

RESULTS AND DISCUSSION

Flavone Content of Ethanolic Extracts. Large variations were observed in the flavone content of the eight *Phellinus* ethanolic extracts (Table 1).

Table 1: Flavonoid Content of Different *Phellinus* Strains

Strain	Yield of ethanol extract	Total flavone content of ethanolic extract	Total flavone content of fruit body
PB-10	26.5 ± 4.9	59.6 ± 2.1	15.8 ± 1.9
PI-12	20.2 ± 7.1	30.4 ± 1.2	6.1 ± 2.7
NFSH	15.1 ± 5.4	30.4 ± 2.7	4.6 ± 2.3
MYSH	17.1 ± 4.7	28.0 ± 1.2	4.8 ± 0.6
CSH	20.0 ± 1.7	32.1 ± 1.5	6.4 ± 3.5
JSH	18.1 ± 7.2	22.4 ± 0.8	4.1 ± 4.3
KSH	17.7 ± 1.8	28.0 ± 2.3	4.9 ± 2.6
PLSH	6.9 ± 3.9	42.5 ± 0.9	2.9 ± 1.7

Values are expressed as % dry weight and represent the mean ±S.D (n = 3)

Highest extract yields (26.5%) and flavone levels (59.6%) were recorded in *P. baumii* PB-10. The flavone content of *P. linteus* PLSH was higher than the remaining samples (up to 42.5%), but the yield of ethanolic extract was the lowest (6.9%). Fruit bodies of *P. baumii* PB-10 had the highest total flavone content (15.8%), and ethanolic extracts of this strain were subjected to further fractionation.

Radical Scavenging Activities of Ethanolic Extracts From different *Phellinus* Fruiting bodies. Ethanolic extracts of *P. baumii* PB-10 exhibited the strongest antioxidant activity with IC_{50} values for superoxide radical and hydrogen peroxide scavenging activity of 3.76 μg/mL and 4.24 μg/mL, respectively (Table 2). Antioxidant activity of *P. linteus* PLSH extracts was also strong with corresponding IC_{50}-values of 5.94 μg/mL and 7.88 μg/mL, whereas IC_{50} values for the other samples were significantly higher. The stronger radical scavenging activity of *P. baumii* PB-10 and *P. linteus* PLSH extracts correlated well with the higher flavone contents and suggested that flavones were the main antioxidants in *Phellinus* fruiting bodies.

Table 2: *In vitro* Antioxidant Activity of Ethanolic Extracts of Different *Phellinus* Strains

Strain/Sample	Scavenging activity	
	Superoxide	H_2O_2
SOD	0.02 ± 0.01^{a}	
Vitamin C		14.2 ± 1.1^{d}
PB-10	6.9 ± 0.7^{b}	5.0 ± 0.4^{a}
PI-12	7.3 ± 0.6^{b}	14.7 ± 0.3^{d}
NFSH	9.4 ± 1.3^{c}	14.6 ± 0.5^{d}
MYSH	8.9 ± 0.9^{b}	16.4 ± 0.7^{e}
CSH	7.2 ± 1.1^{b}	10.9 ± 0.8^{c}
JSH	9.7 ± 2.1^{c}	14.9 ± 1.2^{d}
KSH	8.7 ± 1.6^{c}	12.9 ± 1.4^{c}
PLSH	5.9 ± 0.8^{b}	7.9 ± 1.7^{b}

All values are the mean (n = 3) IC_{50} values (μg/mL) ±S.D. Superoxide dismutase (SOD) and vitamin C served as positive controls. Significant differences determined by the Tukey HSD test ($P<0.05$) are indicated by different letters (a-e).

Table 3: Radical-scavenging activities of extracts of *Phellinus baumii*

Extract standard antioxidants	Total flavone content (% dry wt)	IC50 (μg/ml)			
		Superoxide anion radical scavenging	Hydroxyl radical scavenging	Hydrogen peroxide scavenging	DPPH radical scavenging
Petroleum ether	1.78±1.12	max	1.16±1.37	max	2870.13±5.59
Chloroform	27.04±3.01	25.91±0.75	0.12±0.01	2.49±0.21	14.77±0.21
Ethyl acetate	89.61±1.32	2.98±0.01	0.041±0.001	0.24±0.07	3.89±0.02
n-BuOH	62.01±0.98	4.39±0.11	0.13±0.01	0.38±0.02	5.12±0.04
Ethanol petroleum	9.55±2.34	26.11±2.21	0.41±0.03	225.30±12.4	27.14±0.42
Vc		7.54±0.07	/	/	/
BHA		/	0.021±0.03	/	/
catechin		/	/	0.11±0.04	4.32±0.09

Max stands for too huge to count. Values are means ± SD of three determinations. Vc, BHA, catechin were used as positive compounds to compare with five different extracts.

Radical Scavenging Activities of Different Solvent Fractions from *Ph. baumii* PB-10. The ethyl acetate fraction showed the most potent scavenging ROS radical activities (Table 3) and indicated that compounds with strongest radical-scavenging activity in this species are of medium polarity. The Petroleum ether fraction had the lowest antioxidant capacity, even the IC50 values on superoxide anion radical scavenging and hydrogen peroxide scavenging were too huge to count. It illustrates the petroleum ether fraction has little antioxidant capacity. Except for Hydroxyl radical scavenging, the n-BuOH fraction showed higher antioxidant capacity than the chloroform fraction. The ethyl acetate fraction and the n-BuOH fraction had a higher flavone content and a lower IC_{50} value than the other extracts (Table 3), further supporting a link between flavone constituents and antioxidant activity.

Reducing potential. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity [19]. The reducing power of five different extracts increased with high concentration (Fig 1). The ethyl acetate and n-BuOH extracts exhibited strongest ability among five different extracts. The petroleum ether phase fraction had the lowest reducing potential.

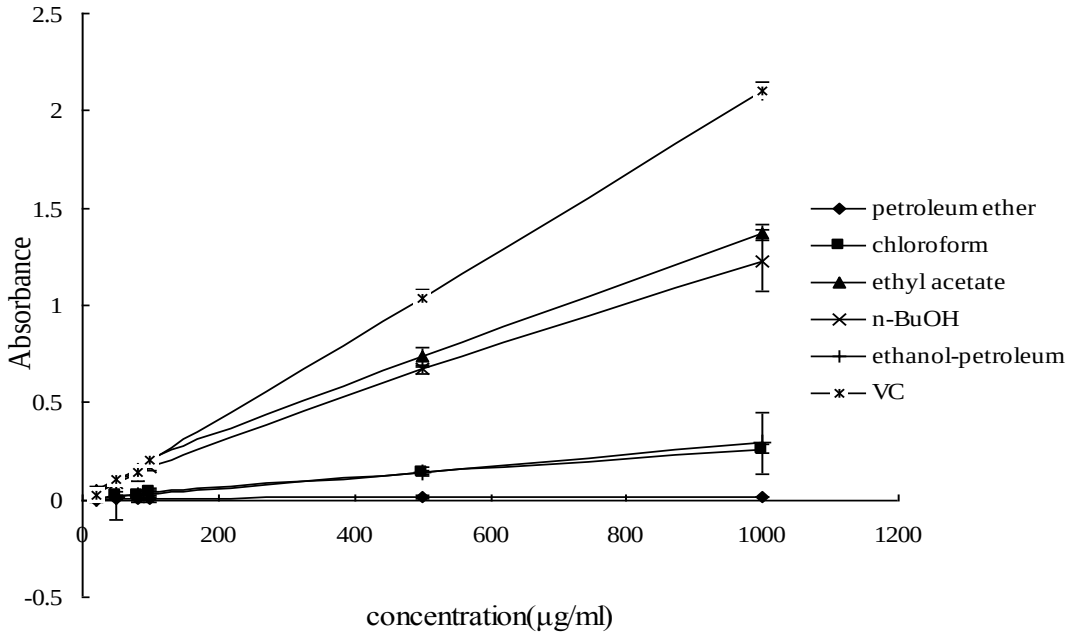

Figure 1: Reducing potential of different extracts from *Phellinus baumii*
Values are means±SD of three determinations, Vc were used as positive compounds

Anti-lipid peroxidation capability. The initiation of lipid peroxidation is carried out mostly by free radicals, such as superoxide, hydroxyl radicals and other reactive oxygen species. Lipid peroxidation causes cellular injury by inactivation of the enzymes and receptors in membrane, and depolymerization of DNA/RNA as well as protein cross-linking and fragmentation [17]. The ethyl acetate extracts exhibited strongest anti-lipid peroxidation capability（Fig 2）and followed by the chloroform extracts. Anti-lipid peroxidation capability of five different extracts increased with high concentration.

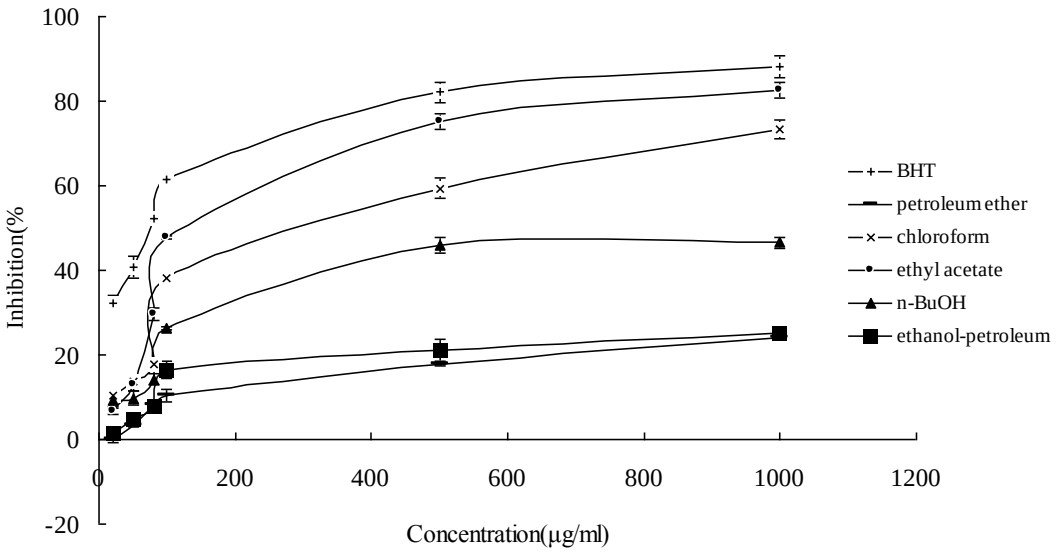

Figure 2: Anti-lipid peroxidation capability of *Phellinus baumii* Values are means±SD of three determinations

Protection of PC12 Cells against Oxidative Damage. Ethanolic extracts of all eight *Phellinus* strains protected PC12 cells against H_2O_2-induced oxidative damage in a dose-dependent manner (Table 4). Highest cell survival rates, 72.47 ±0.3% at low concentrations (10 μg/mL) and 90.73 ±3.6% at high concentrations (150 μg/mL), were recorded with ethanolic extracts of *P. baumii* PB-10. Ethyl acetate and n-BuOH extracts derived from ethanolic extracts of *P. baumii* PB-10, also protected PC12 cells against H_2O_2-induced oxidative damage in a dose-dependent manner (Fig. 3). In addition to involvement of direct free radical scavenging activity, a reduction in free radical generation may also contribute to the protective action of ethanolic extracts [20].

Table 4: Protective Effect of Ethanolic Extracts of Different *Phellinus* Strains Against H_2O_2-induced Oxidative Damage to PC12 Cells

Strain/Sample	**Cell viability (%) at different sample concentrations**		
	10 μg/mL	**50 μg/mL**	**150 μg/mL**
PB-10	72.47 ± 0.3^{b}	80.10 ± 1.7^{a}	90.73 ± 3.6^{a}
PI-12	57.10 ± 1.8^{c}	72.15 ± 1.8^{b}	81.14 ± 1.3^{c}
NFSH	55.77 ± 3.5^{c}	72.89 ± 2.1^{b}	83.06 ± 2.9^{c}
MYSH	56.43 ± 0.5^{c}	69.88 ± 1.2^{b}	81.11 ± 2.3^{c}
CSH	64.01 ± 1.6^{b}	71.10 ± 0.7^{b}	80.67 ± 2.5^{d}
JSH	51.80 ± 1.3^{d}	63.64 ± 3.1^{c}	70.33 ± 3.0^{e}
KSH	59.40 ± 6.7^{c}	67.83 ± 2.2^{b}	86.03 ± 2.8^{b}
PLSH	68.87 ± 2.9^{b}	75.30 ± 6.4^{a}	85.56 ± 2.8^{b}
Control		100.00 ± 6.7	
H_2O_2		$44.17 \pm 2.8^{*}$	
NGF （100 μg/mL）		93.52 ± 2.8^{a}	

All values are the mean (n = 3) ±S.D. NGF served as the positive control. Significant differences determined by the Tukey HSD test ($P<0.05$) are indicated by different letters (a-e) in each concentration column; *compared with control group: $p<0.01$.

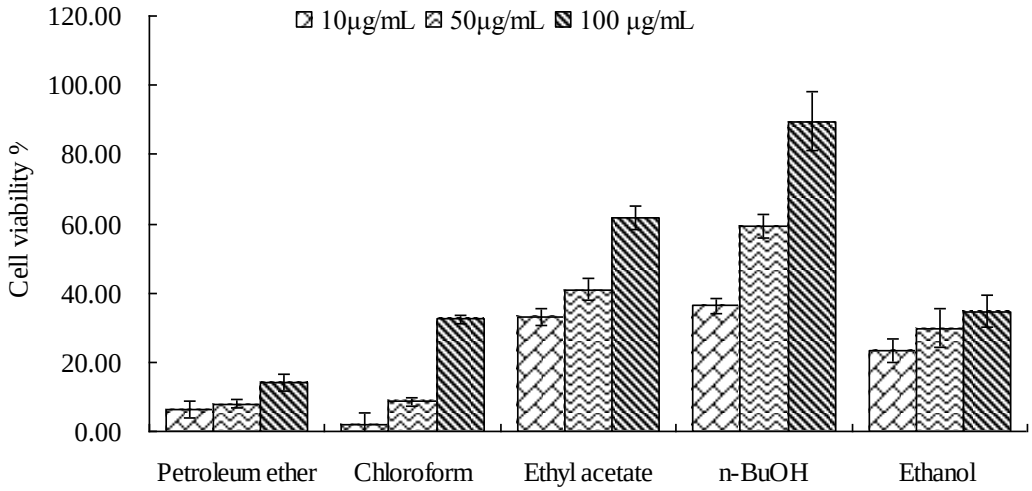

Figure 3: Protection of PC12 cells against H_2O_2-induced oxidative damage by extracted fractions from *Phellinus baumii* PB-10 fruit bodies.

Conclusion

Oxidative damage involving reactive oxygen species (ROS) and other free radicals are involved in a variety of pathological events. Excessive generation of ROS can damage proteins, carbohydrates, polyunsaturated fatty acids, and DNA, thereby leading to oxidative stress and to a variety of degenerative processes and diseases such as ageing, neurological disorders, inflammation, atherosclerosis, coronary heart disease and certain cancers [21-23].

Of the strains examined in our study, PB-10 (*P. baumii*) appeared to have the highest potential value for antioxidant and anti-aging. Our results showed the ethyl acetate and n-BuOH extracts derived from *Ph. Baumii* ethanol extract exhibited stronger antioxidant capacity. This order is similar to the total flavonoids contents of the extracts. Several published reports [24-26] showed the radical-scavenging activity of extracts could be related to the nature of phenolics, thus contributing to their electron transfer/hydrogen donating ability. Further works should be performed on the isolation and identification of the components in the extracts, especially in the ethyl acetate and n-BuOH extracts. In addition, further investigation of individual flavonoid compounds, their *in vivo* antioxidant activity and the different antioxidant mechanisms should be studied.

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