

Antioxidant effect of *Inonotus obliquus*

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Abstract

The mushroom *Inonotus obliquus* (Fr.) Pilát (Hymenochaetaceae), has been widely used as a folk medicine in Russia, Poland and most of the Baltic countries. The purpose of this study was to elucidate the antioxidant capacities of *Inonotus obliquus*. Four extracts from the fungus were evaluated for antioxidant activity against the 1,1-diphenyl-2-picrylhydrazyl (DPPH), superoxide, and peroxy radicals. The polyphenolic extract had a strong antioxidant activity, and the extract containing triterpenoids and steroids presented a relatively strong antioxidant effect. The polysaccharide extract, however, was inactive. The protective effects of these four extracts were assessed against hydrogen peroxide-induced oxidative stress using a human keratinocyte cell line, HaCaT. Our results show that the polyphenolic extract protected these cells against hydrogen peroxide-induced oxidative stress, while the polysaccharide, triterpenoid and steroid extracts were ineffective. Additionally, the remnant polyphenolic and low molecular weight polysaccharide extracts showed a weakly protective effect at a concentration of 50 µg/ml. Our results indicate that *Inonotus obliquus* has the capacity to scavenge free radicals at concentrations higher than 5 µg/ml and that the polyphenolic extract can protect cells against oxidative stress.

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1. Introduction

The mushroom *Inonotus obliquus* (Fr.) Pilát (Hymenochaetaceae), is a black parasitic fungus that grows on living trunks of the mature birch, and is mainly found at latitudes of 45°N–50°N. Traditionally, it has been used for the treatment of gastrointestinal cancer, cardiovascular disease and diabetes since the 16th century in Russia, Poland

and most of the Baltic countries (Huang, 2002). In Western Siberia, *Inonotus obliquus* has been used to alleviate worms, tuberculosis, liver or heart diseases, stomach ailments, and also as an internal cleansing agent. However, its pharmacological actions have not been well documented, in spite of its increasing usage.

It has long been recognized that many naturally occurring substances in plants have antioxidant activities. Of these substances, the phenolics which are widely distributed, have the ability to scavenge free radicals by single-electron transfer (Hirano et al., 2001). Recently, it was reported that the melanin complex obtained from *Inonotus obliquus* contains a strong antioxidant and exhibits genoprotective activity (Babitskaia et al., 2000). In addition, it has been shown that it protects against the effects of gamma radiation (Rasina, 2002) and increases catalase activity in HeLa S3 tumor cells (Rzymowska, 1998). Mushrooms usually contain a wide variety of free radical scavenging molecules, such as polysac-

Abbreviations: AAPH, 2,2'-azobis(2-amidinopropane)dihydrochloride; DCF, 2,7-dichlorofluorescein-diacetate; DPBS, Dulbecco's phosphate-buffered saline; DPPH, 1,1-diphenyl-2-picrylhydrazyl; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NBT, nitroblue tetrazolium chloride

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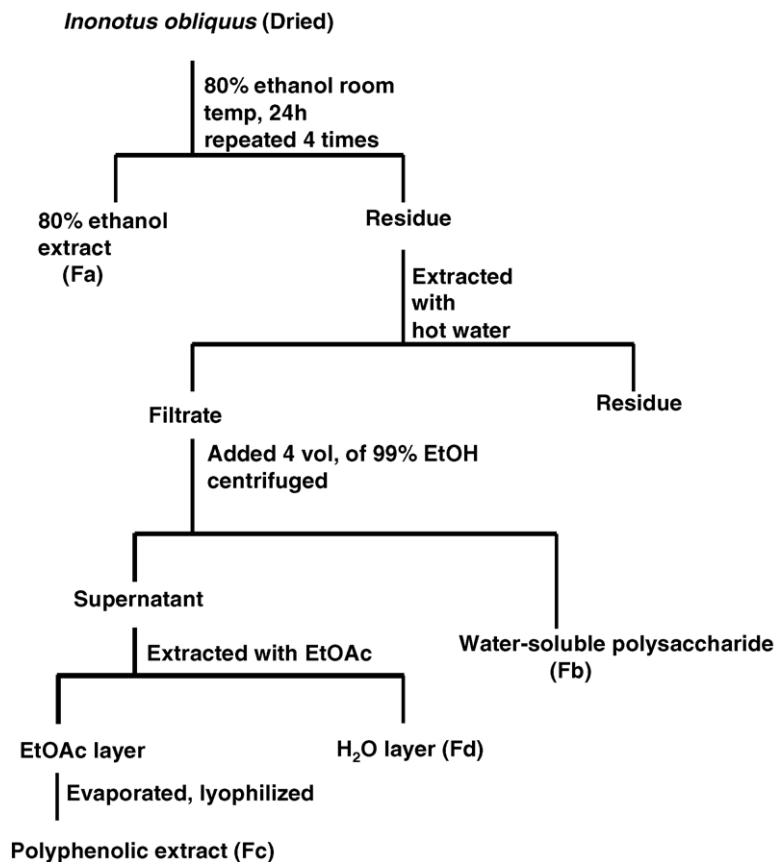


Fig. 1. Preparation of *Inonotus obliquus* extracts.

charides and polyphenols (Liu et al., 1997; Mau et al., 2002). However, little is known about the cytoprotective effects of *Inonotus obliquus* against oxidative stress.

In this study, the antioxidant activity of the extracts obtained from *Inonotus obliquus* was evaluated and compared with that of L-ascorbic acid. Extracts of the soluble polysaccharide and polyphenolic components of the mushroom were prepared according to previously described methods (Mizuno et al., 2000; Thang et al., 2001). This study was undertaken in order to evaluate the antioxidant activity of *Inonotus obliquus*, as assessed by its ability to scavenge free radicals and to protect human keratinocytes from oxidative stress.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), Ham's F-12, fetal bovine serum (FBS, Hyclone, Logan, UT, USA), and Dulbecco's phosphate-buffered saline (DPBS) were purchased from Gibco Ltd. (Grand Island, NY, USA). 1,1-Diphenyl-2-picrylhydrazyl (DPPH), nitroblue tetrazolium chloride (NBT), hypoxanthine, xanthine oxidase, and 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH), hydrogen peroxide, L-ascorbic acid, MTT (3-(4,5-dimethylthiazol-

2-yl)-2,5-diphenyl tetrazolium bromide) were obtained from Sigma Co. (St. Louis, MO, USA) and 2,7-dichlorofluorescein diacetate (DCF) was purchased from Calbiochem (San Diego, CA, USA), while analytical grade ethanol and ethyl acetate were obtained from Merck (Darmstadt, Germany).

2.2. Cell line

Human HaCaT keratinocytes (kindly provided by Dr. N.E. Fusenig, DKFZ, Heidelberg, Germany) were incubated in DMEM supplemented with 10% FBS, 1 mM sodium pyruvate, 50 µg/ml streptomycin and 50 µg/ml penicillin at 37 °C in 5% CO₂.

2.3. MTT assay

Cell viability was determined by the MTT assay (Pagè et al., 1988). For the MTT assay, 20 µl of MTT solution (5 mg/ml) was added to each well of a 96-well plate, and incubated for 4 h. The supernatant was removed, and the formazan crystals produced were dissolved in 200 µl of dimethylsulfoxide, and quantified by measuring their optical density at 540 nm using an ELISA reader (TECAN, Salzburg, Austria).

2.4. Mushroom and extraction procedure

Powdered *Inonotus obliquus* (Fr.) Pilát (100 g) was extracted with 80% ethanol at room temperature overnight, and then freeze-dried (Fa, 3.53 g). The residual fraction was dissolved in distilled water in a boiling water bath for 4 h. The aqueous phase was evaporated and reduced to half its volume and then mixed with 95% ethanol (1:4, v/v), and the precipitated fraction was freeze-dried (Fb, 8.5 g). The aqueous phase was then evaporated to remove the ethanol and mixed with ethyl acetate (2:1, v/v). The upper and lower aqueous phases were then evaporated and lyophilized (Fc, 1.53 g; Fd, 3.9 g, respectively) (Fig. 1). The freeze-dried extracts were reconstituted in 80% ethanol (Fa, 100 mg/ml) and DPBS (Fb, Fc, Fd fraction, each 20 mg/ml). These stock solutions were sterilized by filtration through a 0.45 µm pore membrane (Sartorius AG, Goettingen, Germany) and kept in the dark at 4 °C.

2.5. Scavenging effect on DPPH radicals

Various concentrations of the stock solutions (diluted to final concentrations of 50, 10, and 5 µg/ml) were mixed with 0.25 mM DPPH in ethanol, to produce a final DPPH concentration of 0.1 mM. The mixture was vigorously shaken and left to stand for 10 min in the dark, and its absorbance was measured at 517 nm. L-Ascorbic acid was used as the control (McCune and Johns, 2002).

2.6. NBT/XO (superoxide scavenging) assay

The scavenging potential of the mushroom extract for superoxide radicals was analyzed using a hypoxanthine/xanthine oxidase generating system coupled with NBT reduction, as previously described (Kirby and Schmidt, 1997), with a slight modification involving the use of 96-well plates. Briefly, the reaction mixture contained 134 µl buffer (50 mM KH₂PO₄/KOH, pH 7.4), 2 µl of 100 mM Na₂EDTA, 20 µl of 3 mM hypoxanthine, 2 µl of 10 mM NBT, and 10 µl of extract. The microplates were read 2.5 min after adding 32 µl of xanthine oxidase (1 unit per 10 ml buffer) at 540 nm using an ELISA reader (TECAN, Salzburg, Austria). The superoxide scavenging activity was expressed as the percentage inhibition compared to the blank (buffer instead of extract). L-Ascorbic acid was used as a positive control.

2.7. DCF/AAPH assay

An azo initiator, AAPH, was used to produce peroxy radicals, and the scavenging activity of the mushroom extracts was monitored via the spectrophotometric analysis of 2,7-dichlorofluorescein-diacetate (Valkonen and Kuusi, 1997). The activation of DCF was achieved by mixing DCF (3.41 µl of 50 µg/ml solution) and NaOH (1.75 ml of 0.01N solution) and allowing the mixture to stand for 20 min before adding 18.25 ml of sodium phosphate buffer (25 mM, pH 7.2) (Cathcart et al., 1983). The reaction mixture contained

10 µl of extract (diluted to final concentrations of 50, 10, and 5 µg/ml), 170 µl activated DCF solution and 20 µl of 600 mM AAPH (adjusted to a final concentration of 60 mM). The reaction was initiated by adding the AAPH solution. After 10 min, the absorbance was read at 490 nm using an ELISA reader (TECAN, Salzburg, Austria). The inhibition rate was determined versus L-ascorbic acid.

2.8. Protective effects of *Inonotus obliquus* extract after treatment with hydrogen peroxide

Human HaCaT cells were seeded in 96-well plates at a density of 6000 cells/well. The cells were grown to near 70–80% confluence, and then synchronized by incubation in DMEM containing 0.5% FBS for 8 h. *Inonotus obliquus* extracts were added at concentrations of 10 and 50 µg/ml, respectively. After 16 h, the cells were washed and 1 mM of hydrogen peroxide was added. The cell viability was measured by means of the MTT assay. To compare the data, the cytotoxic effects of *Inonotus obliquus* extracts were also evaluated. L-Ascorbic acid and catalase were used as positive controls.

2.9. Statistical analysis

The significance of the differences between the results was assessed using the Student's *t*-test, and significance was accepted for *p*-values <0.01.

3. Results

3.1. Scavenging of DPPH radicals by *Inonotus obliquus*

Four extracts were prepared from *Inonotus obliquus* as described in the Section 2 (Fig. 1). The different extracts showed variable DPPH radical-scavenging activities. The Fa and Fc extracts exerted free radical scavenging effects in a dose-dependent manner. In particular, the Fc (polyphenolic) extract showed strong antioxidant activity at concentrations higher than 5 µg/ml, but its effect was less than that of L-ascorbic acid (Table 1). The Fa extract, which contained triterpenoids and steroids, also had a relatively strong antioxidant effect. The Fb extract, containing soluble polysaccharides, and the Fd extract, containing remnant polyphenolic compounds and low molecular weight polysaccharides, were almost inactive (Table 1).

3.2. NBT/XO (superoxide scavenging) assay

The superoxide radical scavenging activity of the extracts was investigated (Table 1). All of the extracts showed dose-dependent superoxide radical scavenging activity. In particular, the Fc extract had the highest scavenging activity, which was higher than that of L-ascorbic acid.

Table 1
Scavenging effects of *Inonotus obliquus* extracts for DPPH, superoxide and peroxy radicals

Radical and extract concentration ($\mu\text{g/ml}$)	Fa	Fb	Fc	Fd	L-Ascorbic acid
DPPH					
5	12.4 \pm 2.6	4.5 \pm 0.9	30.6 \pm 1.8	4.9 \pm 0.9	75.2 \pm 1.0
10	22.2 \pm 3.3	3.8 \pm 2.9	60.1 \pm 4.2	5.3 \pm 2.6	88.1 \pm 0.3
50	58.7 \pm 6.4	11.7 \pm 0.9	81.8 \pm 1.0	11.0 \pm 0.5	90.8 \pm 0.2
Superoxide radical					
5	19.9 \pm 11.7	9.8 \pm 9.9	55.1 \pm 1.7	33.5 \pm 6.8	26.3 \pm 4.9
10	28.8 \pm 12.9	38.1 \pm 12.3	80.9 \pm 2.9	55.1 \pm 4.3	44.9 \pm 8.8
50	61.9 \pm 3.3	73.3 \pm 4.3	91.5 \pm 1.4	75.0 \pm 3.5	78.8 \pm 1.0
Peroxy radical					
5	10.1 \pm 7.8	5.4 \pm 9.3	42.9 \pm 7.5	14.8 \pm 9.4	89.9 \pm 2.3
10	20.2 \pm 4.7	3.5 \pm 10.9	63.1 \pm 2.6	23.3 \pm 11.1	94.6 \pm 1.9
50	55.5 \pm 2.6	34.7 \pm 7.2	77.0 \pm 4.3	66.3 \pm 12.4	95.6 \pm 1.3

The scavenging effects were expressed as the percentage inhibition (mean \pm S.D., $n=4$) compared to the blank (buffer instead of extract). L-Ascorbic acid was used as a positive control. Fa: triterpenoids and steroids; Fb: polysaccharides; Fc: polyphenolic extract; Fd: remnant polyphenolic compounds and low molecular weight polysaccharides.

3.3. DCF/AAPH assay

The peroxy radical scavenging effects were also examined. All of the extracts showed dose-dependent activity. In particular, the Fc extract presented the strongest effect, although its scavenging effect was lower than that of L-ascorbic acid (Table 1).

3.4. Cytotoxic effects of *Inonotus obliquus* and hydrogen peroxide

The Fa and Fc extracts of *Inonotus obliquus* were cytotoxic at concentrations higher than 100 $\mu\text{g/ml}$. However, the Fb and Fd extracts were only cytotoxic at concentrations exceeding 400 $\mu\text{g/ml}$ (Fig. 2). Thus, we used concentrations lower than 100 $\mu\text{g/ml}$ for this experiment. To assess the protective effects of the extracts against hydrogen peroxide-induced cell death, hydrogen peroxide was added to cultured cells. The MTT assay showed that hydrogen peroxide was not cytotoxic below

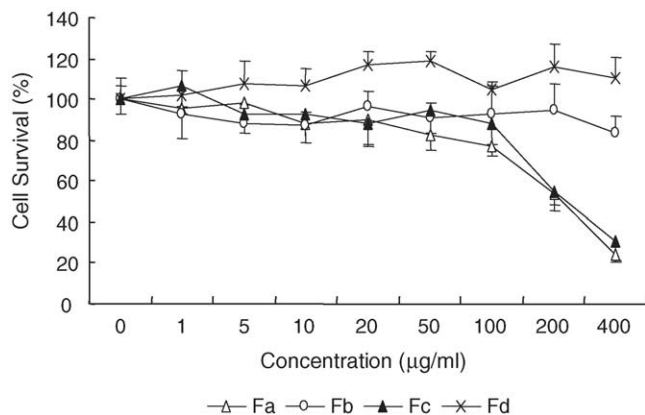


Fig. 2. Cytotoxic effects of *Inonotus obliquus* extracts on HaCaT cells. Cells were treated with the extracts for 24 h. Cell viability was measured by the MTT assay. Data are averages \pm S.D. (bars) of triplicate determinations.

0.5 mM, while treatment with 1 mM hydrogen peroxide resulted in significant cell death (Fig. 3). Based on these data, the cells were treated with 1 mM hydrogen peroxide and the extracts were added in order to assess their protective effects with regard to cell survival.

3.5. Protective effects of *Inonotus obliquus* extract

Hydrogen peroxide (1 mM) decreased the cell viability to 45.6% of that of the control. Catalase was strongly protective against hydrogen peroxide-induced cell death, but L-ascorbic acid showed only a negligible effect. The protective effects of *Inonotus obliquus* were also examined. The Fa and Fb extracts were not protective against hydrogen peroxide-induced oxidative stress. However, the Fc extract was strongly effective at a concentration of 50 $\mu\text{g/ml}$, and this effect was comparable to that of catalase at a concentration of 0.4 mg/ml. The Fd extract was slightly protective at a concentration of 50 $\mu\text{g/ml}$ (Fig. 4).

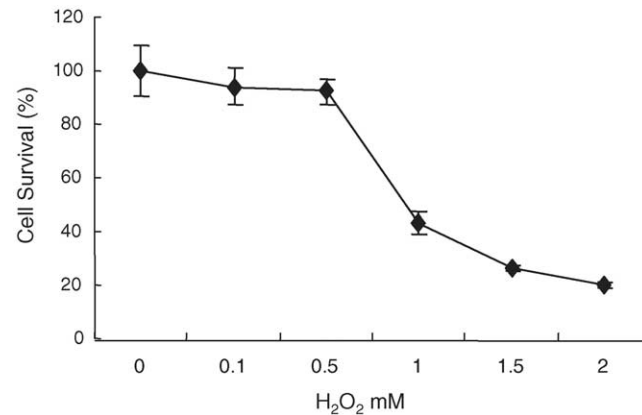


Fig. 3. MTT reduction assay of H₂O₂ cytotoxicity. HaCaT cells were treated with H₂O₂ at different concentrations for 6 h. Data are averages \pm S.D. (bars) of triplicate determinations.

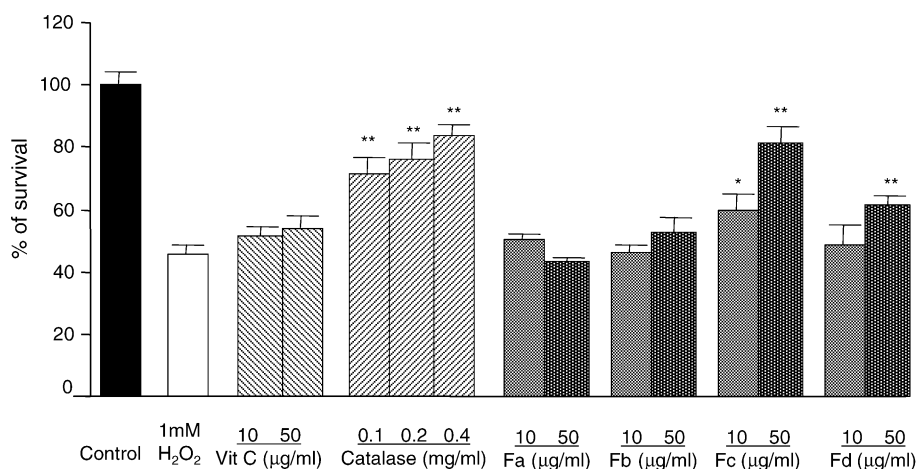


Fig. 4. Protective effects of *Inonotus obliquus* extracts against H₂O₂ (1 mM) induced oxidative stress as determined by the MTT assay. (**p* < 0.01, ***p* < 0.001 vs. H₂O₂ alone).

4. Discussion

In the present study, we found that *Inonotus obliquus* exerts an antioxidant activity and protects cells against oxidative stress. These findings provide a pharmacological explanation for some of its uses in folk medicine. *Inonotus obliquus* has long been used to improve overall health and prevent various diseases, such as cancer, cardiovascular disease and diabetes. In the literature, it has been reported that the antioxidant activity of plants is responsible for their therapeutic effect against cancer, cardiovascular disease and diabetes (Anderson et al., 2004; Stanner et al., 2004). Thus, the action of *Inonotus obliquus* seems to be at least partially associated with its antioxidant effect.

Traditionally, *Inonotus obliquus* has been taken in the form of a hot water extract prepared from a small piece of the mushroom (1–2 g) or one tablespoon of crushed mushroom. This produces an aqueous extract, which is taken at a dose of three cups per day. In this study, we showed that the polyphenolic extract had the strongest antioxidant activity among the four kinds of extracts, as well as exhibiting a strong protective activity against hydrogen peroxide-induced cell damage at a concentration of 10–50 µg/ml. Our study showed that 1.5 g of the Fc extract can be obtained from 100 g of total weight of the mushroom and that the Fc extract was not cytotoxic at these doses. These data showed that only a small amount of the polyphenolic components could be obtained by the traditional methods of extract preparation.

The different extracts of *Inonotus obliquus* showed variable radical scavenging activities. The Fc extract, which contains polyphenolic components, showed strong anti-oxidant activity, while the Fa extract, which contained triterpenoids and steroids including lanosterol, inotodiol, trametenolic acid and ergosterol peroxide (Kahlos et al., 1989), also had a relatively strong antioxidant effect. Triterpenoids and steroids have been previously isolated from *Inonotus obliquus* (He et al., 2001), but their activities have not previously been reported. In our experiment, the Fa extract showed a rela-

tively high DPPH radical scavenging activity. These results suggest that the triterpenoids and steroids in the Fa extract may account for the free radical scavenging effect of *Inonotus obliquus* (Kim et al., 1999). Mau et al. (2002) reported that the methanolic extracts obtained from several medicinal mushrooms, such as *Ganoderma lucidum* and *Ganoderma tsugae*, showed high DPPH free radical scavenging activity. However, this scavenging activity (67.6–74.4%) was only obtained at a relatively high concentration (0.64 mg/ml). In contrast, the scavenging activities of the Fa (58.7%) and Fc (81.8%) extracts of *Inonotus obliquus* were effective at a lower concentration (50 µg/ml) in our study. On the other hand, the Fb polysaccharide extract and the Fd extract, containing remnant polyphenolic compounds and low molecular weight polysaccharides, were almost inactive.

The superoxide radical scavenging activity of the *Inonotus obliquus* extracts was also generally quite high. This was especially the case for the Fc extract, whose activity was higher than that of L-ascorbic acid. It is well known that polyphenolic compounds are able to efficiently scavenge superoxide radicals (Valentao et al., 2002). These phenolic compounds may react with the superoxide radical via a one-electron transfer mechanism or by a hydrogen abstraction mechanism to form the corresponding semiquinone (Wang et al., 1996). Furthermore, polyphenolic crude extracts are known to have a certain inhibitory activity towards xanthine oxidase (Costantino et al., 1992).

Among the four extracts, the polysaccharide extract, Fb, also showed superoxide radical scavenging activities. It has been reported that the polysaccharide extracts of *Ganoderma lucidum* and *Grifola umbellata* possess superoxide radical scavenging activity (Liu et al., 1997). The superoxide radical scavenging activity of polysaccharide extracts appears to depend on the amount of peptides present in the form of polysaccharide–peptide complexes. For example, lentinan and schizophyllan, which contain only trace amounts of peptides in the polysaccharide samples, have almost no scavenging effect, whereas polysaccharide krestin and polysac-

charopeptide, which are obtained from mushrooms, such as *Ganoderma lucidum* and *Grifola umbellata* which have lower polysaccharide/peptide ratios, exhibited the strongest scavenging effects (Liu et al., 1997). However, the mechanism underlying the free radical scavenging activity exerted by polysaccharides is still not fully understood.

The protective effect exerted by the extracts on hydrogen peroxide-induced cell death was assessed. The results showed that the polyphenolic extract showed strong antioxidant activity and was the most strongly protective against hydrogen peroxide-induced cell damage. The effect of the polyphenolic extract at a concentration of 50 µg/ml was comparable to that of catalase at a concentration of 0.4 mg/ml. In contrast, L-ascorbic acid showed negligible effects. The Fa and Fb extracts did not show any protective effect against hydrogen peroxide-induced oxidative stress, although they showed some free radical scavenging ability. These results suggest that hydrogen peroxide-induced cell death is related not only to free radicals, but also to unresolved signaling pathways.

Our results and many previous reports show that the polyphenolic extracts of various plant and fungal species, including *Inonotus obliquus*, have strong antioxidant activity. However, the prooxidant and cytotoxic effects of these phenolic components have been also reported in the literature (Liu et al., 1997). High concentrations of phenolic compounds may inhibit cell proliferation, and simultaneous exposure to hydrogen peroxide and phenolics has been shown to lead to the amplification of proliferation inhibition (Liu et al., 1997). However, in our study, a significant protective effect was observed upon pretreatment with the polyphenolic extract of *Inonotus obliquus*. This discrepancy may arise from several factors, such as the extract concentration, experimental design, and the relationship between polyphenolic compounds and hydrogen peroxide.

In conclusion, the polyphenolic extract shows the strongest antioxidant activity among the four kinds of extracts obtained from *Inonotus obliquus*, and can protect cells against oxidative stress. It has been reported that the fungal melanin complex of *Inonotus obliquus* has strong antioxidant activity. These findings suggest that the Fc extract may contain a strongly antioxidant melanin complex or related polyphenolics (Babitskaia et al., 2000).

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