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Antimutagenic effects of subfractions of Chaga mushroom (*Inonotus obliquus*) extract

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ABSTRACT

Inonotus obliquus is a mushroom commonly known as Chaga that is widely used in folk medicine in Siberia, North America, and North Europe. Here, we evaluated the antimutagenic and antioxidant capacities of subfractions of *Inonotus obliquus* extract. The ethyl acetate extract was separated by vacuum chromatography into three fractions, and the fraction bearing the highest antimutagenic activity was subsequently separated into four fractions by reversed phase (ODS-C_{18}) column chromatography. The most antimutagenic fraction was then separated into two subfractions (subfractions 1 and 2) by normal phase silica gel column chromatography. Ames test analysis revealed that the subfractions were not mutagenic. At 50 μg/plate, subfractions 1 and 2 strongly inhibited the mutagenesis induced in *Salmonella typhimurium* strain TA100 by the directly acting mutagen MNNG (0.4 μg/plate) by 80.0% and 77.3%, respectively. They also inhibited 0.15 μg/plate 4NQO-induced mutagenesis in TA98 and TA100 by 52.6–62.0%. The mutagenesis in TA98 induced by the indirectly acting mutagens Trp-P-1 (0.15 μg/plate) and B(α)P (10 μg/plate) was reduced by 47.0–68.2% by the subfractions, while the mutagenesis in TA100 by Trp-P-1 and B(α)P was reduced by 70.5–87.2%. Subfraction 1 was more inhibitory than subfraction 2 with regard to the mutagenic effects of 4NQO, Trp-P-1, and B(α)P. Subfractions 1 and 2 also had a strong antioxidant activity against DPPH radicals and were identified by MS, ^{1}H NMR and ^{13}C NMR analyses as 3β-hydroxy-lanosta-8, 24-dien-21-al and inotodiol, respectively. Thus, we show that the 3β-hydroxy-lanosta-8, 24-dien-21-al and inotodiol components of *Inonotus obliquus* bear antimutagenic and antioxidative activities.

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1. Introduction

Recent studies have revealed the presence of natural bioactive materials in many different plant species worldwide and the antimutagenicity of these materials is currently under active investigation. In particular, the extracts of various mushrooms have been found to have anti-inflammatory, anti-tumor and anti-mutagenic properties [1,2].

The Chaga mushroom (*Inonotus obliquus*) is a white rot fungus that belongs to the Hymenochaetaceae family of Basidomycetes. Chaga mushrooms have been used in folk medicine for treating cancer in Russia, Western Siberia, Asia, and North America [3]. It has been shown that Chaga mushrooms contain many polyphenolic compounds and show various biological activities, including anti-bacterial [4], hepato-protective [5] and anti-tumor [6–8] properties.

In a previous study, we reported that the ethanol extract, ethyl acetate fraction, water fraction, and water-soluble and insoluble polysaccharides I and II of *Inonotus obliquus* have strong antimutagenic and antioxidative effects and inhibit genotoxicity [2,9]. We used crude fractions in our previous experiment [2]. We think that the crude fraction may have several bioactive compounds. We suggested that further studies are needed to determine whether the individual bioactive compounds in the ethyl acetate fraction could have the antimutagenic effects and further investigations characterizing the chemical structure of the individual bioactive components in the ethyl acetate fraction are needed.

Here, in an effort to identify the antimutagenic compounds in Chaga mushrooms, we subfractionated the ethyl acetate fraction of

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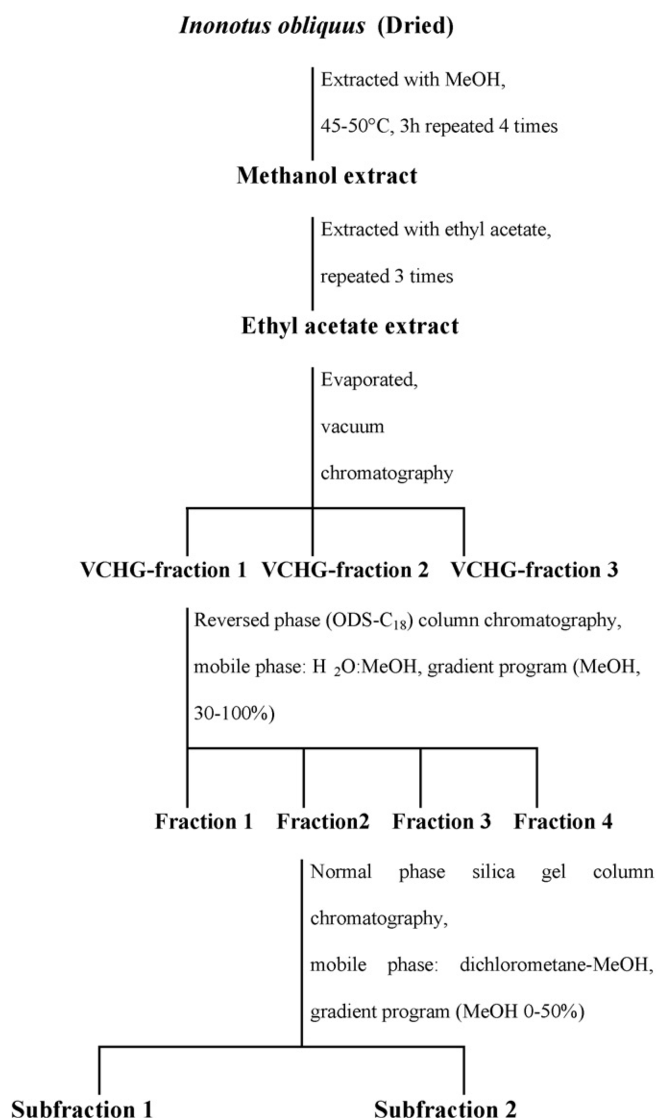

Fig. 1. Preparation of *Inonotus obliquus* subfractions.

these mushrooms by sequential vacuum chromatography, reversed phase (ODS-C_{18}) column chromatography, and normal phase silica gel column chromatography (Fig. 1). We used the subfractions for this study and the purity was confirmed by thin-layer chromatography (TLC), mass spectrometry (MS), ^{1}H nuclear magnetic resonance (^{1}H NMR) and ^{13}C nuclear magnetic resonance (^{13}C NMR) analyses (data not shown). The compounds 1 and 2 (subfractions 1 and 2) had a purity of over 99.5%. We tested the subfractions 1 and 2 each containing only a single compound for *in vitro* antioxidative and antimutagenic activities. The chemical structures of the compounds in the final subfractions bearing the antimutagenic property were characterized by MS, ^{1}H NMR and ^{13}C NMR analyses.

2. Materials and methods

2.1. Materials

D-biotin, 4-nitroquinoline-1-oxide (4NQO), N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), β-NADP, glucose-6-phosphate (G-6-P), gentamycin sulfate, benzo(α)pyrene [B(α)P], 3-amino-1,4-dimethyl-5H-pyrido-(4,3-b)indol (Trp-P-1), and L-histidine were purchased from Hwa-Kwang Chemical (Japan). Nutrient broth was purchased from Difco Laboratory (USA). 2,2-diphenyl-1-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), ascorbic acid, and α-tocophenol were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The rat liver enzyme S-9 (1254 Aroclor induced in 0.154 M KCl) was purchased from Molecular Toxicology Inc. (Boone, NC28607, USA).

2.2. Extraction of inonotus obliquus and isolation of its active antimutagenic compounds

The *Inonotus obliquus* specimen used in this experiment was imported from Russia, dried, powdered, and stored at −20 °C. The powdered specimen (500 g) was extracted twice with 3 L of methanol (99.8%) at 45–50 °C for 3 h. The methanol fraction (45 g) was then rotary evaporated, emulsified, dissolved in water, and extracted three times with ethyl acetate (H_2O:EtOAc, 5:7, v/v). The ethyl acetate fraction (12 g) was obtained after rotary evaporation and the dried extract was reconstituted in methanol. This stock solution was subjected to vacuum chromatography (N–2N, Eyela, Tokyo, Japan) with silica gel (60 g, 230–400 mesh) mixed with dichloromethane. The mobile phases used were A, dichloromethane and B, methanol, with a gradient of increasing methanol (0–100%) in dichloromethane and re-equilibration of the column with 100% A for 3 min prior to the next run (Fig. 1). The success of the fractionation was confirmed by TLC. Of the three ethyl acetate extract fractions, the most bioactive fraction, namely, VCHG-fraction 1, was selected and subjected to reversed phase (ODS-C_{18}) column chromatography using a reversed phase column (ODS-C_{18}) containing LiChroprep RP-18 (25–40 μm) mixed with H_2O:MeOH (1:30, v/v) in a gradient program ranging from 30% methanol to 100% methanol (mobile phase A: methanol; mobile phase B: ultra pure water). Four fractions were isolated, of which fraction 2 contained the most bioactivity. Fraction 2 was further fractionated by chromatography with a normal phase silica gel column (2.4 cm × 15 cm) containing silica gel (200 g, 70–230 mesh; Merck Co.) mixed with dichloromethane eluted by a mobile phase gradient ranging from 100% dichloromethane to 50% methanol. The flow rate was 1.0 mL/min (Fig. 1). This resulted into two subfractions designated as subfractions 1 and 2, both of which showed bioactivity. The compounds in subfractions 1 and 2 were crystallized and analyzed by determining their MS (JEOL), ^{1}H NMR and ^{13}C NMR (Bruker DRX 500 MHz) spectra.

2.3. Confirmation for the ames test

The LT-2 TA98 and TA100 histidine-demanding auxotrophs of *Salmonella typhimurium* were obtained from B.N. Ames, California (USA). The histidine requirement, presence of the rfa and uvrB mutations, and R-factor genetics of these strains were determined according to the method of Maron and Ames [10].

2.4. Metabolic activation system

The rat liver enzyme S-9 was prepared at +4 °C immediately before use and maintained at this temperature until it was added to the overlay agar. The final composition of the S-9 mixture was as follows: $MgCl_2$–KCl salt solution (1.65 M KCl + 0.4 M $MgCl_2$) (0.2 mL), 0.2 M sodium phosphate buffer pH 7.4 (5 mL), 0.1 M nicotinamide adenine dinucleotide (0.4 mL), 1 M glucose-6-phosphate (G-6-P) (0.05 mL), S-9 fraction (1 mL), sterile distilled water (3.35 mL).

2.5. Mutagenicity assay

The mutagenicity of the *Inonotus obliquus* extract subfractions was assayed according to the method of Ames using *Salmonella typhimurium* strains TA98 and TA100 [10]. A solution of 0.5 mL of S-9 mixture, 0.1 mL of overnight-cultured *Salmonella typhimurium* TA98 or TA100, and 0.1 mL of the *Inonotus obliquus* specimen was prepared and preincubated at 37 °C for 20 min. Subsequently, 2 mL of molten top agar supplemented with L-histidine and D-biotin at 45 °C was added to the mixture. The mixture was then gently mixed and poured onto minimal glucose agar plates. The plates were inverted and incubated at 37 °C for 48 h. To test the mutagenicity of the subfractions from *Inonotus obliquus* extracts, each experiment was performed at least in duplicate. Colony counting occurred after 48 h and the inhibition ratio was calculated by using the following formula:

$$\text{Inhibition ratio}(\%) = \left[\frac{(A-C)}{(A-B)}\right] \times 100$$

where *A* is the number of histidine revertants induced by the mutagen alone, *B* is the number of revertants induced in the presence of sample solution and solvent (negative control), and *C* is the number of histidine revertants induced by the mutagen in the presence of the *Inonotus obliquus* sample.

2.6. Antimutagenicity assay

The antimutagenicity of the *Inonotus obliquus* subfractions was assayed by using the Ames test as described above except mutagen was added before the preincubation step. The mutagens used were the directly acting mutagens MNNG and 4NQO, and the indirectly acting mutagens Trp-P-1 and B(α)P, which require the S-9 mixture for metabolic activation. In the assay, 50 μL of the *Inonotus obliquus* subfractions at different concentrations were added to 50 μL of direct or indirect

mutagens and, if bioactivation was necessary, 250 μL of S-9 mixture was also added. Subsequently, 100 μL of preincubated *S. typhimurium* was added to the mixture, 0.2 M sodium phosphate buffer was added to bring the final volume to 700 μL, and the mixture was incubated at 37 °C for 20 min. The remainder of the protocol proceeded according to the mutagenicity test described above. Each sample was assayed using triplicate plates per run, and the data are presented as the means of three experiments conducted using *Inonotus obliquus* subfractions from different batches.

2.7. DPPH radical-scavenging activity

The ability of the *Inonotus obliquus* subfractions to scavenge DPPH radicals was measured according to the method of Hatano et al. [11] with slight modifications. Different concentrations (5–500 μg/mL) of the subfractions, ascorbic acid, tocopherol and BHA were placed in different test tubes (all at 4 mL) and mixed with 1 mL of methanolic solution containing DPPH radicals (1.5×10^{-4} M). The mixture was shaken vigorously and left to stand in the dark for 30 min. The DPPH radical levels were measured at 517 nm. The radical scavenging activity was measured as the decrease in the absorbance of DPPH and was expressed as the EC_{50} value, which is defined as the amount (μg/mL) of the sample that scavenges 50% of the DPPH radicals in the experimental system. This value was calculated from the log-dose inhibition curve. Ascorbic acid, tocopherol and BHA served as standards.

2.8. Statistical analysis

The data are expressed as means ± SD (standard deviation) and are the average values of three to five values per experiment. The analyses were performed by using the SPSS package (Version 10.0, SPSS, Chicago, USA). The experiments were repeated at least twice to confirm the results. Analysis of variance (ANOVA) was conducted, and Duncan's multiple-range tests were used to determine the significance of differences between groups. The level of statistical significance was set to $P < 0.05$.

3. Results and discussion

3.1. Analysis of the mutagenicity of Inonotus obliquus subfractions by using the Ames test

As shown in Fig. 1, the ethyl acetate fraction of *Inonotus obliquus* was separated into three fractions by vacuum chromatography. Analysis of the antimutagenicity of these fractions by using the Ames test revealed the first fraction had the most antimutagenicity (data not shown). This fraction was then further separated into four fractions by reversed phase (ODS-C_{18}) column chromatography, of which the second fraction showed the greatest antimutagenicity (data not shown). This fraction was in turn separated by normal phase silica gel column chromatography into two subfractions.

The subfractions were spotted on TLC and were analyzed for confirmation of single compound purity and the subfractions 1 and 2 showed a single spot on TLC (data not shown). The compounds 1 and 2 (subfractions 1 and 1) had a purity of over 99.5%. The purity was also confirmed by MS, ^{1}H NMR and ^{13}C NMR analyses. The subfractions 1 and 2 from the ethyl acetate fraction were only single compounds. We used the pure single compound for this study.

These subfractions were analyzed further. First, we examined their mutagenicity by using the Ames test employing the TA98 and TA100 strains of *Salmonella typhimurium*. As shown in Table 1, in the absence of the *Inonotus obliquus* subfractions, there were 18 ± 3 and 176 ± 7 TA98 and TA100 revertants per plate, respectively. These spontaneous mutation frequencies did not change in the presence of the *Inonotus obliquus* subfractions, even at the highest concentration of 50 μg/plate. Thus, the *Inonotus obliquus* subfractions are not mutagenic.

3.2. Analysis of the antimutagenicity of Inonotus obliquus subfractions against directly acting mutagens

We analyzed the antimutagenic properties of the *Inonotus obliquus* subfractions against the direct mutagens MNNG and 4NQO by using the Ames method. The *Salmonella typhimurium* strain TA100 is susceptible to MNNG-induced mutagenesis while both TA100 and TA98 are susceptible to 4NQO-induced mutagenesis. Subfractions 1 and 2 at 50 μg/plate inhibited the mutations induced by MNNG (0.4 μg/plate) in *S. typhimurium* TA100 by 80.0% and 77.3%, respectively (Fig. 2), which indicates a relatively strong antimutagenic effect. Subfractions 1 and 2 at 50 μg/plate also inhibited the mutagenicity of 4NQO (0.15 μg/plate) in TA98 by 61.3% and 52.6%, respectively, and in TA100 by 62.0% and 52.6%, respectively (Fig. 3).

Table 1
Mutagenicity of the *Inonotus obliquus* subfractions.

Dose (μg/plate)	His^{+} revertants/plate[a]	
	TA98	TA100
Spontaneous	18 ± 3	176 ± 7
Subfraction 1		
12.5	17 ± 3	176 ± 8
25.0	18 ± 4	174 ± 5
37.5	20 ± 1	178 ± 6
50.0	18 ± 1	176 ± 5
Subfraction 2		
12.5	16 ± 2	178 ± 8
25.0	19 ± 4	179 ± 1
37.5	18 ± 1	177 ± 7
50.0	17 ± 3	176 ± 8

[a] Each value is the mean ± SD of triplicates.

3.3. Analysis of the antimutagenicity of Inonotus obliquus subfractions against indirectly acting mutagens

We determined the ability of the subfractions to prevent the mutagenicity of the indirect mutagens Trp-P-1 and B(α)P. Both TA98 and TA100 are susceptible to the mutagenic effects of Trp-P-1 and B(α)P when cultivated in their presence with the S-9 rat enzyme system. The *Inonotus obliquus* subfractions 1 and 2 at 50 μg/plate reduced the mutagenic effect of 0.15 μg/plate Trp-P-1 in *S. typhimurium* TA98 by 68.2% and 48.4%, respectively (Fig. 4). They also inhibited the mutagenic activity of Trp-P-1 in *S. typhimurium* TA100 by 82.4% and 73.3%, respectively (Fig. 4). Subfractions 1 and 2 at 50 μg/plate also inhibited the mutagenesis

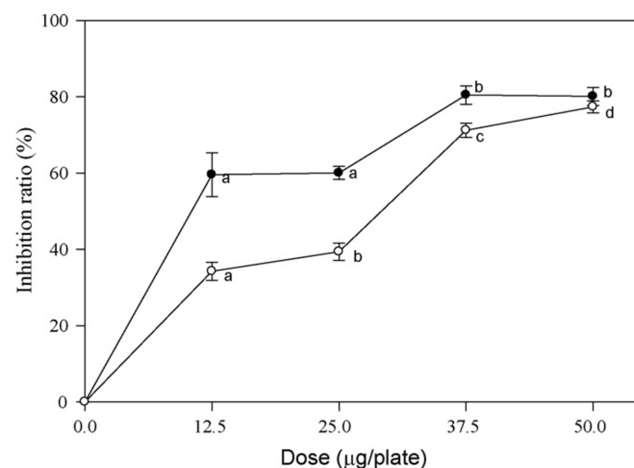

Fig. 2. Ability of *Inonotus obliquus* subfractions 1 and 2 to prevent MNNG-induced mutations in *Salmonella typhimurium* TA100. MNNG was used at 0.4 μg/plate. ●: subfraction 1; ○: subfraction 2. The results are expressed as means ± SD ($n = 3$). (a–d) Means with different letters differ significantly ($P < 0.05$), as determined by Duncan's multiple-range test.

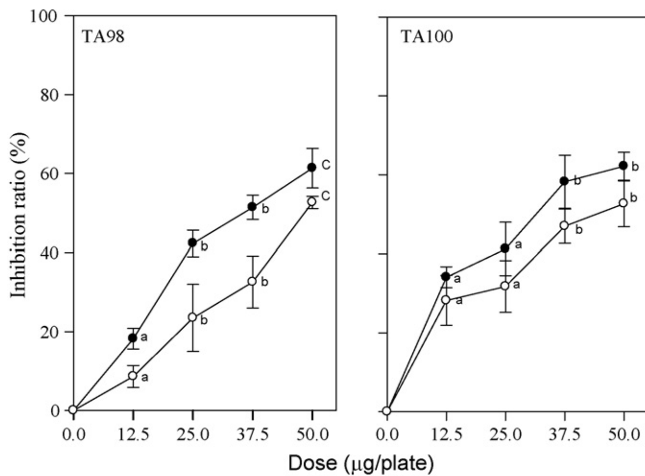

Fig. 3. Ability of *Inonotus obliquus* subfractions 1 and 2 to prevent 4NQO-induced mutations in *Salmonella typhimurium* TA98 and TA100. 4NQO was used at 0.15 μg/plate. ●: subfraction 1; ○: subfraction 2. The results are expressed as means ± SD (n = 3). (a–c) Means with different letters differ significantly (P < 0.05), as determined by Duncan's multiple-range test.

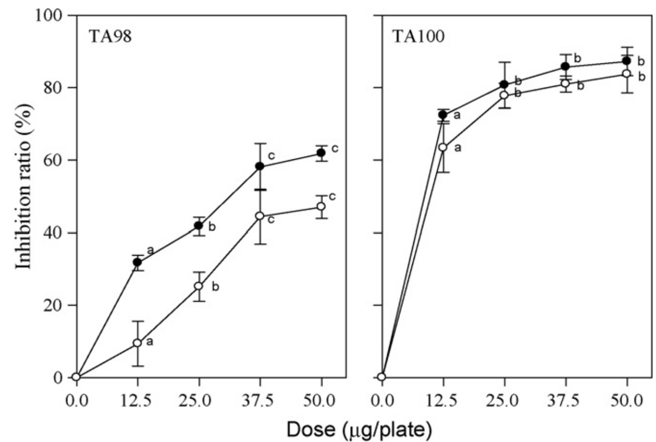

Fig. 5. Ability of *Inonotus obliquus* subfractions 1 and 2 to prevent B(α)P-induced mutations in *Salmonella typhimurium* TA98 and TA100. B(α)P was used at 10 μg/plate. ●: subfraction 1; ○: subfraction 2. The results are expressed as means ± SD (n = 3). (a–c) Means with different letters differ significantly (P < 0.05), as determined by Duncan's multiple-range test.

induced by B(α)P (10 μg/plate) in *S. typhimurium* TA98 by 61.8% and 47.0%, respectively, and in *S. typhimurium* TA100 by 87.2% and 83.6%, respectively (Fig. 5).

Several anti-cancer drugs or cancer treatment compounds have been found in natural products. The mushroom *Inonotus obliquus* examined in this experiment is a kind of cancer-causing germ of black oak trees in Russia and its bioactive properties have been studied since 1958. Bulatov et al. [12] and Ham et al. [2] have found that *Inonotus obliquus* extract bears antitumorigenic and antimutagenic properties. Here, we showed for the first time that the pure compounds (subfractions) isolated from the ethyl acetate fraction of *Inonotus obiquus* relatively strongly inhibit the mutagenic activity of both directly and indirectly acting mutagens in a dose-dependent manner. This suggests that these subfractions can protect the DNA or RNA in cells from mutagens. Alternatively, with regard to the indirectly acting mutagens, the subfractions may help inactivate these mutagenic precursors by hindering their transformation into carcinogens.

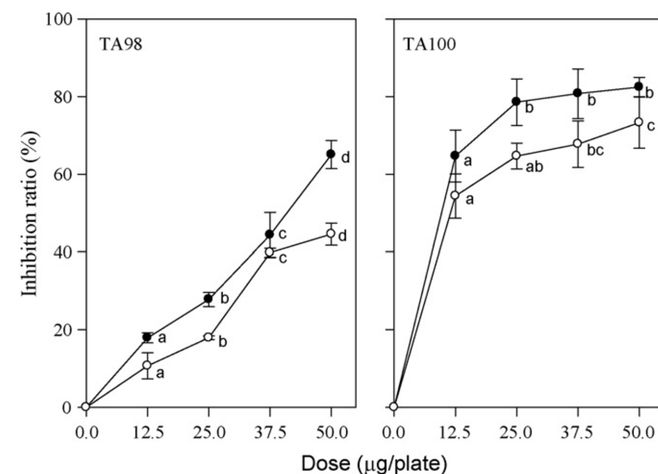

Fig. 4. Ability of *Inonotus obliquus* subfractions 1 and 2 to prevent Trp-P-1-induced mutations in *Salmonella typhimurium* TA98 and TA100. Trp-P-1 was used at 0.15 μg/plate. ●: subfraction 1; ○: subfraction 2. The results are expressed as means ± SD (n = 3). (a–d) Means with different letters differ significantly (P < 0.05), as determined by Duncan's multiple-range test.

Ji et al. [13] found that *Agaricus blazei Murill* mushroom extract had a strong antimutagenic effect, particularly against the direct mutagen 4NQO. In particular, the methanol extract of *Agaricus blazei Murill* (200 μg/plate) inhibited 4NQO mutagenicity in TA100 very strongly (94.7%). Ham et al. [2] also noted that the ethanol, ethyl acetate, and water extracts of *Inonotus obliquus*, along with its water soluble and insoluble polysaccharide I and II, inhibited the mutagenicity of directly and indirectly acting mutagens by more than 90%. These observations are relatively consistent with our own observations. Thus, subfractions of *Inonotus obliquus* extracts possess a strong antimutagenic effect and may be useful as an ingredient in functional anticancer food.

3.4. *DPPH radical scavenging activity of the Inonotus obliquus subfractions*

In general, compounds that strongly inhibit the mutagenicity of indirectly and directly acting mutagens also have a high free radical scavenging activity. Consequently, we determined the antioxidative activity of the *Inonotus obliquus* subfractions by using DPPH, which is a stable free radical that has been widely used as a substrate to evaluate the antioxidative activity of samples [14,15]. We observed that subfraction 1 scavenged the DPPH radicals at higher levels than subfraction 2, as their IC_{50} values were 69.1 ± 8.1 and 360.4 ± 12.1 μg/mL, respectively (Table 2). However, the DPPH radical scavenging abilities of the subfractions were significantly lower than those of tocopherol, BHA and L-ascorbic acid (12.9 ± 0.6, 14.8 ± 0.7 and 22.2 ± 0.8 μg/mL, respectively), which served as positive controls (Table 2).

That *Inonotus obiquus* subfractions bear DPPH radical scavenging activity is consistent with a previous report showing that the

Table 2
EC_{50}^{DPPH} values of *Inonotus obliquus* subfractions.

Antioxidant	EC_{50}[a] (μg/mL)
Subfraction 1	69.1 ± 8.1
Subfraction 2	360.4 ± 12.1
Tocopherol	12.9 ± 0.6
BHA	14.8 ± 0.7
Ascorbic acid	22.2 ± 0.8

[a] EC_{50} is the concentration required to reduce the DPPH levels by 50% after 30 min.

ethanol extract, ethylacetate fraction, and water fraction of *Inonotus obliquus* and its insoluble polysaccharides I and II have DPPH radical scavenging activity [9]. Moreover, Lee et al. [16] and Cui et al. [17] have reported that the pure compounds (polyphenols) isolated from the methanolic extract of *Inonotus obiquus* have DPPH radical-scavenging activity, as do its 80% ethanol extract, water-soluble polysaccharide fraction, water layer, and polyphenolic extract.

3.5. Structural analysis of the Inonotus obliquus subfractions

As shown in Fig. 1, the subfractions containing pure compounds were obtained after reversed phase (ODS-C_{18}) column chromatography of the ethyl acetate extract of *Inonotus obliquus*, followed by normal phase silica gel column chromatography (Fig. 1). The chemical structures of the compounds constituted by subfractions 1 and 2 were determined by comparing their MS, ^{1}H-NMR and ^{13}C-NMR spectra to published library spectra and spectra in the literature [18–20] (data not shown). These analyses revealed that subfractions 1 and 2 are the triterpenes 3β-hydroxy-lanosta-8, 24-dien-21-al and inotodiol, respectively. Thus, 3β-hydroxylanosta-8,24-diene-21-al and inotodiol appear to be bioactive compounds of *Inonotus obliquus* that are responsible, at least in part, for the antimutagenic and antioxidative properties of this mushroom.

Our observations are consistent with those in other studies. Shivrina [21] reported that the steroids and aromatic polyphenol compounds found in *Inonotus obliquus* had strong bioactivity. Moreover, *Inonotus obliquus* appears to have an abundance of triterpenes, including lanosterol and inotodiol [22] and betulin [18]. Loviagina and Shivrina [23] also suggested that the inotodiol component of *Inonotus obliquus* has anticancer properties, while its lanosterol, ergosterol and triterpene alcohol components may delay tumorigenicity. Moreover, they found that the triterpene acids in *Inonotus obliquus* may inactivate cancer cells.

Conflict of interest statement

None.

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