

Original Research

# Identification of Major Antioxidant Compounds from the Edible Mushroom *Basidiomycetes-X* (Echigoshirayukidake)

Toshio Sakamoto<sup>1,2,\*</sup>, Zhenshi Li<sup>2</sup>, Ayaka Nishida<sup>2</sup>, Asako Kadokawa<sup>2</sup>, Takayuki Yoshida<sup>3</sup>, Naoki Wada<sup>1,2</sup>, Seiichi Matsugo<sup>2</sup>, Yutaka Nakamura<sup>4</sup>, Shinji Sato<sup>4</sup>, Tetsuya Konishi<sup>4,5</sup>

<sup>1</sup>School of Biological Science and Technology, College of Science and Engineering, Kanazawa University, Kakuma, 920-1192 Kanazawa, Japan

<sup>2</sup>Division of Natural System, Graduate School of Natural Science and Technology, Kanazawa University, Kakuma, 920-1192 Kanazawa, Japan

<sup>3</sup>Kanazawa Kids' Science Center, Naga-machi, 920-0865 Kanazawa, Japan

<sup>4</sup>Faculty of Applied Life Sciences, Niigata University of Pharmacy and Applied Life Sciences, Higashijima, Akiha-ku, 956-8603 Niigata, Japan

<sup>5</sup>Office HALD Food Function Research, Inc., Yuzawa, 949-6102 Niigata, Japan

\*Correspondence: [tsakamot@staff.kanazawa-u.ac.jp](mailto:tsakamot@staff.kanazawa-u.ac.jp) (Toshio Sakamoto)

Academic Editor: Baohong Zhang

Submitted: 26 November 2021 Revised: 13 January 2022 Accepted: 17 January 2022 Published: 6 April 2022

## Abstract

*Basidiomycetes-X*, of which Japanese vernacular name is Echigoshirayukidake, is a local speciality mushroom found and cultivated in Japan that has been distributed as a precious cuisine material or as a functional food with medicinal properties. Antioxidant activity-guided isolation of major ingredients in *Basidiomycetes-X* revealed the presence of ergosterol, *trans*-10,*cis*-12-octadecadienoic acid (a conjugated linolenic acid, 10(E),12(Z)-CLA) and 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP). Approximately 21% of the 2,2-diphenyl-1-(2,4,6-trinitrophenyl)hydrazino radical (DPPH) scavenging activities in the methanolic extract were related to 10(E),12(Z)-CLA, while approximately 6.2% of the activity was related to ergosterol. DDMP was present in both methanolic and water extracts, and the activity related to DDMP was conspicuously detected in water extracts. Moreover, uridine and adenosine were identified as major components of *Basidiomycetes-X*. The ingredients identified in *Basidiomycetes-X* are expected to be involved in biological functions observed in this mushroom, which is an attractive functional food resource.

**Keywords:** Adenosine; Conjugated linoleic acid (CLA); 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP); Ergosterol; Uridine; Radical scavenger

## 1. Introduction

Edible mushrooms are of interest for their nutritional value as well as their pharmacological characteristics [1], such as containing low-energy indigestible polysaccharides and having high antioxidant capacity [2]. Thus, mushroom extracts are used to promote human health. The antioxidant properties of mushroom extracts have been extensively studied; however, the antioxidative effects of the extracts are thought to be attributed to tocopherols, phenolic compounds, carotenoids, and ascorbic acid [3–9]. Mushroom polysaccharides such as  $\beta$ -glucan are also expected to be bioactive compounds with immunotherapeutic properties, but their action mechanisms remain to be elucidated [10], although the presence of the  $\beta$ -glucan receptor is currently reported on the surface of the cell membrane of immune cells such as macrophages and dendritic cells [11].

*Basidiomycetes-X* (Echigoshirayukidake) was originally discovered in Uonuma district, Niigata prefecture, Japan as an edible mushroom [12] and found to be a fungus strain genetically close to *Ceraceomyces tessulatus*. Since the water-soluble extract of *Basidiomycetes-X* protects lipopolysaccharide-induced hepatic oxidative damage in mice [13], functional studies have been conducted as follows. Oral administration of the aqueous ex-

tract of *Basidiomycetes-X* inhibits atopic dermatitis in a mouse model [14]. Recent studies in *Basidiomycetes-X* have shown potential anti-obesity functions [15,16] and hepatoprotective functions to prevent and ameliorate non-alcoholic steatohepatitis (NASH) in mice [17]. The dry powder of *Basidiomycetes-X* is expected to contain functional ingredients to improve fatty liver through antioxidation and inhibition of lipid peroxidation without serious side reactions [18]. *Basidiomycetes-X* water extracts exhibit high antioxidant abilities, including total phenolic content, DPPH radical scavenging activity,  $\text{Fe}^{3+}$ -reducing ability,  $\text{Cu}^{2+}$ -reducing ability, and  $\text{Fe}^{2+}$ -chelating activity (Matsugo S, Sakamoto T, Nishida A, Wada N, Konishi T. Pyrrole Compound. PCT/JP2018/043401 11/26/2018). We have previously identified pyrrole alkaloid derivatives as candidate active components in *Basidiomycetes-X* [19], however, these compounds have very little antioxidant activity detected.

In the present study, we further identified antioxidant ingredients in *Basidiomycetes-X* extracts assessed by their radical scavenging activity using DPPH as a substrate. As a result, ergosterol and conjugated linoleic acid (CLA) were found to be the major lipophilic antioxidative components, and 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-



4-one (DDMP) was found to be a hydrophilic antioxidative component. Moreover, pyrimidine and purine nucleosides, including uridine and adenosine, were identified as major components in *Basidiomycetes-X* water extracts.

## 2. Materials and Methods

### 2.1 Characterization of DPPH Radical Scavengers by TLC

Mycology Techno Co., Ltd. (Niigata, Japan) provided *Basidiomycetes-X* dry powder as starting materials. One gram of *Basidiomycetes-X* dry powder was suspended in 10 mL of the extraction solvents of water, methanol, chloroform-methanol (1:1, v/v) and 2-propanol, respectively. These suspensions were allowed to stand at room temperature for 4 h and mixed briefly every hour during the extraction. Debris was removed by centrifugation at  $890 \times g$  for 5 min and 10  $\mu\text{L}$  of the extracts were applied onto a TLC plate with a fluorescent dye (TLC Silica gel 60 F<sub>254</sub>, Merck, Darmstadt, Germany) and developed using chloroform-methanol-acetic acid (95:5:3, v/v) as a mobile phase. Under UV illumination at 254 nm, compounds that inhibited fluorescence from the dye on the TLC plate were detected. After drying, the TLC plate was immersed in 1.25 mM DPPH solution in methanol for activity staining to detect the components showing DPPH radical scavenging activity. The band intensity was measured using an image analysis software (ImageJ, developed by Rasband W at NIH, Bethesda, MD).

### 2.2 Purification of Compound I (Ergosterol)

Five-hundred grams of *Basidiomycetes-X* dry powder were divided into 4 portions, and each 125 g was suspended in 500 mL of 2-propanol and stirred for 20 min using a magnetic stirrer at room temperature. The first extracts were recovered by filtration using filter paper. Each residue on the filter paper was suspended again in 2-propanol (400 mL). The 2-propanol extraction was repeated four times, and in total, 4345 mL of 2-propanol extracts were recovered. The 2-propanol extracts were evaporated using a rotary evaporator, the dry matters were dissolved in 65 mL of chloroform, and chloroform-insoluble solid was removed by filtration using filter paper and condensed to 5 mL with a rotary evaporator. The chloroform-soluble fraction was applied to a silica gel column (Wakogel C-200, 75–150  $\mu\text{m}$ ,  $1.8 \times 15$  cm) and eluted with chloroform-methanol-acetic acid (95:5:3, v/v) as a developing solvent. The elute was recovered and the solvent was changed to 2 mL of chloroform after evaporation with a rotary evaporator. The chloroform-soluble fraction was applied to an alumina column (Wako active alumina, 200–300 mesh,  $1.8 \times 15$  cm) and eluted with chloroform as a developing solvent. The chloroform elute was recovered and the solvent was changed to 2 mL of hexane-ethyl acetate (8:2, v/v). The insoluble solid was removed by filtration through a 0.45- $\mu\text{m}$  PTFE syringe filter (RJF1345NH, Reptile), and the hexane-ethyl acetate soluble fraction was further purified using an HPLC system

with a normal phase column (Wakopak Wakosil 10 SIL,  $4.0 \times 300$  mm). The mobile phase was hexane-ethyl acetate (8:2, v/v) at a flow rate of  $0.6 \text{ mL min}^{-1}$ . The ergosterol-containing fraction showing a characteristic UV absorption spectrum with four peaks at 263, 272, 282 and 293 nm was recovered. The final product of ergosterol (4.5 mg) was dissolved in chloroform (**Supplementary Table 1**). The amount of ergosterol was spectrophotometrically determined using an absorption coefficient of  $0.97 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$  at 262 nm [20].

### 2.3 Measurement of Ergosterol Content by HPLC

One gram of *Basidiomycetes-X* dry powder was suspended in 4 mL of methanol and stirred for 20 min using a magnetic stirrer at room temperature. The methanol-insoluble solid was removed by filtration through filter paper. The filtrate was collected and the residue on the filter paper was once again resuspended in 3.2 mL of methanol. The methanol extraction was repeated three times. Debris was removed by filtration through a 0.45- $\mu\text{m}$  PTFE syringe filter and the ergosterol-containing methanol extract (20  $\mu\text{L}$ ) was analyzed using an HPLC system with a pump (PU-2087 Plus Intelligent Prep. Pump, JASCO) equipped with a reversed-phase column (Cosmosil 5C18-MS,  $4.6 \times 150$  mm, Nacalai). The mobile phase was 100% methanol at a flow rate of  $0.6 \text{ mL min}^{-1}$  for isocratic elution. The UV spectrum was recorded with a JASCO photodiode array detector (MD-2018 Plus). A<sub>281</sub> was traced to draw a chromatogram and ergosterol was identified by its UV absorption spectrum with characteristic four peaks at 263, 272, 282 and 293 nm. The amount of ergosterol was determined from a standard curve constructed with known amounts (20 to 80 ng) of the authentic ergosterol standard (Tokyo Chemical Industry Co., Ltd.).

### 2.4 Purification of Compound II (CLA)

*Basidiomycetes-X* powder (500 g) was used as the starting material, and 2-propanol extracts were prepared as described in the purification of ergosterol section above. The 2-propanol extracts were evaporated using a rotary evaporator, and the dry matters (2.4 g) were dissolved in 100 mL of chloroform. Chloroform-insoluble materials were removed by filtration using filter paper, after which the filtrate was evaporated using a rotary evaporator, leading to the recovery of 1.4 g of chloroform-soluble materials. After dissolving in chloroform, the chloroform-soluble sample was applied to a silica gel column (Wakogel C-200, 75–150  $\mu\text{m}$ ,  $1.8 \times 15$  cm) and eluted with chloroform-methanol-acetic acid (95:5:3, v/v). The unabsorbed fraction was collected, evaporated (977 mg) and dissolved in chloroform (2 mL). The chloroform solution was applied to an alumina column (Wako active alumina, 200–300 mesh,  $1.8 \times 15$  cm), washed with 100% chloroform and eluted with chloroform-methanol-acetic acid (95:5:3, v/v). The elute was collected, evaporated (225 mg) and dissolved in

hexane-ethyl acetate-acetic acid (60:40:1, v/v, 2 mL). The sample (2 mL) was applied to a silica gel column (Wakogel C-200, 75–150  $\mu\text{m}$ ,  $1.8 \times 15\text{ cm}$ ) and eluted with hexane-ethyl acetate-acetic acid (60:40:1, v/v). The fractions showing the characteristic UV absorption spectrum with an absorption maximum at 233 nm were collected, evaporated (151 mg) and dissolved in chloroform (0.5 mL). After adjusting the solvent to methanol-chloroform-water (90:5:5, v/v), the sample was filtered through a 0.45- $\mu\text{m}$  PTFE syringe filter (RJF1345NH, Rephile) and then purified using an HPLC system with a reversed-phase column (CAPCELL PAK C18 AQ S5,  $10 \times 250\text{ mm}$ , Shiseido). The mobile phase was 90% (v/v) methanol with 0.1% (v/v) acetic acid at a flow rate of  $3.0\text{ mL min}^{-1}$ .  $A_{233}$  was monitored to detect the target compound. The peak fraction at 25 min was collected, evaporated (29 mg) and dissolved in chloroform-methanol (3:40, v/v). After filtration through a 0.45- $\mu\text{m}$  PTFE syringe filter (RJF1345NH, Rephile), the filtrate was further purified using the same HPLC system with a reversed-phase column (CAPCELL PAK C18 AQ S5,  $10 \times 250\text{ mm}$ , Shiseido). In the second chromatography, the mobile phase was 85% (v/v) methanol with 0.1% (v/v) acetic acid at a flow rate of  $3.0\text{ mL min}^{-1}$ .  $A_{233}$  was monitored to detect compound II. The peak fraction at 61 min was collected, evaporated and dissolved in chloroform as the final product (11.4 mg) (**Supplementary Table 2**). Purified compound II was used for HPLC analysis and MS analysis.

Because the purified CLA was degraded during storage at  $4^\circ\text{C}$  for 2–3 months, CLA was purified again from *Basidiomycetes-X* dry powder (100 g) to obtain enough quantity and quality sample for the NMR analysis. *Basidiomycetes-X* dry powder was suspended in a mixed solvent containing chloroform (250 mL), methanol (500 mL), and acetic acid (0.1 N, 200 mL) and stirred for 2 h at room temperature. The insoluble solid was filtered off, and chloroform (250 mL) and acetic acid (0.1 N, 250 mL) was added to the filtrate. This solution was shaken in a separatory funnel and separated into two phases. The lower layer was collected, and the organic solvents were removed by evaporation under vacuum. The dissolved part in ethyl acetate-hexane (2:8, v/v) was passed through a SNAP ultra 10 g HP-Sphere column (Biotage Isolea one, gradient elution by hexane and ethyl acetate). The fractions eluting before ergosterol were collected and dried *in vacuo*. The crude CLA (approximately 170 mg) was dissolved in 10 mL of methanol-chloroform-water (90:5:5, v/v) and further purified by reversed-phase HPLC. Debris was removed by filtration through a 0.45- $\mu\text{m}$  PTFE syringe filter (RJF1345NH, Rephile) and the filtrate was purified using an HPLC system with a reversed-phase column (Inertsustain, 5  $\mu\text{m}$ ,  $14 \times 150\text{ mm}$ , GL Sciences). The mobile phase was 87.5% (v/v) methanol with 0.1% (v/v) acetic acid at a flow rate of  $5.5\text{ mL min}^{-1}$ .  $A_{233}$  was monitored to detect CLA. The peak fraction at 20 min was collected, evaporated

(39 mg) and dissolved in 4.3 mL of methanol-chloroform (3:40, v/v). Debris was removed by filtration through a 0.45- $\mu\text{m}$  PTFE syringe filter (RJF1345NH, Rephile), the filtrate was purified using an HPLC system with a reversed-phase column (CAPCELL PAK C18 AQ S5,  $10 \times 250\text{ mm}$ , Shiseido) again. In the second chromatography, the mobile phase was 85% (v/v) methanol with 0.1% (v/v) acetic acid at a flow rate of  $3.0\text{ mL min}^{-1}$ .  $A_{205}$  was monitored to detect CLA. The peak fraction at 43 min was collected, evaporated and dissolved in chloroform as the final product (11 mg). The purified CLA was used for the NMR analysis.

### 2.5 Measurement of CLA Content by HPLC

One gram of *Basidiomycetes-X* dry powder was suspended in 10 mL of methanol, and stirred for 24 h using a magnetic stirrer at room temperature. Debris was removed by filtration through filter paper and a 0.45- $\mu\text{m}$  PTFE syringe filter (RJF1345NH, Rephile). The CLA-containing methanol extract (20  $\mu\text{L}$ ) was analyzed using an HPLC system with a pump (PU-2087 Plus Intelligent Prep. Pump, JASCO) equipped with a reversed-phase column (Cosmosil 5C18-MS,  $4.6 \times 150\text{ mm}$ , Nacalai). The mobile phase was 90% (v/v) methanol with 0.1% (v/v) acetic acid at a flow rate of  $0.6\text{ mL min}^{-1}$  for isocratic elution. The UV spectrum was recorded with a JASCO photodiode array detector (MD-2018 Plus).  $A_{232}$  was traced to draw a chromatogram. CLA was identified by its characteristic UV absorption spectrum with an absorption maximum at 232 nm.

The authentic standards *trans*-10,*cis*-12-octadecadienoic acid (10(E),12(Z)-CLA) and *cis*-9,*trans*-11-octadecadienoic acid (9(Z),11(E)-CLA) were purchased from Cayman Chemical Company (Ann Arbor, MI 48108, USA). The concentration of 10(E),12(Z)-CLA in ethanol was spectrophotometrically determined using an absorption coefficient of  $28,700\text{ L mol}^{-1}\text{ cm}^{-1}$  at 232 nm [21], and 140.8 ng of 10(E),12(Z)-CLA was injected into the HPLC system as an external standard.

### 2.6 HPLC Analysis of *Basidiomycetes-X* Water Extract

*Basidiomycetes-X* dry powder (50 g) was suspended in water (500 mL) and stirred for 24 h using a magnetic stirrer at room temperature. After filtration through filter paper under reduced pressure, debris was additionally removed by filtration through a 0.45- $\mu\text{m}$  PTFE syringe filter (RJF1345NH, Rephile). The water extract was analyzed using an HPLC system equipped with a reversed-phase column (Cosmosil 5C18-MS,  $4.6 \times 150\text{ mm}$ , 5  $\mu\text{m}$ , Nacalai). The mobile phase was changed for linear gradient elution using a pump (PU-2087 Plus Intelligent Prep. Pump, JASCO). During the initial 5 min, 0.1% (v/v) acetic acid was eluted, and the methanol concentration in the eluent increased linearly up to 99.9% (v/v) methanol with 0.1% (v/v) acetic acid over 95 min. After a 10-min elution of 99.9% (v/v) methanol with 0.1% (v/v) acetic acid, the methanol concentration in the eluent decreased linearly to 0% (v/v)

methanol with 0.1% (v/v) acetic acid over 5 min and stabilized at 0.1% (v/v) acetic acid for 10 min. The flow rate was maintained at 0.6 mL min<sup>-1</sup> during the elution. The UV spectrum was recorded with a JASCO photodiode array detector (MD-2018 Plus).

### 2.7 Detection of DPPH Radical Scavengers in Water Extract

One gram of *Basidiomycetes-X* dry powder was suspended in 10 mL of water and stirred for 2 h using a magnetic stirrer at room temperature. Debris was removed by centrifugation at 22,200 × g for 5 min, filtration through filter paper and subsequent filtration through a 0.45-μm PTFE syringe filter (RJF3245NH, Reophile). The water extract was fractionated using a preparative HPLC system equipped with a reversed-phase column (CAPCELL PAK C18 AQ S5, 10 × 250 mm, Shiseido). The mobile phase changed stepwise from 7.5% (v/v) methanol with 0.1% (v/v) acetic acid during the first 30 min, to 20% (v/v) methanol with 0.1% (v/v) acetic acid during the next 40 min, to 40% (v/v) methanol with 0.1% (v/v) acetic acid during the next 20 min, and to 99.9% (v/v) methanol with 0.1% (v/v) acetic acid during the final 15 min. The flow rate was kept at 3.0 mL min<sup>-1</sup> during elution with a pump (PU-2087 Plus, JASCO). A<sub>260</sub> was recorded with a JASCO UV/VIS detector (UV-2075 Plus). Twelve peak fractions detected with absorption at 260 nm and 7 interval fractions without absorption at 260 nm were collected, and condensed using a rotary evaporator or a lyophilizer. Each condensed fraction was dissolved in 10 μL of methanol and spotted onto a TLC plate (TLC Silica gel 60 F<sub>254</sub>, Merck). After separation using hexane-ethyl acetate (4:1, v/v) as a developing solvent, the plate was dried at room temperature for 20 min. A 375 μM DPPH solution in methanol was sprayed evenly on the plate and DPPH radical scavengers were detected. The spot intensity detected by activity staining was measured using an image analysis software (ImageJ, developed by Rasband W at NIH, Bethesda, MD).

### 2.8 Purification of Compound III (DDMP)

Seventy grams of *Basidiomycetes-X* dry powder were divided into 7 portions, and each 10 g was suspended in 100 mL of methanol and stirred for 24 h using a magnetic stirrer at room temperature. Debris was removed by filtration through filter paper, the methanol extract was evaporated with a rotary evaporator, after which the dry materials (12.8 g) were dissolved in 140 mL of water. The water-insoluble solid was removed by filtration through filter paper. The filtrate was divided into 7 portions, and methanol (20 mL) and chloroform (20 mL) were added to the filtrate (20 mL). After mixing vigorously using a 100-mL separatory funnel and separated into two phases, the upper aqueous phase was collected. After evaporation with a rotary evaporator, the dry materials were dissolved in 93 mL of water. Debris was removed by filtration through a 0.45-μm PTFE syringe filter (RJF1345NH, Reophile). The DDMP-containing aqueous

fraction was purified using an HPLC system equipped with a reversed-phase column (CAPCELL PAK C18 AQ S5, 10 × 250 mm, Shiseido). The mobile phase was 0.1% (v/v) acetic acid at a flow rate of 3.0 mL min<sup>-1</sup> for isocratic elution. A<sub>290</sub> was monitored to detect the target compound and the peak fraction at 30 min was recovered. After removal of the solvent, 178 mg of dry material was dissolved in 2 mL of water and filtered through a 0.45-μm PTFE syringe filter (RJF1345NH, Reophile). The filtrate was additionally purified by the second chromatographic preparation using a reversed-phase column (CAPCELL PAK C18 AQ S5, 10 × 250 mm, Shiseido). The mobile phase was 0.1% (v/v) acetic acid at a flow rate of 3.0 mL min<sup>-1</sup> for isocratic elution. A<sub>260</sub> was monitored to detect compound III and the peak fraction at 30 min was recovered. After removal of the solvent, 13.4 mg of dry material was obtained as the final product.

### 2.9 Measurement of DDMP Content by HPLC

One gram of *Basidiomycetes-X* dry powder was suspended in 10 mL of methanol and stirred for 24 h using a magnetic stirrer at room temperature. The methanol-insoluble solid was removed by filtration through filter paper. Debris was additionally removed by centrifugation at 21,000 × g for 2 min and subsequent filtration through a 0.45-μm PTFE syringe filter (RJF1345NH, Reophile). The methanol extract (20 μL) was analyzed using an HPLC system with a pump (PU-2087 Plus Intelligent Prep. Pump, JASCO) equipped with a reversed-phase column (Cosmosil 5C18-MS, 4.6 × 150 mm, Nacalai). The mobile phase was 0.1% (v/v) acetic acid for the initial 5 min and changed linearly up to 99.9% (v/v) methanol with 0.1% (v/v) acetic acid over 95 min for gradient elution. The flow rate was constant at 0.6 mL min<sup>-1</sup>. The UV absorption spectrum was recorded with a JASCO photodiode array detector (MD-2018 Plus) and A<sub>296</sub> was traced to draw a chromatogram. A known amount of purified DDMP (400 ng) was used as the external standard.

### 2.10 Purification of Compound IV (Uridine) and Compound V (Adenosine)

*Basidiomycetes-X* dry powder (100 g) was added to 800 mL of water and stirred for 12 h using a magnetic stirrer at room temperature. The water-insoluble solid was removed by centrifugation at 2000 × g for 3 min and subsequent filtration through filter paper to obtain clear aqueous extract. The extraction using distilled water was repeated 4 times, and 2010 mL of the water extract was collected in total. The water extract was condensed using a rotary evaporator, and the resulting dry materials (64.85 g) were dissolved in 450 mL of methanol. The methanol-insoluble solid was removed by filtration through filter paper and the filtrate was condensed to 100 mL using a rotary evaporator. Equal volumes of water and chloroform were added to the 100-mL condensed methanol soluble fraction, mixed vigorously using a 500-mL separatory funnel, and waited

to separate into two phases. The lower organic phase was removed. The chloroform extraction was repeated 3 times and the clear aqueous phase (125 mL) was collected. The aqueous phase was recovered and condensed using a rotary evaporator, and the resulting dry materials were dissolved in 50 mL of methanol. The methanol-soluble fraction was maintained at  $-30\text{ }^{\circ}\text{C}$  for 12 h and the precipitate was removed by filtration through filter paper. After evaporation by a rotary evaporator, the dry materials (10.85 g) were dissolved in 25 mL of water and debris was removed by filtration through a syringe filter ( $0.45\text{ }\mu\text{m}$ , RJF1345NH, Rephile). The filtrate was passed through a reversed-phase column (Cosmosil 5C18-MS-II,  $4.6 \times 150\text{ mm}$ , Nacalai) to remove the substances adsorbed to the column. The mobile phase was 15% (v/v) methanol with 0.1% (v/v) acetic acid at a flow rate of  $1.0\text{ mL min}^{-1}$ . The elution of the target compound was monitored by  $A_{260}$ , and the fraction at retention times of 0–30 min was collected as fraction 1. The solvent of fraction 1 was removed using a rotary evaporator and the resulting materials were dissolved in 20 mL of water. After filtration through a syringe filter ( $0.45\text{ }\mu\text{m}$ , RJF1345NH, Rephile), the sample was purified using a preparative HPLC system equipped with a reversed-phase column (CAPCELL PAK C18 AQ S5,  $10 \times 250\text{ mm}$ , Shiseido). The mobile phase was 7.5% (v/v) methanol with 0.1% (v/v) acetic acid at a flow rate of  $3.0\text{ mL min}^{-1}$ . The elution of the target compound was monitored by  $A_{260}$ . The fraction at retention times of 8.87–10.99 min was collected as fraction 2, and the fraction at retention times of 22.6–25.9 min was collected as compound V.

Fraction 2 was condensed using a rotary evaporator and the resulting materials were dissolved in 10 mL of water. After filtration through a syringe filter ( $0.45\text{ }\mu\text{m}$ , RJF1345NH, Rephile), the water-soluble fraction was further purified using another HPLC system equipped with a reversed-phase column (Inertsil ODS-3,  $4.6 \times 150\text{ nm}$ ,  $5\text{ }\mu\text{m}$ , GL Sciences). The mobile phase was 0.1% (v/v) acetic acid at a flow rate of  $1.0\text{ mL min}^{-1}$ . The elution of the target compound was monitored by  $A_{260}$ , and the fraction at retention times of 16.5–19.5 min was collected as compound IV. Compound IV (10.6 mg, **Supplementary Table 3**) and compound V (14.0 mg, **Supplementary Table 4**) were obtained after the removal of the solvent with a lyophilizer.

### 2.11 Measurement of Uridine Content by HPLC

One gram of *Basidiomycetes-X* dry powder was suspended in 20 mL of water and stirred for 2 h using a magnetic stirrer at room temperature. Debris was removed by centrifugation at  $22,200 \times g$  for 2 min and the supernatant was diluted by the addition of a 3-fold volume of water to obtain a  $12.5\text{ mg mL}^{-1}$  *Basidiomycetes-X* water extract. After filtration through a syringe filter ( $0.45\text{ }\mu\text{m}$ , RJF1345NH, Rephile), the water extract (20  $\mu\text{L}$ ) was analyzed using an HPLC system with a pump (PU-2087 Plus Intelligent Prep. Pump, JASCO) equipped with a reversed-

phase column (Inertsil ODS-3,  $4.6 \times 150\text{ nm}$ ,  $5\text{ }\mu\text{m}$ , GL Sciences). The mobile phase was 7.5% (v/v) methanol with 0.1% acetic acid at a flow rate of  $0.6\text{ mL min}^{-1}$  for isocratic elution. The UV absorption spectrum was recorded with a JASCO photodiode array detector (MD-2018 Plus), and  $A_{262}$  was traced to draw a chromatogram. The amount of uridine was determined from a standard curve constructed with known amounts (60 to 400 ng) of the authentic standard uridine, which was purchased from Sigma-Aldrich. The concentration of the uridine standard solution was determined using the absorption coefficient of uridine ( $10,100\text{ L mol}^{-1}\text{ cm}^{-1}$  at 262 nm).

### 2.12 Measurement of Adenosine Content by HPLC

One gram of *Basidiomycetes-X* dry powder was suspended in 20 mL of water and stirred for 2 h using a magnetic stirrer at room temperature. Debris was removed by centrifugation at  $22,200 \times g$  for 2 min and the supernatant was diluted by the addition of an equal volume of water to obtain  $25\text{ mg mL}^{-1}$  *Basidiomycetes-X* water extract. After filtration through a syringe filter ( $0.45\text{ }\mu\text{m}$ , RJF1345NH, Rephile), the water extract (20  $\mu\text{L}$ ) was analyzed using an HPLC system with a pump (PU-2087 Plus Intelligent Prep. Pump, JASCO) equipped with a reversed-phase column (Inertsil ODS-3,  $4.6 \times 150\text{ nm}$ ,  $5\text{ }\mu\text{m}$ , GL Sciences). The mobile phase was 15% (v/v) methanol with 0.1% (v/v) acetic acid at a flow rate of  $0.6\text{ mL min}^{-1}$  for isocratic elution. The UV absorption spectrum was recorded with a JASCO photodiode array detector (MD-2018 Plus), and  $A_{260}$  was traced to draw a chromatogram. The amount of adenosine was determined from a standard curve constructed with known amounts (66 to 435 ng) of the authentic standard adenosine, which was purchased from Sigma-Aldrich. The concentration of the adenosine standard solution was determined using the absorption coefficient of adenosine ( $14,900\text{ L mol}^{-1}\text{ cm}^{-1}$  at 260 nm).

### 2.13 MS Analysis

EI-MS and EI HR-MS analyses were performed at the Research Institute for Instrumental Analysis at Kanazawa University using a mass spectrometer (JMS-700; JEOL Ltd., Tokyo). FAB-MS and FAB HR-MS analyses were performed using the JEOL JMS-700 mass spectrometer with glycerol or YOKUDEL-FAB-Matrix (JEOL) as a matrix.

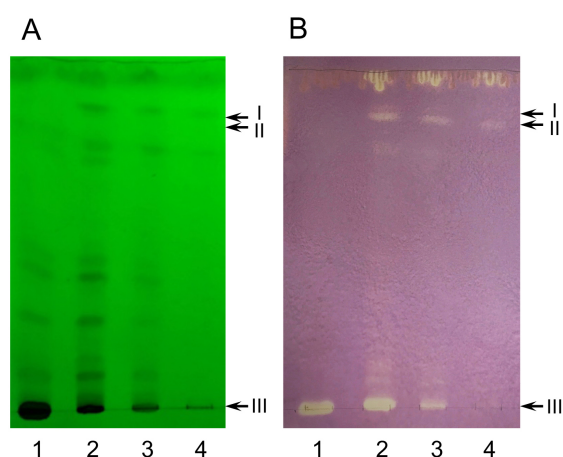
### 2.14 Spectroscopic Analysis

The UV absorption spectra were recorded with a Hitach spectrophotometer (U-3900). During HPLC analyses, the UV/VIS spectra were recorded with a JASCO photodiode array detector (MD-2018 Plus). NMR analyses were performed using JEOL NMR spectrometers (ECA600 or ECS400) with 3-(trimethylsilyl)-1-propanesulfonic acid- $d_6$  sodium salt (TMP) as an internal standard to determine 0 ppm.

### 3. Results

#### 3.1 Characterization of Lipophilic Antioxidants in *Basidiomycetes-X*

Fig. 1. shows the TLC separation of DPPH radical scavengers in the *Basidiomycetes-X* extracts using different extraction solvents. Almost all of the water-soluble DPPH radical scavengers were retained at the origin under the conditions that we used for TLC separation (Fig. 1B, Lane 1). Compounds with DPPH radical scavenging activities at  $R_f$  0.89 and  $R_f$  0.86 were detected in the methanolic extract (Fig. 1B, Lane 2), and their relative intensities were 6.2% ( $R_f$  0.89) and 21% ( $R_f$  0.86), respectively, although approximately 55% of the DPPH radical scavengers were retained at the origin (Fig. 1B, Lane 2). The bands at  $R_f$  0.89 and  $R_f$  0.86 were specifically detected in the 2-propanol extract (Fig. 1B, Lane 4). The lipophilic DPPH radical scavengers in *Basidiomycetes-X* can be extracted using 2-propanol and separated by column chromatography. Thus, we focused on compound I at  $R_f$  0.89 and compound II at  $R_f$  0.86 and characterized them further.



**Fig. 1. Detection of DPPH radical scavengers in *Basidiomycetes-X* extracts after separation by TLC.** *Basidiomycetes-X* extracts were prepared using water (Lane 1), methanol (Lane 2), chloroform-methanol (1:1, v/v) (Lane 3) and 2-propanol (Lane 4). After centrifugation, 10  $\mu$ L of the extracts were applied to TLC plates (Silica gel 60 F<sub>254</sub>, Merck) and separated using chloroform-methanol-acetic acid (95:5:3, v/v) as a mobile phase. UV-absorbing compounds that inhibited fluorescence from a dye on the TLC plate were detected under UV illumination at 254 nm (Panel A). DPPH radical scavengers on the plate were detected by staining with 1.25 mM DPPH solution in methanol (Panel B). The positions of ergosterol (I), CLA (II) and DDMP (III) are indicated.

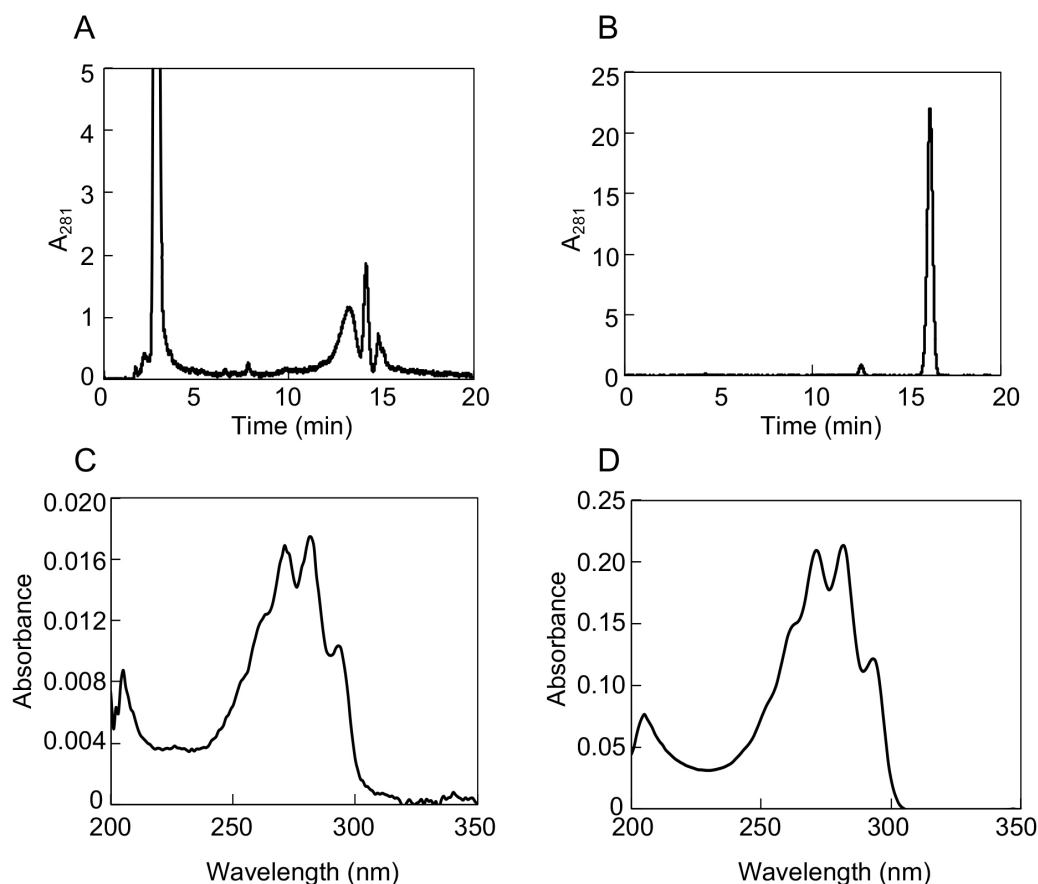
#### 3.2 Identification of Ergosterol

As described above (section 2.2), 4.5 mg of compound I at  $R_f$  0.89 was obtained from 500 g of *Basidiomycetes-*

*X* dry powder (**Supplementary Table 1**). Purified compound I showed a characteristic UV absorption spectrum with four absorption peaks at 263, 272, 282 and 293 nm (Fig. 2D), and a molecular ion with a  $m/z$  of 397 was detected by EI-MS analysis. These features were identical to those of ergosterol. Compound I eluted identically to the commercially available ergosterol standard when analyzed using our HPLC system (data not shown). **Supplementary Table 5** displays the NMR spectroscopic data of compound I to compare with the ergosterol standard. The  $^{13}\text{C}$  and  $^1\text{H}$  NMR signals were essentially identical to each other (**Supplementary Table 5**, Ref. [22]). Taken together, these results identified compound I at  $R_f$  0.89 with DPPH radical scavenging activity as ergosterol. The ergosterol content in the *Basidiomycetes-X* powder was measured by methanol extraction and the following HPLC analysis. Its content ( $167 \mu\text{g g}^{-1}$ ) was similar to a previous report of ergosterol contents in edible mushrooms [23]. The calculated recovery was 5.4% of compound I during our preparation starting from 500 g of the dry powder.

#### 3.3 Identification of CLA

As described above (section 2.4), 11.4 mg of compound II at  $R_f$  0.86 was obtained from 500 g of *Basidiomycetes-X* powder (**Supplementary Table 2**). Purified compound II displayed a characteristic UV absorption spectrum with a single absorption maximum at 233 nm (Fig. 3D), and the strong UV absorption at 233 nm suggests a conjugated double bond in the acyl group, which is a characteristic feature of a conjugated linoleic acid (CLA) [21]. The molecular ion with a  $m/z$  280 was detected by both FAB-MS and EI-MS analyses. A  $m/z$  280.2401 detected by FAB HR-MS analysis of compound II indicates the molecular formula of  $\text{C}_{18}\text{H}_{32}\text{O}_2$  with errors of +0.8 ppm and -0.1 mmu. Purified compound II eluted at identical retention times to the CLA standards *trans*-10,*cis*-12-octadecadienoic acid and *cis*-9,*trans*-11-octadecadienoic acid when analyzed using our HPLC system (data not shown). To identify the isomer of CLA, NMR analysis was performed. **Supplementary Table 6** displays the NMR spectroscopic data of compound II, and these signals were identical or highly similar to those of *trans*-10,*cis*-12-octadecadienoic acid. These results identified compound II at  $R_f$  0.86 with high DPPH radical scavenging activity as *trans*-10,*cis*-12-octadecadienoic acid (10(E),12(Z)-CLA). The CLA content in the *Basidiomycetes-X* powder was measured by methanol extraction and the following HPLC analysis. Its content ( $198 \mu\text{g g}^{-1}$ ) was similar to that of ergosterol ( $167 \mu\text{g g}^{-1}$ ), consistent with the HPLC analysis result of the 2-propanol extract in which ergosterol and CLA were detected as the main components (data not shown). The calculated recovery was 11.5% of compound II during our preparation starting from 500 g of the dry powder.



**Fig. 2. HPLC analysis of compound I (ergosterol) from *Basidiomycetes-X*.** The methanol extract (A, C) and the purified ergosterol (B, D) were analyzed by an HPLC system equipped with a reversed-phase column (Cosmosil 5C-18-MS,  $4.6 \times 150$  mm, Nacalai) using 100% methanol as the mobile phase at a flow rate of  $0.6 \text{ mL min}^{-1}$  for isocratic elution using a pump (PU-2087 Plus Intelligent Prep. Pump, JASCO). Chromatograms detected by  $A_{281}$  of the methanol extract (A) and purified ergosterol (B). The UV absorption spectrum was recorded with a JASCO photodiode array etector (MD-2018 Plus). Absorption spectra at 14.2 min of the methanol extract (C) and at 16.2 min of purified ergosterol (D).

### 3.4 Characterization of Water-Soluble Antioxidants in *Basidiomycetes-X*

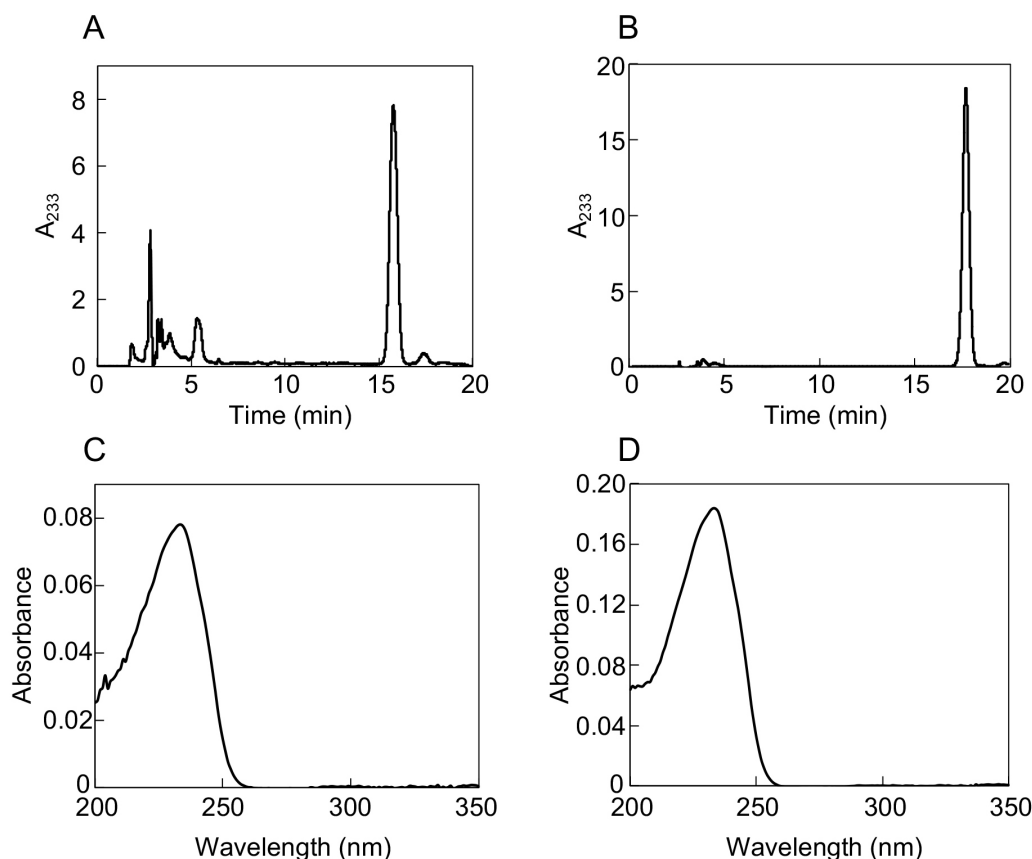
In the *Basidiomycetes-X* water extract, DPPH radical scavengers that stayed at the origin were detected (Fig. 1, Lane 1), which suggests the presence of hydrophilic components of antioxidants in *Basidiomycetes-X*. Thus, the water extract was characterized to seek water-soluble antioxidants as described above (section 2.6). Fig. 4 shows a chromatogram of the *Basidiomycetes-X* water extract analyzed using an analytical HPLC system. Six prominent peaks were detected by monitoring the UV absorbance at 260 nm, two of which were expected to be pyrrole alkaloid derivatives according to their UV absorption spectra and retention times (Fig. 4, Table 1, Ref. [19]).

The separation of DPPH radical scavenging activities in *Basidiomycetes-X* water extract using preparative HPLC was performed as described above (section 2.7), and the fraction containing a compound with a UV absorption maximum at 297 nm showed approximately 17% of the total DPPH radical scavenging activity separated by the HPLC

system. When analyzed using an analytical HPLC system, the compound with an absorbance maximum at 297 nm with DPPH radical scavenging activity was identical to peak 2, which is shown in Fig. 4. Thus, the compound, named compound III, was purified and identified as a water-soluble antioxidant in *Basidiomycetes-X*. Moreover, peaks 1 and 3, which are shown in Fig. 4, were further characterized as the main components, compound IV and compound V, respectively, in the water extract of *Basidiomycetes-X*.

### 3.5 Identification of DDMP

As described above (section 2.8), compound III (13.4 mg) was obtained from *Basidiomycetes-X* dry powder (70 g). Purified compound III displayed a characteristic UV absorption spectrum with a single absorption maximum at 296 nm (data not shown). A  $m/z$  of 144.0433 detected by EI HR-MS analysis of compound III indicates the molecular formula of  $\text{C}_6\text{H}_8\text{O}_4$  with errors of +7.2 ppm and +1.0 mmu. **Supplementary Table 7** displays the NMR spectroscopic data of compound III, and these signals were identi-



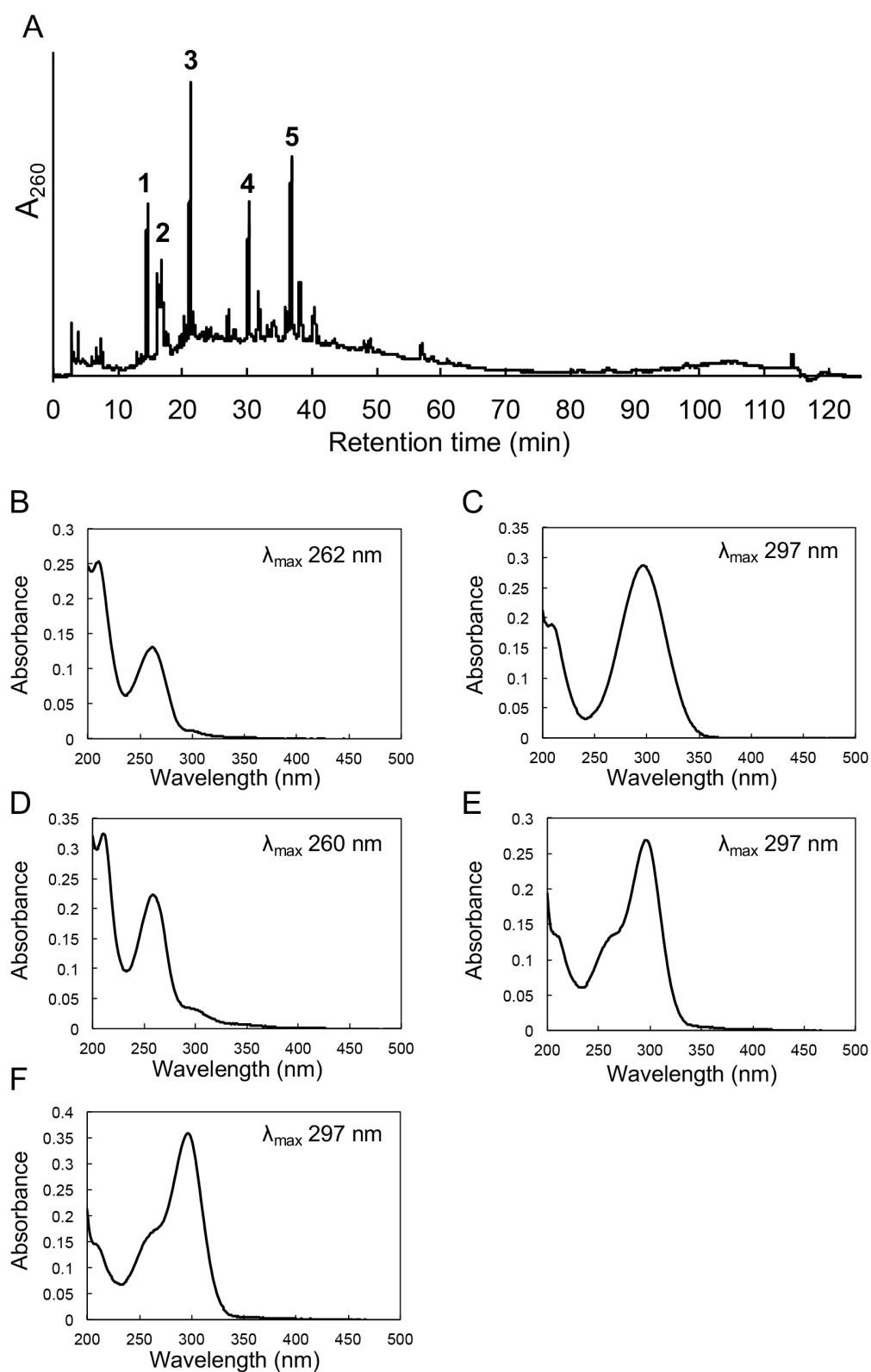
**Fig. 3. HPLC analysis of purified compound II (CLA) from *Basidiomycetes-X*.** The 2-propanol extract (A,C) and purified compound II (B,D) were analyzed by an HPLC system equipped with a reversed-phase column (Cosmosil 5C-18-MS,  $4.6 \times 150$  mm, Nacalai) using 90% methanol with 0.1% acetic acid as the mobile phase at a flow rate of  $0.6 \text{ mL min}^{-1}$  for isocratic elution using a pump (PU-2087 Plus Intelligent Prep. Pump, JASCO). Chromatograms detected by  $A_{233}$  of the 2-propanol extract (A) and purified compound II (B). The UV absorption spectrum was recorded with a JASCO photodiode array detector (MD-2018 Plus). Absorption spectra at 15.7 min of the 2-propanol extract (C) and at 17.7 min of the purified CLA (D). Ergosterol eluted at 9.5 min after changing the developing solvent to 100% methanol. Thus, it is not included in the chromatogram of panel A.

cal or highly similar to those of 2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one (DDMP) [24]. Taken together, these results identified compound III with DPPH radical scavenging activity as DDMP (Fig. 5). The DDMP content in the *Basidiomycetes-X* powder was measured by methanol extraction and the following HPLC analysis. Its content of approximately  $3500 \mu\text{g g}^{-1}$  suggests a calculated recovery of 5.5% of compound III during our preparation starting from 70 g of the dry powder.

### 3.6 Identification of Uridine

Compound IV was purified from 100 g of *Basidiomycetes-X* powder as described above (section 2.10), and 10.6 mg of the final product was obtained (Supplementary Table 3). The HPLC chromatogram of purified compound IV and its UV absorption spectrum with a characteristic absorption maximum at 262 nm were identical to those of the uridine standard (data not shown). FAB-MS analysis of purified compound IV showed molecular ion fragments with

$m/z$  245 and  $m/z$  113, which were thought to be uridine and uracil, respectively. A  $m/z$  of 245.0783 detected by FAB HR-MS analysis of compound IV indicates the molecular formula of  $\text{C}_9\text{H}_{13}\text{N}_2\text{O}_6$  with errors of +3.8 ppm and +0.9 mmu. Supplementary Table 8 displays the NMR spectroscopic data of compound IV to compare with the uridine data. The  $^{13}\text{C}$  and  $^1\text{H}$  NMR signals were essentially identical to one another. Taken together, these results identified compound IV as uridine. The uridine content in the *Basidiomycetes-X* powder was measured by water extraction and the following HPLC analysis. Its content of approximately  $769 \mu\text{g g}^{-1}$  suggests a calculated recovery of 14% of compound IV during our preparation starting from 100 g of the dry powder. The level of uridine in the *Basidiomycetes-X* powder was similar to the content in the enzymatically hydrolyzed extracts of the edible Korean mushrooms *Pleurotus ostreatus*, *Agaricus bisporus* and *Flammulina velutipes* [25].

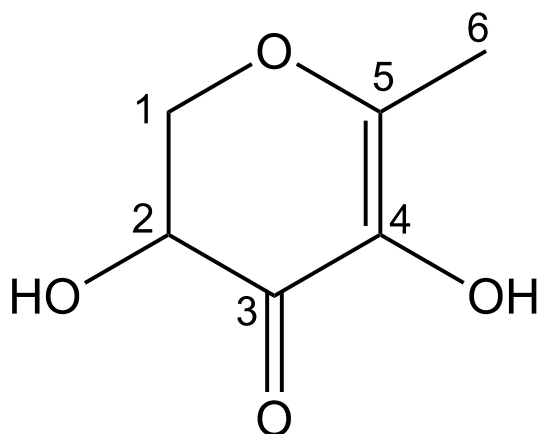


**Fig. 4.** HPLC analysis of a water-soluble extract from *Basidiomycetes-X*. A chromatogram detected by  $A_{260}$  (A) and UV-VIS spectra of peak 1 (B), peak 2 (C), peak 3 (D), peak 4 (E) and peak 5 (F) are shown. The extract from *Basidiomycetes-X* was separated by an HPLC system with a reversed-phase column (Cosmosil 5C18-MS,  $4.6 \times 150$  mm, Nacalai) and eluted by gradient elution as described (section 2.6).

**Table 1. Summary of water-soluble compounds identified in *Basidiomycetes-X*.**

Peak no.	Retention time (min)	Absorption maximum and shoulder (nm)	Compound	Reference
1	14.5	262	Uridine	This study
2	16.2	297	2,3-dihydro-3,5-dihydroxy-6-methyl-4 <i>H</i> -pyran-4-one (DDMP)	This study
3	21.2	260	Adenosine	This study
4	30.2	297 (260)	4-[2-formyl-5-(hydroxymethyl)-1 <i>H</i> -pyrrole-1-yl] butanamide	[19]
5	36.8	297 (260)	4-[2-formyl-5-(hydroxymethyl)-1 <i>H</i> -pyrrol-1-yl] butanoic acid	[19]

Water-soluble compounds with UV absorption were analyzed by our HPLC system as described (section 2.6). The chromatogram and the UV absorption spectra are shown in Fig. 4.



**Fig. 5. Structure of compound III (DDMP) purified from *Basidiomycetes-X*.**

### 3.7 Identification of Adenosine

Compound V was purified from 100 g of *Basidiomycetes-X* powder as described above (section 2.10), and 14.0 mg of the final product was obtained (**Supplementary Table 4**). The HPLC chromatogram of purified compound V and its UV absorption spectrum with a characteristic absorption maximum at 260 nm were identical to those of the adenosine standard (data not shown). FAB-MS analysis of purified compound V showed molecular ion fragments with  $m/z$  268 and  $m/z$  136, which were thought to be adenosine and adenine, respectively. A  $m/z$  of 268.1037 detected by FAB HR-MS analysis of compound V indicates the molecular formula of  $C_{10}H_{14}N_5O_4$  with errors of  $-3.3$  ppm and  $-0.9$  mmu. **Supplementary Table 9** displays the NMR spectroscopic data of compound V to compare with the adenosine data. The  $^{13}C$  and  $^1H$  NMR signals were essentially identical to each other. Taken together, these results identified compound V as adenosine. The adenosine content in the *Basidiomycetes-X* powder was measured by water extraction and the following HPLC analysis. Its content of approximately  $424 \mu g g^{-1}$  suggests a calculated recovery of 33% of compound V during our preparation starting from 100 g of the dry powder. The level of adenosine in the *Basidiomycetes-X* powder was similar to the content in the Chinese medicinal mushroom DongChongXiaCao, *Cordy-*

*ceps* sp. [26].

## 4. Discussion

*Basidiomycetes-X* (Echigoshirayukidake) is a local speciality mushroom in Niigata prefecture in Japan, which has been distributed as a precious cuisine material or as a resource for functional food and medicine. Thus far, several beneficial health functions, such as potential antioxidant-inflammation activities, anti-obesity function, prevention of lipidemia, liver fat accumulation, atopic inflammation, and amelioration of NASH, have been reported (for a review, [12]). In this study, antioxidative compounds, including ergosterol, conjugated linoleic acid (CLA) and 2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one (DDMP), were identified in *Basidiomycetes-X*. Ergosterol and CLA were relatively hydrophobic, and thus, 2-propanol was used as the suitable extraction solvent (Fig. 1). DDMP is a hydrophilic radical scavenger that is water-soluble and unable to separate using normal-phase chromatography (Fig. 1). The DPPH radical scavengers retained at the origin (Fig. 1) were expected to be hydrophilic radical scavengers in water-soluble extracts, and we identified DDMP as one of the major water-soluble radical scavengers. Uridine and adenosine were detected as major components in the water extract comparable to DDMP by reversed-phase HPLC (Fig. 4). Table 2 summarizes the contents of ergosterol, (10*E*, 12*Z*)-CLA, DDMP, uridine and adenosine, which were identified in *Basidiomycetes-X* powder in this study. Pyrrole alkaloid derivatives have been reported as other candidate active components in *Basidiomycetes-X* (Fig. 4, Tables 1,2, Ref. [19]). These compounds are expected to be involved in the reported pharmacological functions of *Basidiomycetes-X* [12], and the function of each compound will be elucidated in future studies.

Ergosterol is provitamin D2, which converts to biologically active vitamin D2 by exposure to UV light [27]. It is also known as the main sterol in the cell membrane of fungi [23,28]. It has been reported that ergosterol contents are positively correlated with antioxidant activities in button mushrooms [29]. In *Basidiomycetes-X* extracts, ergosterol significantly contributed to the total antioxidant capacity (Fig. 1). Ergosterol acts as an anti-inflammatory substance in lipopolysaccharide-stimulated macrophages *in vitro* [28].

**Table 2. Contents of the Identified Compounds in *Basidiomycetes-X*.**

Compound	Content	Reference
	$\mu\text{g g}^{-1}$	
Ergosterol	$167 \pm 14$	This study
(10E, 12Z)-CLA	$198 \pm 6$	This study
DDMP	$3500 \pm 16$	This study
Uridine	$769 \pm 54$	This study
Adenosine	$424 \pm 39$	This study
Pyrrole alkaloid I <sup>a</sup>	$825 \pm 39$	[19]
Pyrrole alkaloid II <sup>b</sup>	$484 \pm 23$	[19]

Each value indicates the average  $\pm$  standard deviation (N = 3).

The contents of ergosterol, (10E, 12Z)-CLA, DDMP, uridine and adenosine in *Basidiomycetes-X* dry powder were measured as described (section 2.3, 2.5, 2.9, 2.11 and 2.12).

<sup>a</sup> 4-[2-formyl-5-(hydroxymethyl)-1H-pyrrol-1-yl] butanoic acid, peak 5 shown in Fig. 4.

<sup>b</sup> 4-[2-formyl-5-(hydroxymethyl)-1H-pyrrole-1-yl] butanamide, peak 4 shown in Fig. 4.

Fungal ergosterol is a direct trigger of macrophage pyroptosis, which is a form of inflammatory programmed cell death [30,31]. Moreover, ergosterol from *Agaricus blazei*, a commercially cultivated mushroom, is identified as an antitumor compound [32], and ergosterol is the main anticancer ingredient in the medicinal mushroom *Amauroderma rude* [22].

Conjugated linoleic acid (CLA) is a C<sub>18</sub> fatty acid with conjugated double bonds in the acyl group, and positional and stereo isomers of CLA are known. CLA is contained in beef and milk, and its health benefits are of interest as a dietary supplement. Numerous physiological properties of CLA, including antiadipogenic, antidiabetogenic, anticarcinogenic, and antiatherosclerotic effects, have been reported [33,34], and it acts as a DPPH radical scavenger *in vitro* [35]. CLA accounts for approximately 1% of the total fatty acids in ruminant milk, and *cis*-9,*trans*-11-octadecadienoic acid (9(Z),11(E)-CLA), which is named rumenic acid, is the most abundant CLA isomer [33,34]. 10(E),12(Z)-CLA was identified in *Basidiomycetes-X* extracts, and approximately 21% of DPPH radical scavenging activities in the methanol extract were attributed to 10(E),12(Z)-CLA (Fig. 1). The biosynthetic pathway of 10(E),12(Z)-CLA in *Basidiomycetes-X* will be elucidated in the future. Both ergosterol and CLA are detected in button mushrooms [29], but the additive or synergistic effects of these compounds are unknown. We demonstrated that purified CLA was degraded during storage at 4 °C for 2–3 months, probably due to oxidation of CLA, although CLA in the crude fraction before HPLC purification and CLA in *Basidiomycetes-X* powder were rather stable. This suggests the presence of stabilizer(s) for CLA, and such antioxidative compound(s) in *Basidiomycetes-X* that need to be verified in future studies.

DDMP is known as a strong antioxidant [36–39] that can be formed from hexose in a nonenzymatic manner [24,40–42], and the thermal degradation of D-glucose to form DDMP has been reported [39,42,43]. DDMP inhibits tyrosinase activity, which is involved in the initial reaction of melanin formation from phenolic substrates and is thus attractive to cosmetic and medical applications [36]. DNA strand-breaking activity and mutagenicity of DDMP have been reported [44], and this can be a harmful function against health. From the point of view of health promotion, the inhibition of colon cancer cell growth by inducing apoptotic cell death via the inhibition of NF- $\kappa$ B [45], the increase of brown adipose tissue sympathetic nerve activity and the body temperature above the interscapular brown adipose tissue [46], and the anti-inflammatory activity by carrageenin-induced rat hind paw edema method [47] are reported as positive functions of DDMP.

Three different kinds of bioactive compounds with radical scavenging activity, ergosterol, 10(E),12(Z)-CLA and DDMP, were identified in the edible mushroom *Basidiomycetes-X*. These compounds are the components of the radical scavengers reported in *Basidiomycetes-X* extracts [13] and are expected to be involved in the medicinal effects reported in *Basidiomycetes-X* [12]. Although they may function additionally or synergistically, further studies are needed to specify their pharmacological functions.

Uridine is a pyrimidine nucleoside consisting of uracil and ribose that are linked via a  $\beta$ -N1-glycosidic bond. Uridine is thought to have helpful functions that regulate various biological systems and can be used as a medicine for the circulatory, respiratory, reproductive, and nervous systems and as an anticancer treatment and antiviral therapy [48]. Diabetes-induced peripheral nerve neuropathy [49] and developmental delays associated with increased nucleotidase activity [50] can be treated with oral uridine. The latest studies to show that supplementation with uridine and pyruvate protects the proliferative capacity of T lymphocytes, cell-mediated immunity effector cells, from mitochondrial toxic antibiotics [51] and that administration of guanosine or uridine can reduce lung inflammation in OVA-induced asthmatic mice [52].

Adenosine is a purine nucleoside consisting of adenine and ribose that are linked via a  $\beta$ -N9-glycosidic bond. Adenosine is not only used as a part of various biomolecular components but also functions as a signaling molecule received by adenosine receptors and has physiological activities. Adenosine receptors belong to the G-protein coupled receptor family and distribute in human tissues widely. They are known to participate in physiopathological responses, which include vasodilation, pain, and inflammation [53]. Therefore, it is believed that adenosine has a variety of pharmacological effects and can be used to treat diseases. Adenosine has been used in clinical medicine for emergency treatment of arrhythmia [54] and is believed to be an anti-inflammatory agent at the A<sub>2A</sub> receptor [55,56].

Recently, it has been reported that the adenosine A<sub>2A</sub> receptor can inhibit the onset of pulmonary inflammation and thrombosis caused by COVID-19 [57]. Adenosine has also been shown to be useful in cancer immunotherapy [58–61]. Moreover, adenosine receptors are highly expressed in the brain, and caffeine functions as an adenosine A<sub>2A</sub> receptor antagonist, which suggests that adenosine is an important short-lived autocrine/paracrine mediator of central nervous system functions [62].

Both pyrimidine and purine nucleosides, uridine and adenosine, respectively, were detected at similar levels in the *Basidiomycetes-X* powder (Table 2), although it is unclear whether these complementary bases interact with each other. We have identified water-soluble compounds that exhibit UV absorption, namely, DDMP, uridine, adenosine, 4-[2-formyl-5-(hydroxymethyl)-1H-pyrrol-1-yl] butanoic acid, and 4-[2-formyl-5-(hydroxymethyl)-1H-pyrrol-1-yl] butanamide (Fig. 4, Tables 1,2, Ref. [19]). There are relatively large amounts of the components (Table 2), and their chemical structures have been determined by direct spectroscopic measurements using purified products (this study, [19]). The *Basidiomycetes-X* water extract contains medicinal ingredients that show anti-obesity effects in mice [15], and the water-soluble compounds identified thus far (Table 1) may be involved in the pharmacological effects in mice. This will be verified as a future study. However, there is a possibility that other compounds without UV absorption may have strong physiological activities. Thus, we will continue to characterize bioactive compounds in the novel mushroom *Basidiomycetes-X* (Echigoshirayukidake) focused on various research approaches.

## 5. Conclusions

DPPH radical scavenging activity-guided characterization of major ingredients revealed the presence of ergosterol, *trans*-10,*cis*-12-octadecadienoic acid (10(E),12(Z)-CLA) and 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP) in *Basidiomycetes-X*. Uridine and adenosine were identified as major components in *Basidiomycetes-X* water extracts. In addition to the previously discovered pyrrole compounds [19], five components were identified (this study), resulting in a total of seven major components in *Basidiomycetes-X* (Table 2). Several beneficial health functions, such as potential antioxidant-inflammation activities, anti-obesity function, prevention of lipidemia, liver fat accumulation, atopic inflammation, and amelioration of nonalcoholic steatohepatitis (NASH), have been reported in *Basidiomycetes-X*. The ingredients identified in *Basidiomycetes-X* are expected to be involved in biological functions observed in this mushroom, which is an attractive functional food resource. We are planning animal experiments using the synthesized compounds to confirm their medicinal effects.

## Abbreviations

DPPH, 2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazino radical.

## Author Contributions

TS, SM and TK designed the research study. TS, ZL, AN, AK, TY, NW and YN performed the research. SS provided help and advice on LC-MS analysis. TS and YN analyzed the data. TS and TK wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

## Ethics Approval and Consent to Participate

Not applicable.

## Acknowledgment

The authors would like to thank Mari Ikurumi and Takumi Nishiuchi for their helpful assistance in the MS analyses.

## Funding

This research was supported by Mycology Techno Co., Ltd. (Niigata, Japan) (joint research contract with Kanazawa University since 2014).

## Conflict of Interest

The authors declare no conflict of interest.

## Supplementary Material

Purification tables for compounds I, II, IV and V (Supplementary Tables 1,2,3,4) and NMR spectroscopic data of compounds I, II, III, IV and V (Supplementary Tables 5,6,7,8,9) are available online. Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.31083/j.fbe1402010>.

## References

- [1] Valverde ME, Hernández-Pérez T, Paredes-López O. Edible Mushrooms: Improving Human Health and Promoting Quality Life. *Int. J. Med. Microbiol.* **2015**; 2015: 1–14.
- [2] Kalač P. A review of chemical composition and nutritional value of wild-growing and cultivated mushrooms. *J. Sci. Food Agric.* **2013**; 93: 209–218.
- [3] Yang J, Lin H, Mau J. Antioxidant properties of several commercial mushrooms. *Food Chem.* **2002**; 77: 229–235.
- [4] Cheung LM, Cheung PCK, Ooi VEC. Antioxidant activity and total phenolics of edible mushroom extracts. *Food Chem.* **2003**; 81: 249–255.
- [5] Puttaraju NG, Venkateshaiah SU, Dharmesh SM, Urs SMN, Somasundaram R. Antioxidant activity of indigenous edible mushrooms. *J. Agric. Food Chem.* **2006**; 54: 9764–9772.
- [6] Elmastas M, Isildak O, Turkecul I, Temur N. Determination of antioxidant activity and antioxidant compounds in wild edible mushrooms. *J. Food Compos. Anal.* **2007**; 20: 337–345.
- [7] Lee Y, Huang G, Liang Z, Mau J. Antioxidant properties of three extracts from *Pleurotus citrinopileatus*. *LWT* **2007**; 40: 823–833.

- [8] Jayakumar T, Thomas PA, Geraldine P. In-vitro antioxidant activities of an ethanolic extract of the oyster mushroom, *Pleurotus ostreatus*. *Innov. Food Sci. Emerg. Technol.* **2009**; 10: 228–234.
- [9] Boonsong S, Klaypradit W, Wilaipun P. Antioxidant activities of extracts from five edible mushrooms using different extractants. *Agric. Nat. Resour.* **2016**; 50: 89–97.
- [10] Wasser SP. Current findings, future trends, and unsolved problems in studies of medicinal mushrooms. *Appl. Microbiol. Biotechnol.* **2011**; 89: 1323–1332.
- [11] Brown GD, Gordon S. A new receptor for  $\beta$ -glucans. *Nature* **2001**; 413: 36–37.
- [12] Konishi T, Watanabe K, Arummugam S, *et al.* Nutritional and therapeutic significance of *Echigoshirayukidake* (*Basidiomycetes-X*), a novel mushroom found in Niigata, Japan. *Glycative Stress Res.* **2019**; 6: 248–257.
- [13] Watanabe T, Nakajima Y, Konishi T. In Vitro and in Vivo Antioxidant Activity of Hot Water Extract of *Basidiomycetes-X*, Newly Identified Edible Fungus. *Biol. Pharm. Bull.* **2008**; 31: 111–117.
- [14] Watanabe K, Karuppagounder V, Sreedhar R, *et al.* *Basidiomycetes-X*, an edible mushroom, alleviates the development of atopic dermatitis in NC/Nga mouse model. *Exp. Mol. Pathol.* **2018**; 105: 322–327.
- [15] Sato S, Sakurai M, Konishi T, Nishikawa K, Tsuno Y. Anti-obesity effect of *Echigoshirayukidake* (*Basidiomycetes-X*) in rats. *Glycative Stress Res.* **2019**; 6: 198–211.
- [16] Khatun MA, Sato S, Konishi T. Obesity preventive function of novel edible mushroom, *Basidiomycetes-X* (*Echigoshirayukidake*): Manipulations of insulin resistance and lipid metabolism. *J. Tradit. Complement. Med.* **2020**; 10: 245–251.
- [17] Watanabe K, Afrin R, Sreedhar R, *et al.* Pharmacological Investigation of *Ceraceomyces tessulatus* (*Agaricomycetes*) in Mice with Nonalcoholic Steatohepatitis. *Int. J. Med. Mushrooms* **2020**; 22: 683–692.
- [18] Yonei Y, Yagi M, Takabe W, Nishikawa K, Tsuno Y. Effect and safety of *Echigoshirayukidake* (*Basidiomycetes-X*) on fatty liver: Stratified randomized, double-blind, parallel-group comparison study and safety evaluation study. *Glycative Stress Res.* **2019**; 6: 258–269.
- [19] Sakamoto T, Nishida A, Wada N, *et al.* Identification of a novel pyrrole alkaloid from the edible mushroom *Basidiomycetes-X* (*Echigoshirayukidake*). *Molecules.* **2020**; 25: 4879.
- [20] te Welscher YM, ten Napel HH, Balagué MM, *et al.* Natamycin blocks fungal growth by binding specifically to ergosterol without permeabilizing the membrane. *J. Biol. Chem.* **2008**; 283: 6393–6401.
- [21] Pitt GAJ, Morton RA. Ultra-violet spectrophotometry of fatty acids. *Prog. Chem. Fats Other Lipids.* **1957**; 4: 227–278.
- [22] Li X, Wu Q, Xie Y, *et al.* Ergosterol purified from medicinal mushroom *Amauroderma rude* inhibits cancer growth in vitro and in vivo by up-regulating multiple tumor suppressors. *Oncotarget* **2015**; 6: 17832–17846.
- [23] Villares A, Mateo-Vivaracho L, García-Lafuente A, Guillamón E. Storage temperature and UV-irradiation influence on the ergosterol content in edible mushrooms. *Food Chem.* **2014**; 147: 252–256.
- [24] Chen Z, Xi G, Fu Y, *et al.* Synthesis of 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one from maltol and its taste identification. *Food Chem.* **2021**; 361: 130052.
- [25] Kim MS, Kim GH. Contents of nucleic acids (nucleosides and mono-nucleotides) in extracts of *Pleurotus ostreatus*, *Agaricus bisporus* and *Flammulina velutipes*. *Korean J. Food & Nutr.* **2010**; 23: 376–380. (In Korean)
- [26] Hsu T, Shiao L, Hsieh C, Chang D. A comparison of the chemical composition and bioactive ingredients of the Chinese medicinal mushroom *DongChongXiaCao*, its counterfeit and mimic, and fermented mycelium of *Cordyceps sinensis*. *Food Chem.* **2002**; 78: 463–469.
- [27] Jäpelt RB, Jakobsen J. Vitamin D in plants: a review of occurrence, analysis and biosynthesis. *Front. Plant Sci.* **2013**; 4: 136.
- [28] Kuo C, Hsieh C, Lin W. Proteomic response of LAP-activated RAW 264.7 macrophages to the anti-inflammatory property of fungal ergosterol. *Food Chem.* **2011**; 126: 207–212.
- [29] Shao S, Hernandez M, Kramer JKG, Rinker DL, Tsao R. Ergosterol profiles, fatty acid composition, and antioxidant activities of button mushrooms as affected by tissue part and developmental stage. *J. Agric. Food Chem.* **2010**; 58: 11616–11625.
- [30] Rodrigues ML. The multifunctional fungal ergosterol. *mBio.* **2018**; 9: e01755-18.
- [31] Koselny K, Mutlu N, Minard AY, Kumar A, Krysan DJ, Wellington M. A genome-wide screen of deletion mutants in the filamentous *Saccharomyces cerevisiae* background identifies ergosterol as a direct trigger of macrophage pyroptosis. *mBio.* **2018**; 9: e01204-18.
- [32] Takaku T, Kimura Y, Okuda H. Isolation of an Antitumor Compound from *Agaricus blazei* Murill and its Mechanism of Action. *J. Nutr.* **2001**; 131: 1409–1413.
- [33] Belury MA. DIETARY CONJUGATED LINOLEIC ACID in HEALTH: Physiological Effects and Mechanisms of Action. *Annu. Rev. Nutr.* **2002**; 22: 505–531.
- [34] Rodríguez-Alcalá LM, Castro-Gómez MP, Pimentel LL, Fontecha J. Milk fat components with potential anticancer activity—a review. *Biosci. Rep.* **2017**; 37: BSR20170705.
- [35] Yu L. Free radical scavenging properties of conjugated linoleic acids. *J. Agric. Food Chem.* **2001**; 49: 3452–3456.
- [36] Takara K, Otsuka K, Wada K, Iwasaki H, Yamashita M. 1,1-Diphenyl-2-picrylhydrazyl Radical Scavenging Activity and Tyrosinase Inhibitory Effects of Constituents of Sugarcane Molasses. *Biosci. Biotechnol. Biochem.* **2007**; 71: 183–191.
- [37] Hwang IG, Kim HY, Woo KS, Lee SH, Lee J, Jeong HS. Isolation and Identification of the Antioxidant DDMP from Heated Pear (*Pyrus pyrifolia* Nakai). *Prev. Nutr. Food. Sci.* **2013**; 18: 76–79.
- [38] Yu X, Zhao M, Liu F, Zeng S, Hu J. Identification of 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one as a strong antioxidant in glucose–histidine Maillard reaction products. *Food Res. International* **2013**; 51: 397–403.
- [39] Kanzler C, Haase PT, Schestkova H, Kroh LW. Antioxidant Properties of Heterocyclic Intermediates of the Maillard Reaction and Structurally Related Compounds. *J. Agric. Food Chem.* **2016**; 64: 7829–7837.
- [40] Mills FD, Weisleder D, Hodge JE. 2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one, a novel nonenzymatic browning product. *Tetrahedron Lett.* **1970**; 15: 1243–1246.
- [41] Shaw PE, Tatum JH, Berry RE. 2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one, a degradation product of a hexose. *Carbohydr. Res.* **1971**; 16: 207–211.
- [42] Kim M, Baltes W. On the Role of 2,3-Dihydro-3,5-dihydroxy-6-methyl-4(H)-pyran-4-one in the Maillard Reaction. *J. Agric. Food Chem.* **1996**; 44: 282–289.
- [43] Li H, Tang X, Wu C, Yu S. Formation of 2,3-dihydro-3,5-dihydroxy-6-Methyl-4(H)-Pyran-4-one (DDMP) in glucose-amino acids Maillard reaction by dry-heating in comparison to wet-heating. *LWT* **2019**; 105: 156–163.
- [44] Hiramoto K, Nasuhara A, Michikoshi K, Kato T, Kikugawa K. DNA strand-breaking activity and mutagenicity of 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP), a Maillard reaction product of glucose and glycine. *Mutat. Res.* **1997**; 395: 47–56.
- [45] Ban JO, Hwang IG, Kim TM, *et al.* Anti-proliferate and pro-apoptotic effects of 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyranone through inactivation of NF- $\kappa$ B in Human Colon Can-

- cer Cells. *Arch. Pharm. Res.* **2007**; 30: 1455–1463.
- [46] Beppu Y, Komura H, Izumo T, *et al.* Identification of 2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one isolated from *Lactobacillus pentosus* strain S-PT84 culture supernatants as a compound that stimulates autonomic nerve activities in rats. *J. Agric. Food Chem.* 2012; 60: 11044–11049.
- [47] Ashwathanarayana R, Naika R. Anti-inflammatory properties of *Pavetta crassicaulis* Bremek. leaf and flower crude extracts and its pure compounds collected from Western Ghats, Karnataka, India. *Asian J. Pharm. Clin. Res.* **2018**; 11: 72–90.
- [48] Connolly GP, Duley JA. Uridine and its nucleotides: biological actions, therapeutic potentials. *Trends Pharmacol. Sci.* **1999**; 20: 218–225.
- [49] Gallai V, Mazzotta G, Montesi S, Sarchielli P, Gatto F. Effects of uridine in the treatment of diabetic neuropathy: an electrophysiological study. *Acta Neurol. Scand.* **1992**; 86: 3–7.
- [50] Page T, Yu A, Fontanesi J, Nyhan WL. Developmental disorder associated with increased cellular nucleotidase activity. *Proc. Natl. Acad. Sci.* **1997**; 94: 11601–11606.
- [51] Battaglia S, De Santis S, Rutigliano M, *et al.* Uridine and pyruvate protect T cells' proliferative capacity from mitochondrial toxic antibiotics: a clinical pilot study. *Sci. Rep.* **2021**; 11: 12841.
- [52] Luo Y, Chen H, Huang R, Wu Q, Li Y, He Y. Guanosine and uridine alleviate airway inflammation via inhibition of the MAPK and NF- $\kappa$ B signals in OVA-induced asthmatic mice. *Pulm. Pharmacol. Ther.* **2021**; 69: 102049.
- [53] Ballesteros-Yáñez I, Castillo CA, Merighi S, Gessi S. The role of adenosine receptors in psychostimulant addiction. *Front. Pharmacol.* 2018; 8: 985.
- [54] Gupta A, Lokhandwala Y, Rai N, Malviya A. Adenosine—a drug with myriad utility in the diagnosis and treatment of arrhythmias. *J. Arrhythm* **2021**; 37: 103–112.
- [55] Trevethick MA, Mantell SJ, Stuart EF, Barnard A, Wright KN, Yeadon M. Treating lung inflammation with agonists of the adenosine a2a receptor: promises, problems and potential solutions. *Br. J. Pharmacol.* **2008**; 155: 463–474.
- [56] Nakav S, Chaimovitz C, Sufaro Y, *et al.* Anti-inflammatory preconditioning by agonists of adenosine A1 receptor. *PLoS ONE.* **2008**; 3: e2107.
- [57] DiNicolantonio JJ, Barroso-Aranda J. Harnessing adenosine a2a receptors as a strategy for suppressing the lung inflammation and thrombotic complications of COVID-19: Potential of pentoxifylline and dipyridamole. *Med. Hypotheses* **2020**; 143: 110051.
- [58] Leone RD, Emens LA. Targeting adenosine for cancer immunotherapy. *J. Immunother. Cancer* **2018**; 6: 57.
- [59] Boison D, Yegutkin GG. Adenosine Metabolism: Emerging Concepts for Cancer Therapy. *Cancer Cell* **2019**; 36: 582–596.
- [60] Vigano S, Alatzoglou D, Irving M, *et al.* Targeting adenosine in cancer immunotherapy to enhance T-cell function. *Front. Immunol.* **2019**; 10: 925.
- [61] Allard B, Allard D, Buisseret L, Stagg J. The adenosine pathway in immuno-oncology. *Nat. Rev. Clin. Oncol.* **2020**; 17: 611–629.
- [62] Jacobson KA, Gao Z-G, Matricon P, Eddy MT, Carlsson J. Adenosine A<sub>2A</sub> receptor antagonists: from caffeine to selective non-xanthines. *Br. J. Pharmacol.* **2020**; 2020: 1–16.