Inhibitory Effects of the Edible Mushroom *Flammulina velutipes* on Lipid Accumulation in 3T3-L1 Cells

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Abstract: We investigated the inhibitory effect of the edible mushroom *Flammulina velutipes* on intracellular lipid accumulation. Among the five fractions of the *F. velutipes* extract, the acetone fraction had an inhibitory effect on intracellular lipid accumulation. Seven known compounds were isolated from the acetone fraction: methyl linoleate, methyl γ-linolenate, ergosterol, ergosta-5,7-dien-3β-ol, ergost-7-en-3β-ol, ergosterol peroxide, and 5,6-epoxy-24(*R*)-methylcholesta-7,22-dien-3β-ol. Ergosterol peroxide exhibited a potent inhibitory effect on the intracellular lipid accumulation. Our results indicate that ergosterol peroxide may be important as an anti-obesity agent since it inhibits the metabolic syndrome.

Keywords: *Flammulina velutipes*, ergosterol peroxide, 3T3-L1 cells, lipid accumulation.

INTRODUCTION

In recent years, edible mushrooms have been widely consumed as nutritious and healthy foods [1]. Mushrooms are well known as a nutritional and low-calorie food because of high content of proteins, carbohydrates, fiber, and low content of fats [2-3]. Among the cultivated edible mushrooms, *Flammulina velutipes,* also called golden needle mushroom, winter mushroom, and enokitake, is one of the most popular edible mushrooms worldwide [4]. *F. velutipes* (called enokitake in Japan), belonging to *Basidiomycotina* of *Eumycota*, is an edible mushroom consumed in Japan [4-6]. *F. velutipes* extracts have been reported to exhibit many biological activities, such as antioxidant, anti-inflammatory, immunomodulatory, antitumor, antiviral, antifungal as well as cholesterol-lowering properties and inhibitory effects on visceral fat accumulation [7-15].

Obesity is a rapidly growing epidemic in industrialized countries, and it is a major risk factor for metabolic disorders such as coronary heart disease, hypertension, diabetes, cancer, respiratory diseases, and osteoarthritis [16-18]. Obesity is a condition in which adipocytes accumulate a large amount of fat and become enlarged. This condition is characterized at the cellular level by an increase in the number and size of adipocytes differentiated from fibroblastic preadipocytes in adipose tissue [19]. Adipocyte differentiation has been extensively examined in cultured preadipocytes, such as 3T3-L1 cells. Therefore, the 3T3-L1 cells serve as a suitable model system for obesity-related research [20-22].

In the present study, the *F. velutipes* extract was chromatographed on a Diaion HP-20 column and eluted into five fractions. Among these, the acetone fraction had inhibitory effects on intracellular lipid accumulation. We describe the inhibitory effect of *F. velutipes* edible mushrooms on intracellular lipid accumulation.

EXPERIMENTAL METHODS

Materials

3T3-L1 fibroblast cells were purchased from the Human Science Research Resources Bank (Osaka, Japan). Isobutyl-3-methylxanthine (IBMX), dexamethasone, insulin, adenine, transferrin, and glutamine were purchased from Wako Pure Chemical Industries Limited (Osaka, Japan). Triiodothyronine was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium were purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Calf serum (CS) was purchased from cell culture technologies. Fetal bovine serum (FBS) and oil red O were purchased from Cosmo Bio. Co., Ltd. (Tokyo, Japan). Penicillin-streptomycin-neomycin (PSN) and penicillin-streptomycin-glutamine (PSG) were purchased from Life Technologies (Burlington, ON). MTT Cell Count Kit (MTT) was purchased from Nacalai Tesque Inc. (Kyoto, Japan).

Plant Material

F. velutipes (Nagano Nokoken G-6 GO) were obtained from Agricultural Technology Institute of Nagano Farmers' Federation (Nagano, Japan).

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Cell Culture

3T3-L1 preadipocytes were plated into 24-well plates and 6-cm dishes and maintained in DMEM supplemented with 10% CS and 1% PSG at 37°C in a humidified 5% CO_2 incubator. For induction of adipose differentiation, the cells were grown to confluence. The cells were then fed with differentiation medium (2.7:1 mixture of DMEM and Ham's F12 containing 10% FBS, 1% PSN, 1.6 µM insulin, 0.0005% transferrin, 180 µM adenine, 20 pM triiodothyronine, 0.25 µM dexamethasone, and 500 µM IBMX. The cells were treated with fresh differentiation medium (in samples) on day 2 and day 4 without dexamethasone and IBMX after induction of differentiation. After 7 days, the cells were fixed on plates with 4% formalin in PBS for 24 h. The intracellular lipid accumulation was measured eight days after induction of differentiation.

Oil-Red O Staining

For oil-red O staining on cultured cells, cells were fixed on plates with 4% formalin in PBS for 24 h, rinsed once with H_2O, and air dried. The fixed cells were stained with oil-red O staining solution (0.5% Oil-red O in isopropanol, diluted 3:2 in H_2O, and then filtered with a 1.0-µm filter) for 15 min. The cells were then rinsed three times with H_2O before visualization and documentation.

The density of lipid content was evaluated by AK09F extraction liquid after oil-red O staining. The absorbance at 540 nm was measured using a microplate reader. The value of DMSO-treated cells was normalized to 100%.

Statistical Analysis

When applicable, results are presented as mean ± SD. Student's *t* test was used to calculate *P* values. Findings were considered significant at $^{*}P < 0.05$ and $^{**}P < 0.01$.

General Experimental Procedure

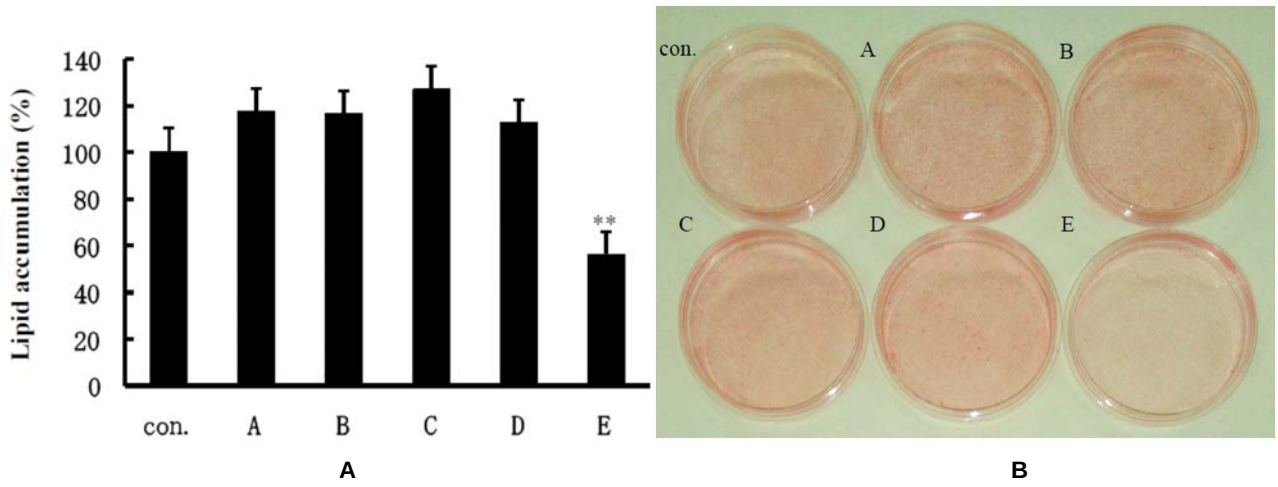
The NMR spectra were measured on a JEOL ECA-600 spectrometer (^{1}H-NMR: 600 MHz, ^{13}C-NMR: 150) in $CDCl_3$ containing tetramethylsilane (TMS) as the internal standard. The MS spectra were recorded on a JEOL CG mate instrument. HPLC was performed on a JASCO PU-2089 apparatus equipped with JASCO UV-2075. Column chromatography was performed on silica gel (Merck Japan, Tokyo, Japan) and Diaion HP-20 (Mitsubishi Chemical Corp., Tokyo, Japan).

Extraction and Isolation

Fruit body of *F. velutipes* (10 kg) was extracted three times with MeOH under ultrasonication. The MeOH extract was concentrated *in vacuo* to provide extracts (691 g). The crude extracts were chromatographed on a Diaion HP-20 column and eluted successively with stepwise gradients of H_2O (4 L), 20% MeOH (2 L), 50% MeOH (3 L), MeOH (3 L), and acetone (3 L), and then each eluate was concentrated *in vacuo* to provide five fractions [H_2O (636.0 g), 20% MeOH (14.52 g), 50% MeOH (12.12 g), MeOH (7.68 g), and acetone (11.11 g)]. The acetone fraction (10.0 g) was chromatographed on a silica gel column and successively eluted with solvents of increasing polarity [*n*-hexane-EtOAc (20:1 → 0:20 v/v)] to provide 14 fractions (Fr. 1–14). Fr. 14 (300 mg) was purified by HPLC [Inertsil ODS-P column, 10 × 250 mm, 5 µm, GL Sciences Inc., Japan (column A)] with 0.1% trifluoroacetic acid (TFA)-MeOH (15:85 v/v) at a flow rate of 5.0 ml/min to yield 1 (36.43 mg) and 2 (76.70 mg). Fr. 5 (200 mg) was purified by reversed-phase HPLC [Inertsil ODS-EP column, 10 × 250 mm, 5 µm, GL Sciences Inc., Japan (column B)] with 0.1% TFA-MeOH (10:90 v/v) at a flow rate of 5.0 ml/min to yield 3 (7.67 mg), 4 (8.78 mg), and 5 (7.19 mg). Fr. 10 (100 mg) was purified by HPLC (column B) with 0.1% TFA-MeOH (12:88 v/v) at a flow rate of 5.0 ml/min to yield 6 (7.82 mg). Fr. 12 (110 mg) was purified by HPLC (column B) with 0.1% TFA-MeOH (12:88 v/v) at a flow rate of 5.0 ml/min to yield 7 (2.02 mg).

RESULTS AND DISCUSSION

The crude extracts chromatographed on a Diaion HP-20 column eluted five fractions. The cytotoxicity of five fractions was evaluated by the MTT assay in 3T3-L1 cells. The results indicated that five fractions were nontoxic at 150 µg/ml (H_2O: 104.0%, 20% MeOH: 98.0%, 50% MeOH: 95.5%, MeOH: 123.5%, and acetone: 105.0%). Thus, the 3T3-L1 cells treated with 150 µg/ml five fractions. Intracellular lipid accumulation was measured eight days after induction of differentiation. Lipid accumulation assay results demonstrated that the acetone fraction had inhibitory effects on intracellular lipid accumulation (Figure **1A**). As shown in Figure **1B**, treatment of acetone fraction inhibited adipose differentiation compared to the other fractions, as determined by oil-red O staining. We focused our efforts on investigating the chemical constituents of the acetone fraction. Seven known compounds, two fatty acids, and five sterols, were isolated from the acetone fraction. By comparing the



A B

Figure 1: A: Effect of five fractions on lipid accumulation in 3T3-L1 cells.

con.: control, A: H_2O fraction, B: 20% MeOH fraction, C: 50% MeOH fraction, D: MeOH fraction, E: acetone fraction. Cells were treated with five fractions (150 μg/ml) on day 2 and day 4. The intracellular lipid content was measured eight days after induction of differentiation. Results are expressed as mean ± SD. of three individual experiments. ***P* < 0.01 *vs*. control. The value of DMSO-treated cells was normalized to 100%.

B: Oil-red O staining of five fractions.

con.: control, A: H_2O fraction, B: 20% MeOH fraction, C: 50% MeOH fraction, D: MeOH fraction, E: acetone fraction Cells were treated with five fractions (150 μg/ml) on day 2 and day 4. The intracellular lipid content was measured eight days after induction of differentiation.

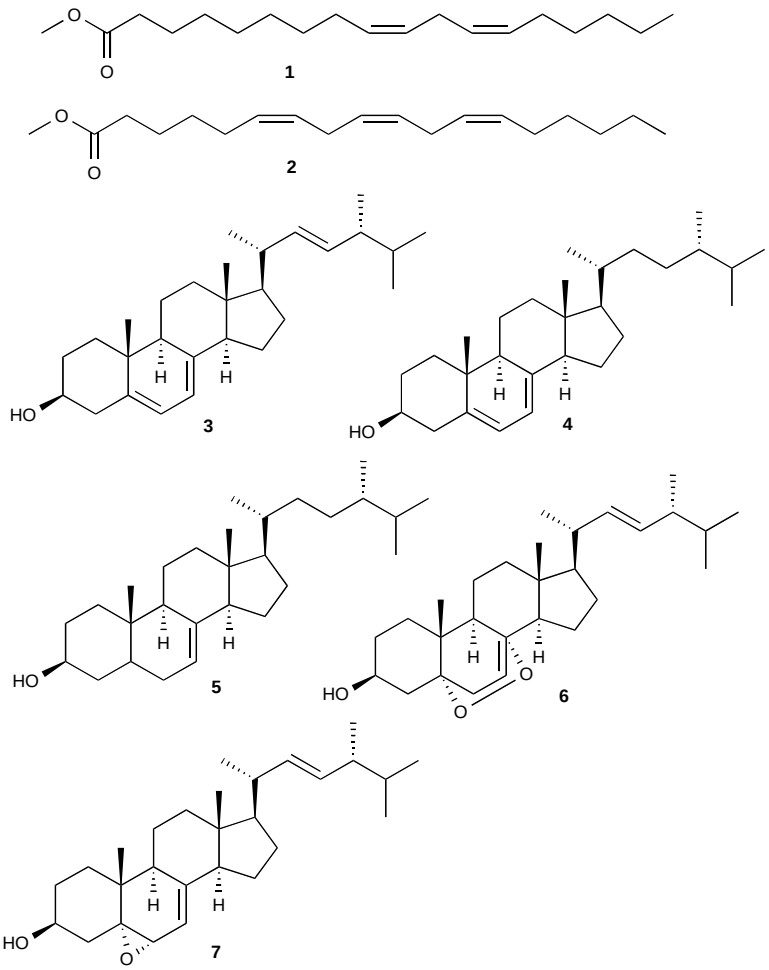


Figure 2: Structures of compounds **1–7**.

1: methyl linoleate, **2**: methyl γ-linolenate, **3**: ergosterol, **4**: ergosta-5,7-dien-3β-ol, **5**: ergost-7-en-3β-ol, **6**: ergosterol-peroxide, **7**: 5,6-epoxy-24(*R*)-methylcholesta-7,22-dien-3β-ol.

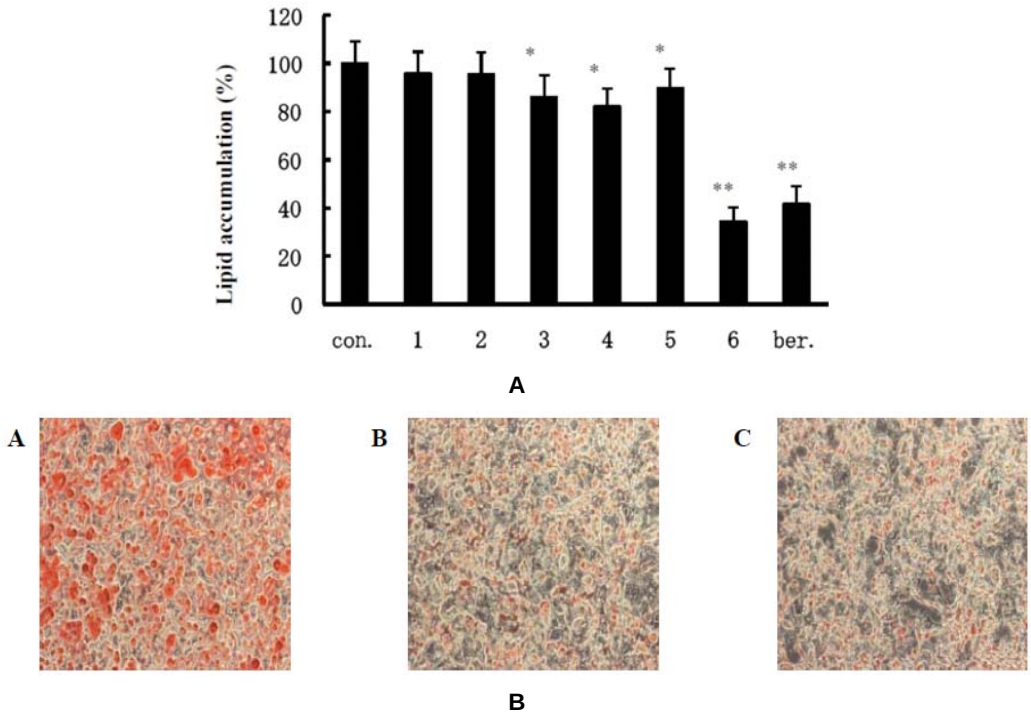


Figure 3: A: Effect of compounds 1–6 on lipid accumulation in 3T3-L1 cells.
con.: control, 1: methyl linoleate, 2: methyl γ-linolenate, 3: ergosterol, 4: ergosta-5,7-dien-3β-ol, 5: ergost-7-en-3β-ol, 6: ergosterol-peroxide, ber.: berberine. Cells were treated with compounds (30 μM) on day 2 and day 4. The intracellular lipid content was measured eight days after induction of differentiation. Results are expressed as mean ± SD. of three individual experiments.*$P < 0.05$ *vs*. control, **$P < 0.01$ *vs*. control. The value of DMSO-treated cells was normalized to 100%.
B: Ablation of ergosterol peroxide, berberine, and control by oil-red O staining. A: control, B: ergosterol peroxide, C: berberine.

spectroscopic data, the structures of the compounds were determined to be methyl linoleate (1), methyl γ-linolenate (2), ergosterol (3), ergosta-5,7-dien-3β-ol (4), ergost-7-en-3β-ol (5), ergosterol-peroxide (6), and 5,6-epoxy-24(*R*)-methylcholesta-7,22-dien-3β-ol (7) (Figure **2**) [23-27].

The inhibitory effect of six compounds, including two fatty acids (1–2) and four sterols (3–6) on intracellular lipid accumulation was examined. Berberine was used as a positive control because it has been reported to have inhibitory effects on intracellular lipid accumulation [28]. The cytotoxicity of six compounds was evaluated by the MTT assay in 3T3-L1 cells. The results indicated that the six compounds were nontoxic at concentrations up to 30.0 μM. The 3T3-L1 cells were treated with compounds 1–6 (30 μM) on day 2 and day 4. The intracellular lipid content was measured eight days after induction of differentiation. Our results demonstrated that six compounds inhibited intracellular lipid accumulation at 30 μM (Figure **3A**). Fatty acids (1–2) had little inhibitory effects on lipid accumulation. As shown in Figure **3B**, ablation of ergosterol peroxide, berberine, and untreated control resulted in inhibited lipid accumulation as shown by oil-red O staining. Ergosterol peroxide inhibited lipid accumulation as well as the positive control. As shown in Figure **4**, ergosterol peroxide inhibited lipid accumulation in a concentration-dependent manner. However, ergosterol, ergosta-5,7-dien-3β-ol and ergost-7-en-3β-ol exhibited a minimum inhibitory effect of lipid accumulation in the positive control. Ergosterol peroxide is more effective than other sterols. These data suggest that the presence of 5α, 8α-epidioxy moiety in the molecule is possibly important for potent inhibitory effect.

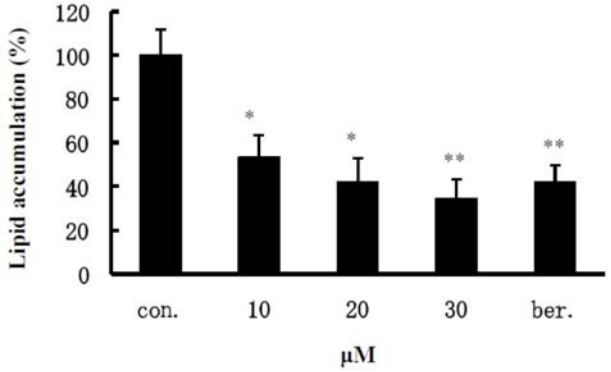


Figure 4: Effect of ergosterol peroxide concentration-dependent manner on lipid accumulation in 3T3-L1 cells.
The intracellular lipid content was measured eight days after induction of differentiation. Results are expressed as mean ± SD. of three individual experiments.*$P < 0.05$ *vs*. control, **$P < 0.01$ *vs*. control. The value of DMSO-treated cells was normalized to 100%.

CONCLUSION

We investigated the anti-obesity effect of the edible mushroom *F. velutipes*. Lipid accumulation assay results demonstrated that the acetone fraction of the mushroom had inhibitory effects on intracellular lipid accumulation. Seven known compounds were isolated from the acetone fraction. Of these, ergosterol, ergosta-5,7-dien-3β-ol and ergost-7-en-3β-ol had a minimum inhibitory effect on intracellular lipid accumulation. Ergosterol peroxide exhibited the highest inhibitory effect on intracellular lipid accumulation as well as positive control. Our results suggest that ergosterol peroxide is important as an anti-obesity agent by inhibiting the metabolic syndrome.

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