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o-Orsellinaldehyde from the Submerged Culture of the Edible Mushroom *Grifola frondosa* Exhibits Selective Cytotoxic Effect Against Hep 3B Cells Through Apoptosis

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The tumoricidal activity of a bioactive metabolite produced by submerged culture in a 2.1-L airlift fermentor of *Grifola frondosa* NTUS was investigated. After 14 days of cultivation, ethyl acetate extracts from the supernatant of culture broth (EES) were analyzed by cell viability assay. The IC_{50} of EES for cytotoxicity against human carcinoma cells (Hep 3B, Hep G2, HeLa, CL1-1) and normal human lung fibroblast MRC-5 was 78.4, 52.7, 77.6, 71.0, and 233.3 μg/mL, respectively. EES was further fractionated and a main cytotoxic compound, HE-5-5, was obtained. The IC_{50} of HE-5-5 based on the cell viability of Hep 3B and MRC-5 cells was 3.6 and 33.1 μg/mL, respectively. Thus, HE-5-5 showed a selective cytotoxic effect against Hep 3B cells and MRC-5. According to the UV, MS, and NMR data, HE-5-5 was identified as *o*-orsellinaldehyde. A DNA fragmentation assay together with the presence of a significant sub-G1 peak by flow cytometry suggested that *o*-orsellinaldehyde might mediate its cytotoxicity through apoptosis.

KEYWORDS: ***o*-Orsellinaldehyde; apoptosis; *Grifola frondosa*; submerged culture**

INTRODUCTION

"Edible and medicinal mushrooms" have increasingly gained worldwide recognition recently. There are at least 270 species of mushrooms that demonstrate medicinal and functional properties, and anticancer properties are the most important among them (*1*). Anticancer substances isolated from mushrooms can be divided into two classes, namely, high molecular weight and low molecular weight compounds. High molecular weight bioactive substances from mushrooms had been identified as β-glucans and have been used as immunomodulation agents in cancer therapy. On the other hand, low molecular weight compounds seem to act by more complicated tumoricidal mechanisms and are less well studied (*2*).

Grifola frondosa is a basidiomycete fungus belonging to the order *Aphyllopherales* and family *Polyporeceae* (*3*). Its fruiting body, also called "maitake" or "huishu hua", has been reported to contain various antitumor β-glucan (*4*, *5*). These β-glucans are considered to be a biological response modifier that can stimulate the immune response significantly and can be used as an adjunct to cancer therapy (*6*). Furthermore, they are also used as an ameliorative agent against the side effects of chemotherapeutic agents. In addition, β-glucans from maitake may also provide some benefit in the treatment of HIV, cancer, hyperlipidemia, hypertension, and hepatitis (*7*−*10*). It usually takes 4−6 months to culture the fruiting body of maitake. In order to decrease the cultivation time, it has been shown that submerged culture, which only takes 1−2 weeks, can be a more efficient way of producing mycelium. Polysaccharides isolated from the submerged culture of *G. frondosa* have been assayed as having antitumor activity in allogenic tumor systems (*11*, *12*).

Over recent years, research on the metabolites of *G. frondosa* for use in cancer therapy has focused on the production of extracellular polysaccharides using submerged culture (*13*, *14*). Even though low molecular weight metabolites are considered to be a novel and valuable source of therapeutically useful substances, there has been only a limited investigation of these in *G. frondosa* (*15*). In this study, we investigated the tumoricidal effects of bioactive metabolites produced by the submerged culture of *G. frondosa* NTUS, which is isolated from Taiwan. The novel cytotoxic effects of the bioactive metabolites from this strain on human carcinoma cells and normal fibroblast cells were studied. Furthermore, the effect of these substances on Hep 3B cell cycle distribution and apoptosis was evaluated.

MATERIALS AND METHODS

Materials. Potato dextrose broth, malt extract, yeast extract, and agar were purchased from Difco (Detroit, MI). Dulbecco's modified eagle media (DMEM), fetal bovine serum (FBS), and trypsin−EDTA were obtained from Gibco (Grand Island, NY). Ethyl acetate, *n*-hexane, and TLC plates were obtained from Merck (Darmstadt, Germany). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), propidium iodide, and all other chemicals were purchased from Sigma (St. Louis, MO).

Microorganism. The fungus *G. frondosa* NTUS used in this study was isolated from the fruiting body of *G. frondosa*. The stock culture was maintained on a potato dextrose agar slant at 25 °C and transferred monthly.

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Cultivation Methods. A piece of agar covered with mycelium from a 7-day stock culture was inoculated into 75 mL of MY medium consisting of 40 g of glucose, 10 g of malt extract, and 5 g of yeast extract per liter of distilled water (pH 4.5) in a 500-mL Hinton flask. Cultivation was carried out on a reciprocal shaker at 120 rpm and 25 °C for 72 h. The culture produced was used as seed culture for the main fermentation. For each main culture, 150 mL of seed culture was inoculated into a 2.1-L airlift fermentor (*16*) loaded with 1.35 L of MY medium and the cultivation was carried out at an aeration rate of 0.5 vvm and 25 °C for 14 days.

Preparation and Fractionation of Ethyl Acetate Extracts. After 14 days of cultivation in the 2.1-L airlift fermentor, the culture broth was centrifuged at 8000*g* for 15 min at 25 °C. Mycelium and supernatant were extracted separately using ethyl acetate. First, mycelium (12 g) was resuspended in 1 L of water and extracted with 1 L of ethyl acetate twice. The ethyl acetate extracts were combined and concentrated using a Rotavapor-R rotary evaporator (BÜCHI Labortechnik AG, Flawil, Switzerland) under reduced pressure at 40 °C. The residue was designated as the ethyl acetate extract from the mycelium or EEM (300 mg). Second, the supernatant was extracted with an equal volume of ethyl acetate twice at room temperature. The ethyl acetate extracts were combined and concentrated under reduced pressure at 40 °C. The residue was designated as the ethyl acetate extract from supernatant, EES (430 mg). The EEM and EES were then resuspended in *n*-hexane. After initial analysis, the EES in *n*-hexane was subjected to separation on a silica gel column (silica gel 60G, Merck, 20 × 2 cm i.d.) by loading in *n*-hexane and eluted using a mixture of *n*-hexane−ethyl acetate, 50:50 (v/v). After silica gel column chromatography, the product was analyzed by thin-layer chromatography. Silica gel plates (silica gel 60 F_{254}, Merck) were developed with *n*-hexane−ethyl acetate, 50:50 (v/v), as the mobile phase. After development, the silica gel plates were treated with 10% H_2SO_4 and the spots were revealed by heating at 110 °C for 5 min. Fractions showing similar profiles were combined to give eight main fractions, and the solvent was removed under reduced pressure at 40 °C. The most cytotoxic fraction was further separated by HPLC system (Shimadzu LC-10A) using a MetaChem Polaris C18-A column (5-μm particles, 150, 250 × 10 mm i.d., Varian Inc., Palo Alto, CA) and eluted with 80% methanol in H_2O. We obtained a pure compound termed HE-5-5, which showed cytotoxicity against Hep 3B cells.

Chemical Structure Assay. The chemical structure of HE-5-5 was determined on the basis of GC−MS and ^{1}H NMR spectra. GC−MS analysis was performed with a HP 6890 series GC system (Hewlett-Packard, Palo Alto, CA) equipped with HP-5-MS fused silica capillary column. ^{1}H NMR spectrum was measured with BRUKER NMR AC 200 spectrometer (Bruker, Billerica, MA) in CD_3OD operating at 300 MHz.

Cell Line and Culture Conditions. MRC-5, a human lung fibroblast cell line, was obtained from the Bioresources Collection and Research Center (Hsinchu, Taiwan). Hep 3B, a human hepatocellular carcinoma cell line, and Hep G2, a human hepatoblastoma cell line, were a gift from Dr. B.H. Chiang (Institute of Food Science and Technology, National Taiwan University, Taipei, Taiwan). HeLa, a human cervical epithelioid carcinoma cell line, and CL1-1, a human lung adenocarcinoma cell line, were a gift from Dr. F.H. Chang (Institute of Biochemistry and Molecular Biology, National Taiwan University, Taipei, Taiwan). All cells were cultured in 10% FBS in DMEM with 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2.

Assay for Biomass. The biomass concentration was measured by dry weight, which involved the filtration of culture broth samples through predried and weighted Whatman No 2 filter papers (Whatman Ltd, Maidstone, UK). The biomass was then washed, dried overnight at 70 °C, and weighed. The biomass was defined as the dry weight of cells (g) per liter of culture broth.

Assay for Cell Viability. Inhibition of cell viability was measured using the MTT assay, which is based on the ability of cells to convert tetrazolium salt into purple formazan (*17*). Briefly, cells were subcultured into a 96-well plate with 5000 cells per well in 100 μL medium. After 24-h incubation, the medium in each well was discarded and replaced with 200 μL of medium containing the test agents dissolved in 0.4% DMSO, each in triplicate. DMSO alone was used as the control. At the end of the incubation, the medium was discarded and 55 μL of MTT solution (0.5 mg/mL) was added to each well. The plates were then incubated for 4 h at 37 °C. The supernatant was removed and 100 μL of DMSO was added to each well to dissolve the formazan. The absorbance at 570 nm was measured using a MRX II microplate reader (DYNEX, Chantilly, VA). The inhibition of cell viability was expressed as a percentage of the proliferation of the control cells.

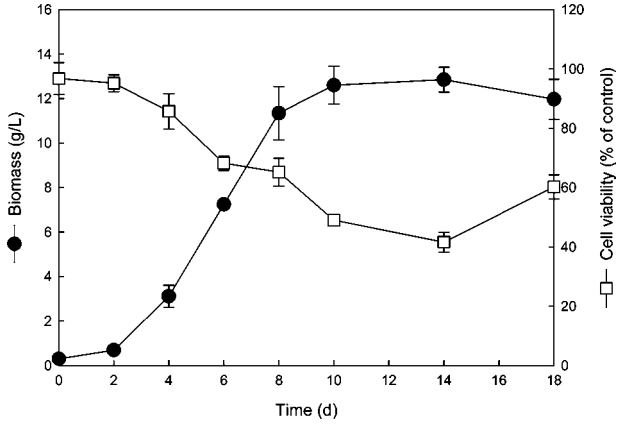

Figure 1. Time course for the production of mycelium and tumoricidal activity extracted from a submerged culture broth of *G. frondosa* NTUS grown in a 2.1-L airlift fermentor; 200 μg/mL of an ethyl acetate extract of culture broth was used to evaluate the tumoricidal activity.

Assay for Nuclear Morphology. After treatment with sample solution for 48 h, Hep 3B cells were washed three times with phosphate-buffered saline (PBS) and fixed in 4% formaldehyde for 30 min. The cells were stained with 1 μg/mL DAPI (4',6-diamidino-2-phenylindole), a DNA-binding fluorescent dye, for 30 min and the stained nuclei were observed by fluorescence microscope (*18*).

DNA Fragmentation Assay. DNA fragmentation was determined to evaluate apoptosis (*19*, *20*). Hep 3B cells treated with sample solution were scraped off the 6-well plates into medium. The cells were pelleted by centrifugation and incubated with 1 mL of lysis buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 10 mM EDTA, 100 g/mL proteinase K, and 0.5% SDS) for 1 h at 50 °C. The samples were then extracted with 1 mL of chloroform/isoamyl alcohol (24:1). The DNA was precipitated with 2.5 volumes of ethanol plus 10% volume of 3 M sodium acetate. After air drying, the precipitated DNA was resuspended in 50 μL of TE buffer supplemented with 0.1 μg/mL RNase A. To detect fragmented DNA, samples were subjected to electrophoresis using 1.5% agarose gels and stained with ethidium bromide.

Assay for Cell Cycle Distribution. Cell cycle distribution was analyzed using a flow cytometer COULTER EPICS XL-MCL (Beckman Coulter, Fullerton, CA). DNA content was analyzed by the propidium iodide staining method (*21*). Hep 3B cells were harvested by trypsinization after treatment with sample solution for 48 h. The cells were washed with ice-cold PBS and then fixed in 70% ethanol. After being washed in PBS, the fixed cells were stained with propidium iodide containing 100 μg/mL RNase for 30 min at room temperature and then analyzed using the flow cytometer. The results were analyzed by Muticycle software (Phoenix Flow systems, San Diego, CA).

Data Analysis. All experiments were performed in triplicate and expressed as mean ± standard deviation. Statistical evaluation was done by Student's *t*-test.

RESULTS

Time Course for the Submerged Culture of *G. frondosa* NTUS. The production of mycelium and bioactive metabolites was carried out in a 2.1-L airlift fermentor. The activity of bioactive metabolites extracted from the culture broth was evaluated using the carcinoma cell line viability assay. The time course profile of mycelial production and the tumorcidal activity is shown in **Figure 1**. The maximum biomass production by

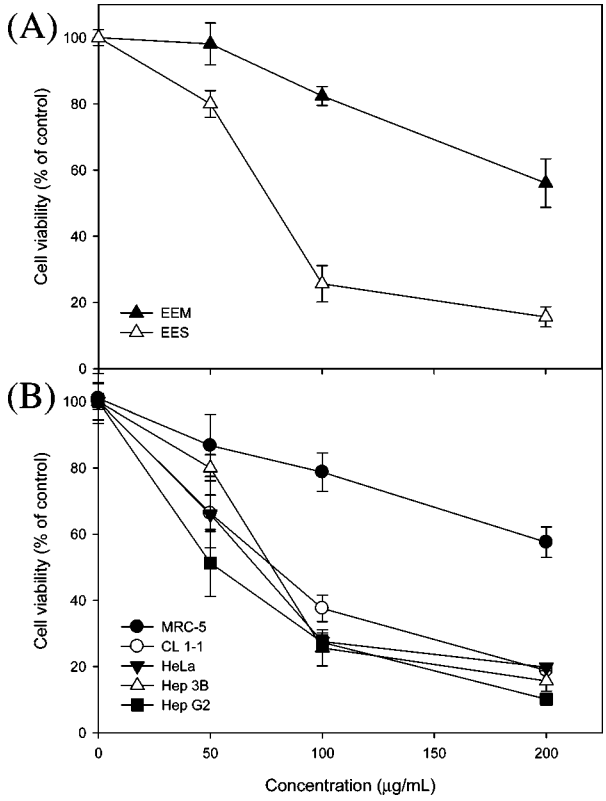

Figure 2. Dose-dependent inhibitory effect of an ethyl acetate extract of the mycelium and culture supernatant against human cell lines: (**A**) cytotoxicity of EEM and EES against Hep 3B cells and (**B**) cytotoxicity of EES against various human cell lines.

the submerged culture of *G. frondosa* NTUS was 12.8 g/L. Hep 3B cells were used to evaluate the tumoricidal activity of the ethyl acetate extracts from culture broth. After 14 days of cultivation, a maximum for the tumoricidal activity against Hep 3B cells of 59.4% was detected.

Cytotoxicity of Ethyl Acetate Extracts from the Submerged Culture of *G. frondosa* NTUS. The ethyl acetate extracts from mycelia (EEM) and supernatant (EES) from 14 day-culture broth were tested using the Hep 3B cells viability assay. The extracellular metabolites from the EES showed notable cytotoxic effects on Hep 3B cells, but those of the EEM were less effective. The IC_{50} for EES and for EEM was 78.4 and 223.8 μg/mL, respectively (**Figure 2**A). **Figure 2B** shows the viability inhibition effects on the various human carcinoma cell lines (Hep 3B, Hep G2, HeLa, CL1-1) and a normal human lung fibroblast cell line (MRC-5) when treated with various concentrations of EES. The inhibition profiles for CL1-1, Hep G2, Hep 3B, and HeLa cells treated with EES were almost the same, and for these cell lines, the IC_{50} for EES at 48 h were 78.4, 52.7, 77.6, and 71.0 μg/mL, respectively. However, the effect of EES on MRC-5 cells was much less and the IC_{50} was only 223.3 μg/mL. EES was further fractionated by silica gel column chromatography, and eight main fractions were collected. The cytotoxicity of the eight fractions on Hep 3B cells is shown in **Figure 3**. It was found that fraction 5 (HE-5) had a significant cytotoxic effect on Hep 3B cells. HE-5 was further separated by HPLC and a pure compound termed HE-5-5 was obtained (**Figure 4**) which showed cytotoxicity against Hep 3B cells. The IC_{50} of HE-5-5 for Hep 3B and MRC-5 cells was 3.6 and 33.1 μg/mL, respectively. (**Figure 5**).

Identification of HE-5-5. The UV, GC−MS, and NMR spectrometric data for HE-5-5 were follows: UV λ_{max} 288, 204, 218, 230; MS (70 eV, two most intense ions each 14 mass units above *m/e* 44) 51 (4), 55 (3); 67 (5), 69 (8); 77 (5), 81 (1); 95 (4); 106 (6); 123 (3); 134 (2); 151 (100); 152 (71); ^{1}H NMR (CD_3OD, 300 MHz) δ 10.06 (s, OH, CHO), 6.20 (d $J = 2$ Hz, H5), 6.09 (d $J = 2$ Hz, H3), 4.62 (s, OH), 2.49 (s, CH_3). These data identified HE-5-5 as *o*-orsellinaldehyde (2,4-dihydroxy-6-methylbenzaldehyde), on the basis of reported data (*22*, *23*). As shown in **Figure 6**, the commercial standard of *o*-orsellinaldehyde purchased from Sigma also showed the same cytotoxic effect against Hep 3B cells with HE-5-5.

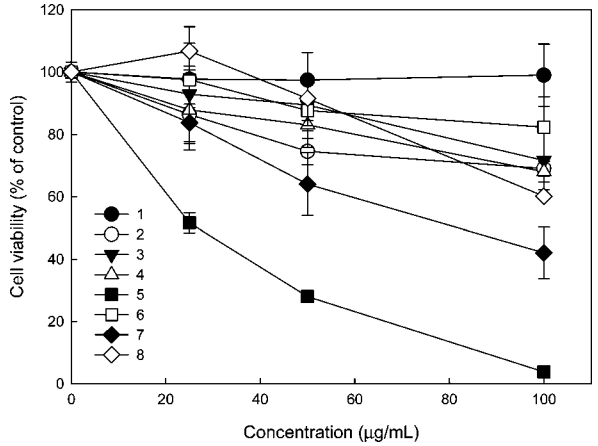

Figure 3. Cytotoxicity against Hep 3B cells of the various fractions obtained from the ethyl acetate extract of the culture supernatant.

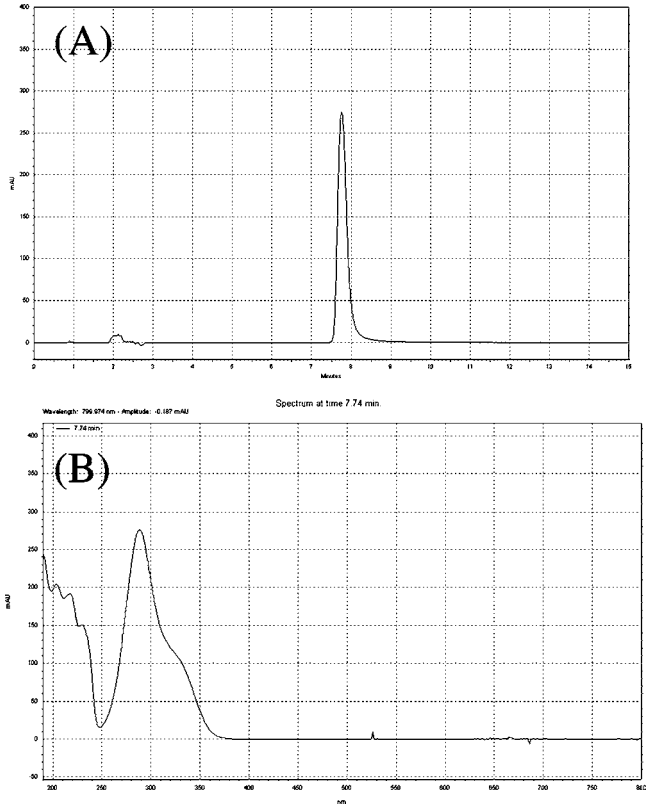

Figure 4. Chromatograms of HE-5-5: HE-5-5 was detected at 288 nm (**A**); the UV spectrum of HE-5-5 (**B**).

Effects of *o*-Orsellinaldehyde on Nuclear Morphology. To investigate whether the reduction in viability was due to apoptosis, the nuclear morphology of *o*-orsellinaldehyde-treated Hep 3B cells was analyzed using the DNA-binding dye DAPI.

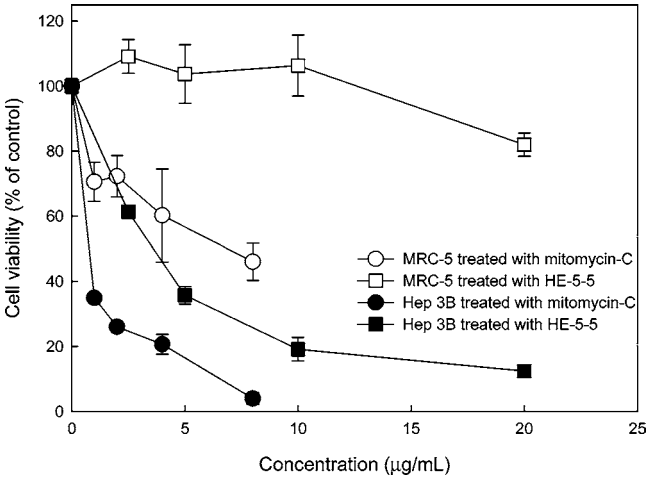

Figure 5. Cytotoxicity of HE-5-5 and mitomycin-C against Hep 3B and MRC-5 cells.

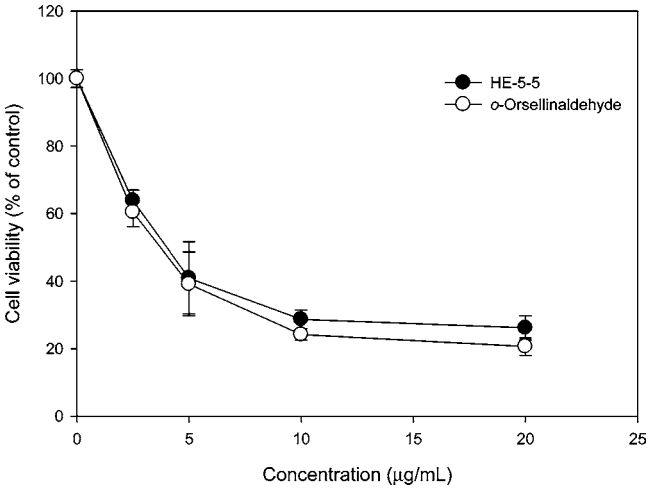

Figure 6. Cytotoxicity of HE-5-5 and *o*-orsellinaldehyde against Hep 3B cells.

As shown in **Figure 7**, Hep 3B cells exposed to 5 g/mL *o*-orsellinaldehyde for 48 h exhibited condensed chromatin and fragmented nuclei. These changes suggest that apoptosis is occurring.

Detection of DNA Fragmentation. DNA fragmentation showing a ladder pattern is a marker of apoptosis. Hep 3B cells were harvested at 48 h after treatment with different concentrations of *o*-orsellinaldehyde, and total DNA was analyzed. As shown in **Figure 8**, DNA fragmentation was detected after treatment with 5 μg/mL of *o*-orsellinaldehyde for 48 h.

Cell Cycle Distribution Among Treated Hep 3B Cells. To investigate the effect of *o*-orsellinaldehyde on cell cycle progression of Hep 3B cells, cell cycle analysis was performed using flow cytometry. As shown in **Figure 9**, there was an induction of a distinct sub-G1 peak after exposure to *o*-orsellinaldehyde for 48 h. The effect was dose-dependent and the proportion of cells in the sub-G1 phase among *o*-orsellinaldehyde-treated Hep 3B cells increased with the dose.

DISCUSSION

For many years, interest has concentrated on the isolation of bioactive polysaccharides from mushrooms. Because cultivation of fruiting bodies requires a long period of time, submerged culture is regarded as a good method for the production of mycelium and bioactive metabolites such as ganoderic acid and various polysaccharides (*24*, *25*). In this study, we were interested in the production of bioactive metabolites from *G. frondosa* NTUS and wished to explore their use in novel biotechnological applications. We used ethyl acetate to prepare

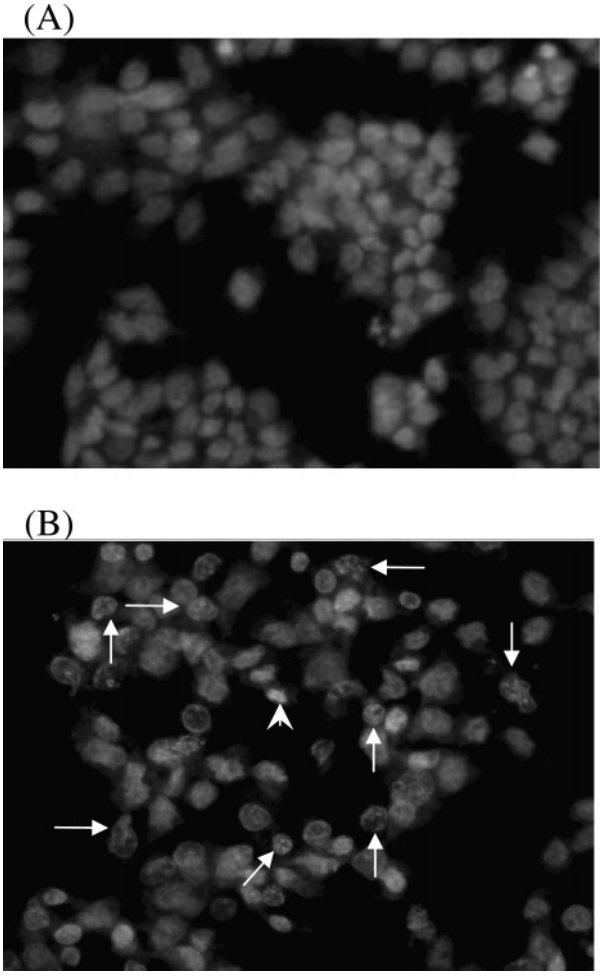

Figure 7. DAPI staining of Hep 3B cells: cells were treated with (**A**) 0.4% DMSO as control or (**B**) with 5 μg/mL *o*-orsellinaldehyde. DAPI stain was showing *o*-orsellinaldehyde-induced condense (big arrowhead) and fragmented (arrow) nuclei.

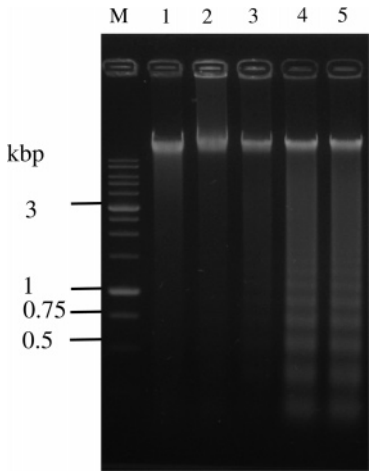

Figure 8. DNA fragmentation analysis of Hep 3B cells after treatment with *o*-orsellinaldehyde for 48 h: (lane M) DNA marker; cells were treated with (lane 1) 0.4% DMSO as control, (lane 2) 2.5 μg/mL *o*-orsellinaldehyde, (lane 3) 5 μg/mL *o*-orsellinaldehyde, (lane 4) 10 μg/mL *o*-orsellinaldehyde, or (lane 5) 20 μg/mL *o*-orsellinaldehyde.

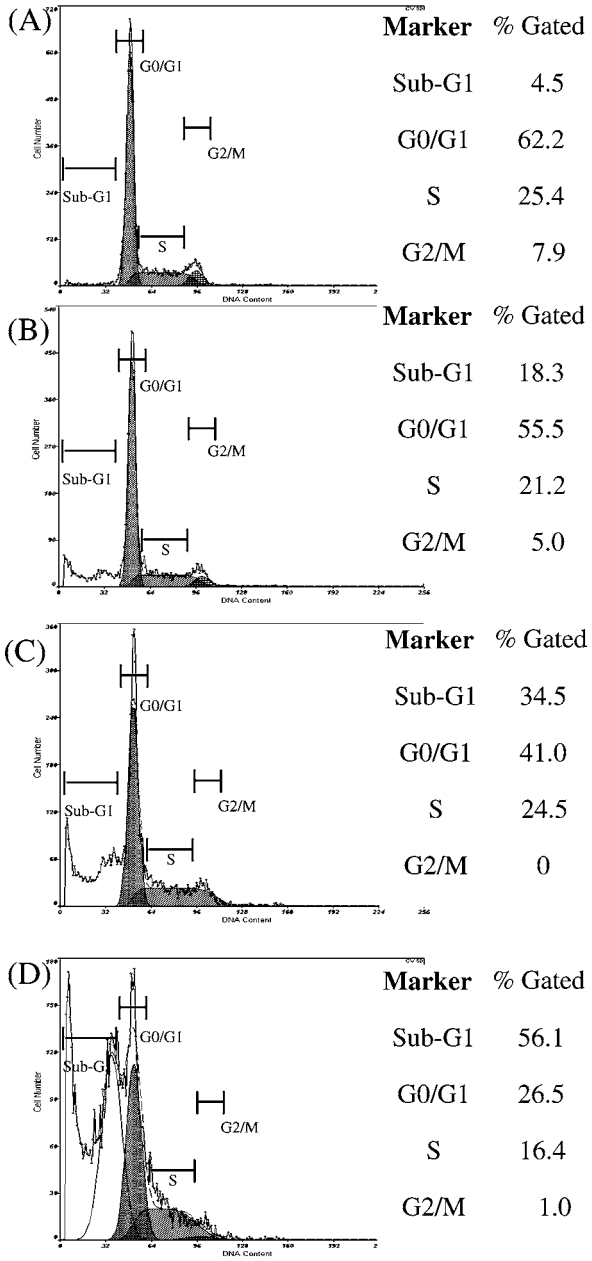

Figure 9. Cell cycle analysis of Hep 3B cells after treatment with *o*-orsellinaldehyde for 48 h: (**A**) control; cells treated with (**B**) 5 μg/mL *o*-orsellinaldehyde, (**C**) 10 μg/mL *o*-orsellinaldehyde, or (**D**) 20 μg/mL *o*-orsellinaldehyde.

crude extracts and attempted to identify crude extracts that directly show antitumor effects against cancer cells. According to the cytotoxic effects on the human hepatocellular carcinoma cells (Hep 3B), the maximum bioactive metabolites were obtained after 14 days of cultivation (**Figure 1**). Mycelium and supernatant crude extracts (EEM, EES) were prepared from submerged culture after 14 days of cultivation and evaluated for their ability to inhibit the growth of Hep 3B cells. The extracellular metabolites EES showed notable cytotoxic effects on CL1-1, Hep G2, Hep 3B, and HeLa cells but had less effect on a human normal lung fibroblast cell line, MRC-5 (**Figure 2**). There have been some reports on the tumoricidal effects of the crude extracts from the submerged culture of edible and medicinal mushrooms; however, most of the tumoricidal metabolites were isolated from mycelium (*26*−*28*). In this study, the main tumoricidal metabolites of *G. frondosa* NTUS were isolated from culture broth supernatant. These results together with the presence of immunomodulatory extracellular polysaccharides support the potential of the use *G. frondosa* NTUS submerged culture in cancer prevention and therapy.

Supernatant extract was further fractionated and the main cytotoxic compound, HE-5-5, was obtained (**Figure 4**). As shown in **Figure 5**, although the tumoricidal activity of HE-5-5 was less than that of mitomycin-C, HE-5-5 showed more selective cytotoxic effect against Hep 3B cells and MRC-5. After structure analysis, HE-5-5 was identified as *o*-orsellinaldehyde, and the commercial standard of *o*-orsellinaldehyde purchased from Sigma also showed the same cytotoxic effect against Hep 3B cells with HE-5-5 (**Figure 6**). According to the results of DAPI-staining (**Figure 7**), the DNA fragmentation assay (**Figure 8**), and the sub-G1/PI analysis (**Figure 9**), it would seem that *o*-orsellinaldehyde might mediate its cytotoxicity through apoptosis. This is the first report about *o*-orsellinaldehyde mediating its cytotoxicity through apoptosis. Besides, we attempted to analyze metabolites extracted from the fruiting body of *G. frondosa*, and *o*-orsellinaldehyde was not found by HPLC assay.

It is well-known that the most important bioactive metabolites from mushrooms are polysaccharides (*3*, *29*), but cancer therapy may need substances with a range of different mechanisms of action. The present study is the first report of bioactive metabolites from a submerged culture of *G. frondosa* that exert a cytotoxic activity through apoptosis. Therefore, further work is required to elucidate the molecular mechanism of apoptosis induced by EES and *o*-orsellinaldehyde.

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