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Cytotoxic Effects of Extracts from *Tremella fuciformis* Strain FB001 on the Human Colon Adenocarcinoma Cell Line DLD-1

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Abstract Cytotoxic effects of extracts from *Tremella fuciformis* strain FB001 were evaluated on the DLD-1 human colon adenocarcinoma cell line and the content of polyphenolic compounds in the extracts were analyzed. Hexane, chloroform, and ethyl acetate subfractions (experimental setting I) exhibited cytotoxic effects on the human colon adenocarcinoma DLD-1 cell line with IC_{50} values of 350, 400, and 450 ppm, respectively. When *T. fuciformis* was extracted sequentially with ether, ethyl acetate, chloroform, and ethanol (experimental setting II), the ether extract demonstrated potent cytotoxicity with an IC_{50} value of 150 ppm, followed by ethyl acetate and chloroform fractions. If the first extraction solvent was chloroform instead of ether (experimental setting III), exposure of the cell line to chloroform, ethyl acetate, and ether extracts at 1,000 ppm led to cell death. High levels of phenolic compounds were estimated for all hydrophobic extracts, which exhibited cytotoxic effects. We propose that this useful information gives additional support to our understanding of the biology and utility of this particular mushroom.

Keywords: extraction methods, white jelly mushroom, *Tremella fuciformis*, cytotoxicity

Introduction

The white jelly mushroom, *Tremella fuciformis*, of the family *Tremellaceae*, class *Hymenomycetes* and division *Basidiomycota* (1-4) is one of approximately 40 fungi species in the genus *Tremella* and found mainly in East asia. The body of *T. fuciformis* is used as a common food and clinically in traditional medicine in China. This mushroom is also an excellent source of industrial enzymes such as beta-D-mannosidase and beta-N-acetyl-D-hexosaminidase (5).

Investigation of the active compounds in the fruiting body of *T. fuciformis* to date has been limited. Soluble extracts of *T. fuciformis*, however, have been studied intensively (6) because of various noted biological effects, such as enhancing immune functions (7), anti-tumor (8), and hypoglycemic activities (9). The polysaccharide fraction of the soluble extracts has been used clinically in China for cancer patients undergoing treatment with chemotherapeutic agents or radiotherapy to enhance native immune function (7) including cytokine-stimulating activity (10, 11). Further, superoxide-scavenging ability was reported for a soluble extract of *T. fuciformis*, however, the active components remain unknown (12).

Polysaccharides in the soluble extract of the mushroom represent the most promising active anticancer agent (13-16). Polyphenols and flavonoids however, in preliminary chemical examination of non-polar solvent extracts of mushrooms, were regarded as the primary active components (17, 18). Several of these compounds have been reported to possess anti-tumor activity (17). The main mechanism of tumor inhibition by the flavonoids is poorly studied, however, one possible role would seem to be a decrease in the glycolytic activity through inhibition of the plasma membrane (Na, K)-ATPase (18).

Recently, potent chemopreventive agents for colon and stomach cancer were reported from *shiitake* mushrooms, *Lentinus edodes* and a wild mushroom in Korea, *Polyozellus multiplex* (19-21). To investigate a possible anticancer property of the *T. fuciformis* mushroom, we have examined the cytotoxic effects of hydrophobic extracts on cell cultures of the DLD-1 human colon adenocarcinoma cell line. In this report, we indicate that among several fractions, cytotoxicity to the test cells could be demonstrated and in a dose dependent manner. These cytotoxic effects were evaluated according to the extraction procedures with relation to the content of the extracts phenolic components.

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Materials and Methods

Materials Dulbecco's modified Eagle's medium (DMEM), RPMI-1640 medium, penicillin, and streptomycin were purchased from Gibco Ltd. (Grand Island, NY, USA). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), trypsin inhibitor from soybean, fetal bovine serum (FBS), dimethyl sulfoxide (DMSO) were obtained from Sigma Co. (St. Louis, MO, USA) and all other reagents and solvents for extraction were of analytical grade.

Identification of *T. fuciformis* The stromatal forms of *T. fuciformis* and the mycelia of *Hypoxylon* sp. were collected from limb of *Quercus* sp. within Haenam, Jeonnam province in the summer, 2003. The DNA sequences in the Internal Transcribed Spacer (ITS) region of the 5.8S ribosomal genes were analyzed and compared

with the gene sequences of *T. fuciformis* and *Hypoxylon* sp. in the EMBL/GenBank database through BLAST searching to identify appropriate strains. Homology of the sequences was determined by using the Clustal X program (22). The universal primer, ITS1 (5'-TCCGTAGG TGAA CCTGCGG-3') and ITS4R (5'-CAGACTT (G/A) TA(C/T) ATGGTCCAG-3') were designed for ITS-5.8S rDNA sequencing (23, 24) and the PCRs were performed as described by Innis and Carbone (24, 25). Total genomic DNA were prepared according to the modified method of Correll *et al.* (23).

Preparation of various mushroom fractions by systemic extraction The cultivation of the fruiting bodies of *T. fuciformis* with *Hypoxylon* sp. was performed according to the method of Chang and Miles (26).

A three-fold experimental design was used to prepare the extracts. The first extraction system (I) was as follows: freeze dried mushroom (100 g) was milled into a powder using food mixer and the mushroom powder then extracted twice with methanol (2 L) overnight at room temperature. The extract was filtered through Whatman filter paper No. 4, and the filtrate evaporated to dryness under reduced pressure and on a rotary evaporator at 40°C. The dried extract was dissolved in 300 mL of 10% methanol, and 300 mL of hexane was added to the flask contents. The mixture was then partitioned into hexane and aqueous layers. After separation of the hexane layer, 300 mL of chloroform was added to the remaining aqueous layer and the chloroform layer then separated from the partitioned solution. Using the same liquid/liquid partition procedure, ethyl acetate, *N*-butanol, and final aqueous extracts were obtained.

Two additional extraction systems (II and III) were also used. Thus, in the second system (II) the milled powder of *T. fuciformis* was extracted twice with 1 L of ether in reflux condenser for 1 hr at 40°C and the residue further extracted sequentially twice with ethylacetate, chloroform, ethanol, and water in the same reflux condenser at 77, 61, 78, and 100°C, respectively (II). In the third system (III), chloroform was used first instead of ether as the initial extraction solvent and ethylacetate, ether, ethanol, and water applied sequentially and in the same manner. All extracts were concentrated and dried for further experimentation. Each experiment was performed in triplicate.

Cell culture The human colon adenocarcinoma cell line (DLD-1) was purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). The cell line was maintained in RPMI 1640 medium containing 10% FBS and 1% antibiotics (penicillin/streptomycin) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Early stationary phase cultures with a typical density of $2\text{-}3\times10^6$ cells/mL and with a typical viability of 90-95% were passed every 2 to 3 days. The cell density at seeding was adjusted at 1×10^5 cells/mL.

Cytotoxicity assays Cytotoxicity testing was performed in triplicate. Cells were grown in 13 mL of culture medium in a T-75 culture bottle for 2 days until approximately 90% confluence was observed. After removing the culture media, the cells were detached from the flask by trypsin treatment. Cells were cultured in microtiter plates and the cytotoxicity was assessed. After counting the number of viable cells, $2\text{-}3\times10^4$ cells were seeded in standard 96-well microtiter plates in a volume of 200 μL per well. The plates were maintained in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Seeding density of the cells was confirmed before the addition of extracts to ensure that cultures did not become confluent.

The cell viability assay was based on cleavage of the tetrazolium salt to form a red formazan dye by live cells. Cell viability was directly quantified in triplicate using a scanning multi-well spectrophotometer. Test extracts dissolved in DMSO at various concentrations (1, 10, 100, and 1,000 ppm) were added into each well; DMSO was used for the control. Seeded cells in microtiter plates were kept in a CO_2 incubator for 48 hr. The culture incubation was terminated by the addition of 100 μL (1 mg/mL) of MTT solution to each well. After the plate was further incubated for 5 hr, 100 μL of 0.01 N HCl/isopropyl alcohol was added to each well to solubilize formazan. The absorbance of solubilized formazan in each well was then read on a microwell-plate reader (3550; Bio-Rad, Hercules, CA, USA) at a wavelength of 590 nm and recorded. The mean absorbance of the control wells represented 100% cell survival and the mean absorbance of treated cells was compared with that of control. Cell survival (%) was determined by averaging three repeat experiments. The mean inhibitory concentration (IC_{50}) represented the concentration at which cell viability was reduced by 50%.

Determination of phenolic compound contents The Folin-Ciocalteu method was applied to determine the total phenolic content of the sample extracts according to modified method of Singleton *et al.* (27). A small aliquot (0.5 mL) of sample extract in methanol was mixed with 1.5 mL distilled water and 0.5 mL of the Folin-Ciocalteu reagent. After a 5 min incubation at room temperature, 0.5 mL Na_2CO_3 (10% aqueous solution) was added to the mixture. The mixture was kept in the dark for 60 min and its absorbance then measured at 640 nm using a UV-visible spectrophotometer (Uvikon 933, Milan, Italy) to determine the contents of phenolic compounds in each extract. Chlorogenic acid was used as a standard for the expression of equivalents by reflecting the phenolic content as the amount of standard (μg) in 1 mg sample. Estimation of the phenolic compounds was carried out in triplicate and the results presented as mean values ± standard deviation.

Results and Discussion

Identification of *T. fuciformis* The sequences of the ITS region in the 5.8S ribosomal genes (rDNA) of isolates were analyzed after polymerase chain reaction (PCR) with the primer pair for the ITS region and the sequence homology (Fig. 1). The sequence of isolated strain FB001 was very similar to that of *T. fuciformis* in the EMBL/GenBank database, with a homology of over 98% (Fig. 1). A second isolate, No FB002, also exhibited high homology with *Annulohhypoxylon stygium* (Fig. 2), one of the symbiotic strains for cultivating *T. fuciformis.* Both

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T. fuciformis    ---GTAGGTGAAACCTGCGGAAGGATCATTTGAGATTACACCGGGCCGCGAGGCCCTTCC 57
FB001            TCCGTAGGTGAA-CCTGCGGAAGGATCATTTGAGATTACACCGGGCCACGAGGCCCTTCC 59

T. fuciformis    AAACACCTGTGCACATCGGACCGCGCCTCCGGGCCGGGCCGCCTTCACACAAACATATGT 117
FB001            AAACACCTGTGCACATCGGACCGCGCCCCCGGGCCGGGCCGCCTTCACACAAACATATGT 119

T. fuciformis    CAAGAACGTAATGCATCATAACATGAAACAACTTTCAACAACGGATCTCTTGGCTCTCGC 177
FB001            CAAGAACGTAATGCATCATAACATGAAACAACTTTCAACAACGGATCTCTTGGCTCTCGC 179

T. fuciformis    ATCGATGAAGAACGCAGCGAATTGCGAAAAGTAATGTGAATTGCAGAATTCAGTGAATCA 237
FB001            ATCGATGAAGAACGCAGCGAATTGCGAAAAGTAATGTGAATTGCAGAATTCAGTGAATCA 239

T. fuciformis    TCGAATCTTTGAACGCACCTTGCGCCTTTTGGTATTCCGAAAGGCATGCCTGTTTGAGTG 297
FB001            TCGAATCTTTGAACGCACCTTGCGCCTTTTGGTATTCCGAAAGGCATGCCTGTTTGAGTG 299

T. fuciformis    TCATGTAGACTCAACCCCCCGGGTTTCTGACCCGGCGGTGTTGGATTTGGGCCCTGCCTC 357
FB001            TCATGTAGACTCAACCCCCTGGGTTTCTGACCCGGCGGTGTTGGATTTGGGCCCTGCCTC 359

T. fuciformis    TCTGGCTGGCCTTAAATGCGTTAGTGGTTTCACGCAGACGTCGTAAGTTACGCGTCGACT 417
FB001            TCTGGCTGGCCTTAAATGCGTTAGTGGTTTCACGCAGACG---------------------- 399

T. fuciformis    GTGGGCCGCTCACAACCCCCTTTACTTTTGCACTCTGGCCTCAAATCAGGTAGGGCTACC 477
FB001            ------------------------------------------------------------

T. fuciformis    CGCTGAACTTAAGCATATCAATAAGCGGA 506
FB001            ----------------------------
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Fig. 1. DNA sequence analysis of *T. fuciformis* isolate No. FB001 in the ITS region of 5.8S ribosomal genes through the BLAST search.

strains were further employed to produce the fruiting bodies.

Preparation of various fractions by systemic extraction

Edible mushrooms have beneficial effects on health and in the treatment of disease believed to be through their immunomodulatory, anti-neoplastic, and lipid-reducing properties (28, 29). For example, water extracts of the *shiitake* fruiting bodies, one of the world's most popular edible mushrooms, have been shown to prevent tumor growth such as colorectal carcinogenesis or colon carcinoma, in mice (19, 20, 30). *P. multiplex*, a Korean wild edible mushroom, was extracted using methanol with water and ethylacetate and an assay of each fraction with MTT revealed tumoristatic effects against human gastric and other cancer cells (21).

So far there have been no studies reported related to the characterization of active cytotoxic components in *T. fuciformis* against a human carcinoma. Therefore, an experimental design was put in place to assess possible cytotoxic components in extracts from *T. fuciformis*. In experimental setting I, the chemical components in the methanol extract of *T. fuciformis* were further fractionated by solvents of increasing polarity yielding the hexane, chloroform, ethyl acetate, *N*-butanol, and water subfractions as described by Lee *et al.* (31) (Fig. 3). As shown in Table 1, a total of 6.0 %(w/w) of cell dry weight was extracted by methanol and most components were assigned to the *N*-butanol and water subfractions. The level of *N*-butanol extract was about half of the total extract and the yield of the water subfractions was 2.69%(w/w). In experimental setting II compared to experimental setting I, every extract was prepared directly from the fruiting body of *T. fuciformis* with various solvents at different elevated temperature according to the boiling temperature (32, 33).

Table 1. Preparation of extracts and component yields according to experimental settings I, II, and III

I		II		III	
Solvents	Yield (%)	Solvents	Yield (%)	Solvents	Yield (%)
Hexane	0.46	Ether	0.34	Chloroform	0.21
Chloroform	0.05	Ethyl acetate	0.90	Ethyl acetate	0.80
Ethyl acetate	0.06	Chloroform	0.96	Ether	0.45
N-butanol	3.17	Ethanol	4.73	Ethanol	4.61
Water	2.69	Water	8.90	Water	10.80
Total	6.43		15.83		16.87

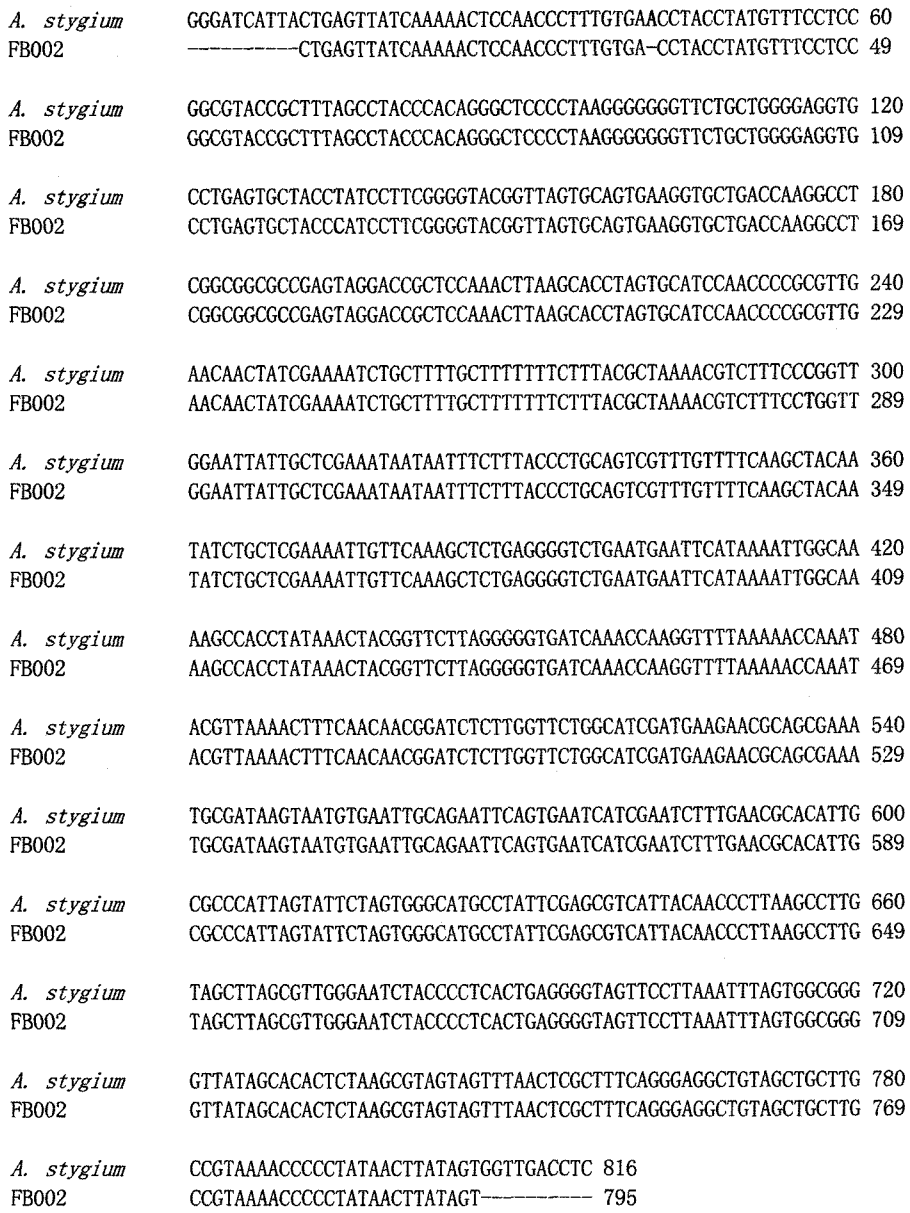

Fig. 2. DNA sequence analysis of *Hypoxylon* spp. isolate No. FB002 in the ITS region of 5.8S ribosomal genes through the BLAST search.

Most components were assigned to the aqueous fraction with a yield of 15.83%. In system II, ether was the initial extraction solvent and 0.34% of components were extracted in this step. The extraction yields using ethyl acetate, chloroform, and alcohol were 0.90, 0.96, and 4.73%, respectively. In the third system (III), chloroform was used instead of ether as a first extraction solvent and ethyl acetate, ether, ethanol, and water were then applied sequentially and in the same manner. The yield of the water fraction was essentially similar to the results of experimental setting II. Most components remained in the ethanol and water fractions and lesser amounts of the components were found in the other hydrophobic fractions of this system.

Cytotoxic effects of extracts from *T. fuciformis* on the DLD-1 cell line Mushroom extracts appear to contain potent chemopreventive agents against colon carcinoma and stomach cancer (20, 21). To elucidate possible similar anticancer activities of the *T. fuciformi* mushroom, the DLD-1 human colon adenocarcinoma cell line was chosen as our experimental model (8) and its viability was examined in the presence of various extracts of *T. fuciformis*. Figure 4 presents data for the cytotoxic effects of all fractions from experimental setting I on the DLD-1 colon adenocarcinoma cell line. The chemical components in the methanol extract were fractionated by solvents of increasing polarity to yield hexane, chloroform, ethyl acetate, *N*-butanol, and water subfractions. Hydrophobic components, which were restricted to the hexane, chloroform, and ethyl acetate subfractions exhibited cytotoxic effect on the DLD-1 cell line with IC_{50} values of 350, 400, and 450 ppm, respectively (Fig. 4). The *N*-butanol and water subfractions did not show any sign of cytotoxic effects on

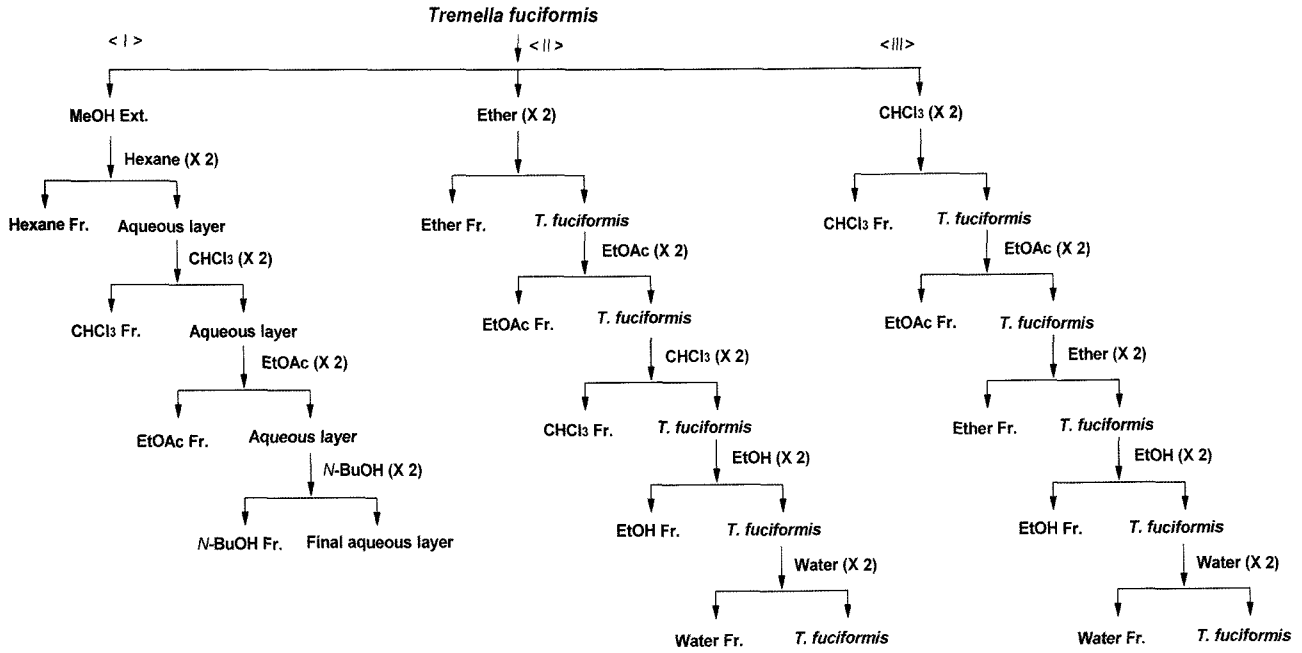

Fig. 3. Preparation of various fractions by systemic extraction.

the cells when applied at less than 1,000 ppm.

As shown in Fig. 5, some extracts in experimental setting II were cytotoxic to the cell line and in a dose dependent manner. The viability of the DLD-1 cell line was shown to be decreased by the addition of ether, ethyl acetate, and chloroform extracts and IC_{50} values for the cells were identified as 150, 800, and 820 ppm, respectively. The cell line was very sensitive to treatment with ether fractions and approximately 40% of the cell viability was decreased by exposure to the ether extract at 50 ppm compared to control.

In experimental setting III, the hydrophobic components were extracted by solvents of increasing polarity, so that the first extraction solvent was chloroform instead of ether, which had been used as a first solvent in setting II. Three extracts including chloroform, ethyl acetate, and ether exhibited relative strong dose-dependent cytotoxic effects on the DLD-1 cell line (Fig. 6) with IC_{50} values of 350, 420, and 420 ppm, respectively. In most cases, the hydrophobic extracts from *T. fuciformis* caused a dose-dependent decline in cell viability, while no noticeable effect on viability could be seen in cultures exposed to ethanol or water extracts.

The discovery of new anti-cancer substances from biological systems has been the target of many and variable studies. These studies include such important parameters as cytotoxicity, genotoxicity, induction of apoptosis, etc. Cytotoxicity is one of the major chemotherapeutic targets of anti-tumor activity (34), and most clinically effective anti-tumor agents possess significant cytotoxic activity *in vitro* systems. Recent publications support the notion that the number of anti-tumor agents from the field of fungi and mushrooms is increasing (35-41). A survey of the cytotoxicity or antitumor activities reported for several mushrooms indicated that the cytotoxic effect related to anti-tumor activity might result from a mushroom's polysaccharides (13-16) found in soluble fractions or the

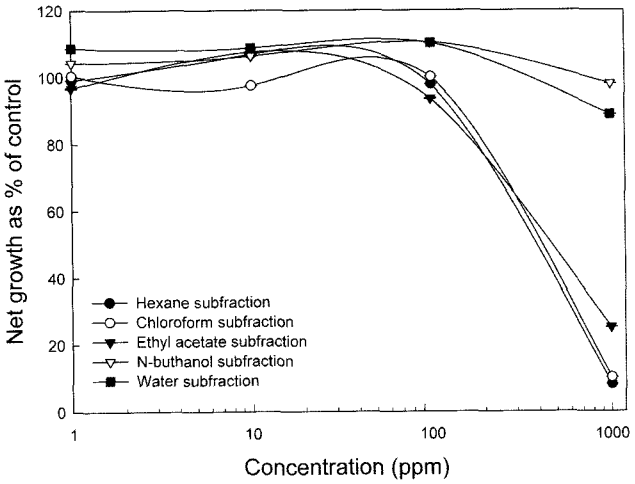

Fig. 4. Cytotoxic effects of extracts from *T. fuciformis* on the DLD-1 human colon adenocarcinoma cell line in experimental setting I. The cytotoxicity test was performed in triplicate.

polyphenols and flavonoids in extracts using hydrophobic solvents (17,18). As presented in our results, hydrophobic components of *T. fuciformis* in extracts using hydrophobic solvents presented dose dependent cytotoxic effects on the viability of the DLD-1 human adenocarcinoma cell line. Therefore, the composition of the *T. fuciformis* extracts should be investigated to understand their possible cytotoxic role. From this perspective, phenolic compounds in the extracts are of especial interest because phenolic compounds and flavonoids are thought to be the major components in several mushrooms (17).

Content of phenolic compounds in the fractions from experimental settings (I, II, III) In previous report, polyphenols and flavonoids were regarded as the main

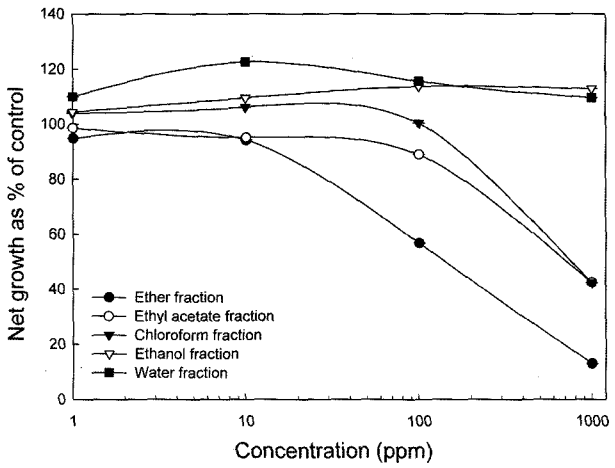

Fig. 5. Cytotoxicity of extracts from *T. fuciformis* against the DLD-1 cell line in experimental setting II.

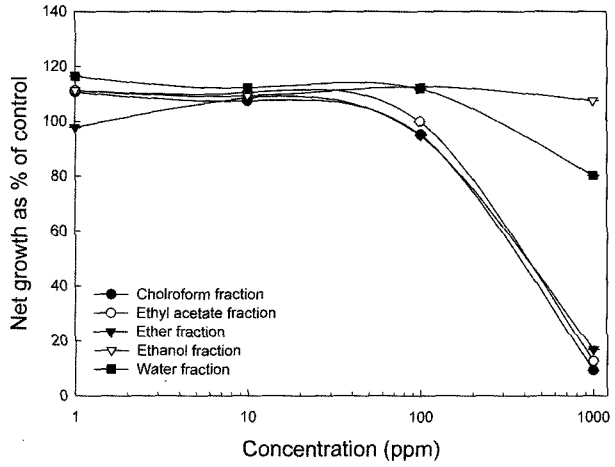

Fig. 6. Cytotoxic effect of extracts from *T. fuciformis* against the DLD-1 cell line experimental setting III.

active components in mushroom extracts when using non-polar solvents such as ethyl acetate and methanol (17, 18). As presented in Table 2, most components remained in ethanol and water fractions for all three of our experimental settings. Relatively lesser amounts of such substances were found in other hydrophobic fractions, in which large portions of phenolic compounds were analyzed as shown in Table 2. Hexane, chloroform, and ethylacetate fractions in experimental setting I exhibited a high content of total phenolic compounds and their chlorogenic acid equivalents were determined to be 39.06, 66.3, and 25.6 μg/mg, respectively. These fractions exhibited high cytotoxic effects on the DLD-1 cell line as shown in Fig. 2. The *N*-butanol and water subfractions contain very little of the phenolic compounds and did not demonstrate a cytotoxic effect against the cells at under 1,000 ppm. In experimental setting II, every extract was prepared directly from the fruiting bodies of *T. fuciformis* at high temperature. Large quantities of hydrophilic components were found in the ethanol and aqueous fractions in which very little of the phenolic compounds were noted. High levels of phenolic compounds were identified in the ether, ethyl acetate, and chloroform fractions which were shown to be cytotoxic to the DLD-cells (Fig. 5). Experimental setting III presented essentially the same results as with setting II relative to the content of total phenolic compounds. Extracts with chloroform, ethyl acetate, and ether fractions in which most phenolic compounds were found had high cytotoxic effects on the DLD-1 cell line at 1,000 ppm.

The survey for cytotoxicity or antitumor activities of several mushrooms indicated that the cytotoxicity related to anti-tumor activity might represent two different classes of substances. A mushroom's polysaccharide, especially high molecular weight glucans and polysaccharide-protein complexes have been considered to have anti-cancer properties. (38-43). Other mushroom derived substances cited for their cytotoxicity are the polyphenols and flavonoids. Polyphenols and flavonoids are major compounds in extracts obtained with non-polar solvents such as ethyl acetate and methanol (17, 18).

Interestingly, only the hydrophobic solvent extracts of *T. fuciformis* exhibited cytotoxicity against the DLD-1 cell line while hydrophilic extracts had very low effect on the cell viability. The cytotoxic effect of extracts did not necessarily coincide with the content of total phenolic compounds in the extract as the correlation coefficients obtained in these studies were quite small in all three experimental settings. This result is thought mainly due to the different types and levels of phenolic compounds in the test fractions. Even though no correlation was observed in the experiments, cytotoxicity was nonetheless measured only in those fractions, which contained phenolic compounds, similar to the results reported by Pomilio *et al.* (18)

The findings reported from this experiment design suggest that hydrophobic extracts from *T. fuciformis* can provider active components with cell cytotoxicities, and

Table 2. Total phenolic content of extracts from *T. fuciformis* according to each experimental setting

I		II		III	
Solvents	Chlorogenic acid eq. (μg/mg)	Solvents	Chlorogenic acid eq. (μg/mg)	Solvents	Chlorogenic acid eq. (μg/mg)
Hexane	39.07	Ether	31.11	Chloroform	46.00
Chloroform	66.31	Ethyl acetate	45.17	Ethyl acetate	41.60
Ethyl acetate	25.61	Chloroform	31.65	Ether	40.82
N-butanol	8.97	Ethanol	5.09	Ethanol	9.14
Water	6.97	Water	5.60	Water	10.59

may be mainly comprised of polyphenols and flavonoids. Further study should be performed to characterize the active components exhibiting cytotoxic effects on the DLD-1 human carcinoma cell line, further extending the consumption and use of *T. fuciformis* as a source for food and traditional medicines.

Acknowledgments

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