

Cytotoxicity Effect of *Agaricus blazei*, *Grifola frondosa* and *Hericium erinaceus* Used in Traditional Medicine

N. Nur Shahirah^{1,2,*}, M.Y. Farida Zuraina¹, R. Nor Fadilah², C.F. Suziana Zaila^{1,2}, L. Florinsiah^{1,2}, M.N. Norfazlina^{1,2} and L. Lek Mun²

¹Faculty of Applied Sciences, Universiti Teknologi MARA, 40450, Shah Alam, Selangor

²Toxicology Laboratory, Faculty of Health Sciences, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur, Wilayah Persekutuan, Malaysia

Abstract: *Agaricus blazei* Murrill (ABM), *Grifola frondosa* (GF) and *Hericium erinaceus* (HE) are mushrooms that are native to China and are widely cultivated in Malaysia for its medicinal uses. They are considered as the most important edible and culinary-medicinal biotechnology species. This study was carried out to determine the *in vitro* toxicity of these medicinal mushrooms and their possible risk to human health. Extracts were prepared from these mushrooms using various solvents. The cytotoxicity of these extracts was determined using the MTT assay [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] in Chinese hamster fibroblast cell line (V79-4). Five different concentrations of the mushroom extracts (2mg/ml, 1mg/ml, 0.5mg/ml, 0.25mg/ml and 0.125mg/ml) were used and cytotoxicity was determined following 24 hours treatment. The results showed that only aqueous extracts of *Agaricus blazei* Murrill display cytotoxicity effects (IC₅₀—1.7mg/ml) compared to the methanol extracts. In conclusion, the aqueous extracts of *Agaricus blazei* Murrill showed weak cytotoxicity *in vitro* compared to methanol extracts, suggesting that these mushrooms are safe to consume.

Keywords: *Agaricus blazei* Murrill, Chinese hamster fibroblast cell line, Cytotoxicity effect, *Grifola frondosa*, *Hericium erinaceus*, MTT assay,

INTRODUCTION

Of the 14,000 to 15,000 species of mushrooms in the world, around 700 have medicinal properties. However it has been estimated that there are about 1800 species of mushrooms that have medicinal attributes. Thus, mushroom have vast prospects as source of medicinals. Although plant extracts have been used in the treatment of diseases over centuries, it is also known that many plants synthesize toxic substances, which in nature act as defence against infections, insects and herbivores. Previous studies have also potentially toxic and carcinogenic [3] and it has also been reported that some traditional medicines may have a genotoxic potential [1, 12, 14]. Assessment of the potential genotoxicity of traditional medicines is indeed an important issue as damage to the genetic material may lead to critical mutations and therefore increase the risk of cancer and other diseases. Lacking of knowledge in combination with widely use of medicinal mushroom, the major purpose of the present study was to evaluate the potential cytotoxicity of the aforementioned mushrooms. These mushrooms had been traditionally believed to have medicinal value as effective in both preventing and treating specific disease and antioxidant activity. Thus, the intake of mushrooms and their extractable

bioactive compounds appear to be effective in cancer prevention and growth inhibition.

Another important fact is the certainty that mushroom extracts, compared with other drugs, show a very low toxicity when regularly consumed, even in high dosages. However, despite of widespread use of these mushrooms as folk medicine to control ailments, research about either they have a genotoxic potential has not been reported. The main purpose of this study to determine the safety and efficacy of the mushrooms between *Agaricus blazei*, *Grifola frondosa* and *Hericium erinaceus*.

MATERIALS AND METHODS

The samples were obtained from Bioresis, Perak whilst Chinese hamster fibroblast cell line (V79-4) was obtained from Toxicology Laboratory UKM. The chemicals used were DMEM high glucose medium, fetal bovine serum 10%, 1% penicillin/streptomycin, [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], DMSO, Trypan blue and Trypsin.

Samples Collection

Selected species of mushrooms (*A. blazei*, *G. frondosa* and *H. erinaceus*) were obtained from Bioresis, Perak. Every sample comprises complete mushroom fruiting bodies (cap, gills, tubes, and stripe) of different sizes. The mushroom samples were washed with running water and kept at <4°C within 24 hour prior to sample preparation.

*Address correspondence to this author at the Faculty of Applied Sciences, Universiti Teknologi Mara, Shah Alam, Selangor, Malaysia;
Tel: +60-013-309-2270; E-mail: nurshahirah0209@gmail.com

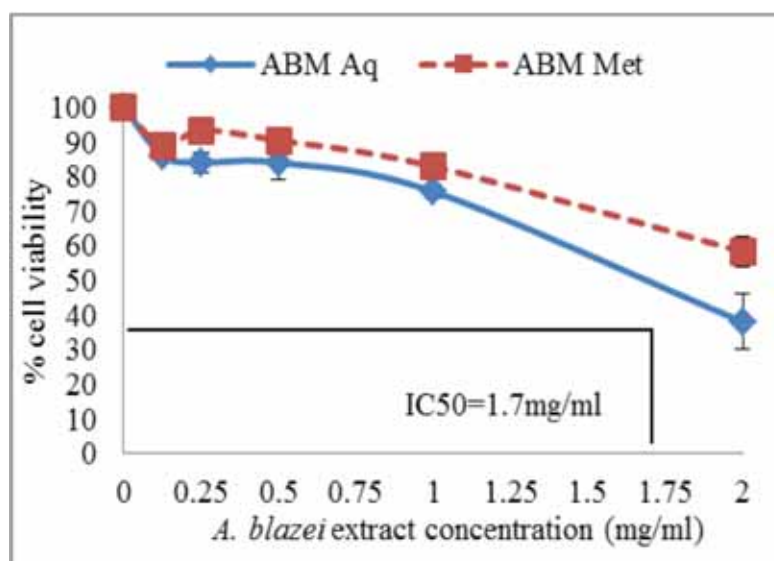


Fig. (1). The cells treated with different concentrations of *Agaricus blazei* (ABM) in aqueous and methanol for 24hour. Data are Means \pm SEM, N = 3. **P < 0.05 vs. control.

Culture of Chinese Hamster Fibroblast Cell Line (V79)

Chinese hamster fibroblast cell line (V79-4) were obtained from Toxicology Laboratory UKM and cultured as described previously (ATCC Catalogue Details No CCL-93). Cells were grown as monolayers in a T-25 cm² culture flask. The medium was supplemented with 2.0 g/l sodium bicarbonate, antibiotics (100 U of penicillin/ml, 100 g of streptomycin/ml) and 10% fetal bovine serum. The cell culture medium and their supplements were purchased from Life Technologies, Gibco BRL Products (Rockville, MD). The cell cultures were maintained in a humidified atmosphere of 5% CO₂ at 37°C and were harvested when they reached 80% confluence, i.e., in their exponential growth phase. For bioassay activity, 70% methanol and aqueous extracts of each sample were dissolved in aqueous to a final concentration of 200 mg/ml. These solutions were then filtered using sterile 0.45 μ m syringe filter. Menadione at 50 μ M was used as positive control for MTT assay.

Plant Material and Extraction Procedures

The methanol extraction method is the modification from the method by Ugochukwu and Babady [15]. *G. frondosa*, *H. erinacium* and *A. blazei* were dried at 45°C in the oven for twenty one days and ground into fine powder. The powder was soaked respectively in 70% methanol at the ratio of 1:10 and shake for 72 hours at room temperature. The combined suspension was filtered using Whatman filter paper No.1. The extract was pooled and concentrated under reduce pressure at 40°C using a rotary evaporator (Buchi R-114). The extract was completely lyophilized by continuous freeze drying operation for 72 hours, yielding a certain amount of crude extract. The extracts were kept in dark at 4°C for further use. Prior to use, the crude extract was dissolved in distilled water [10].

The extraction aqueous method that was a modification from the method by Sakanaka [13]. For aqueous extraction, the ground powder (200 g) of the mushroom was soaked in 500 ml of distilled water for 24 h and stored at 4°C in the dark to prevent microbial activity. The mixture was then

filtered freeze-dried and the dry extract kept at 4°C in an air-tight jar prior to the bioassays.

MTT Cytotoxicity Assay

The viability of the V79-4 cells was used to determine the cytotoxicity effect of each of the mushroom extract as described previously [9]. The cell monolayers in exponential growth were harvested and 5 x 10³ cells in 100 μ l were placed into each well of the 96-well plates (NunclonTM, VWR International Inc., MD). The plates were incubated for 24 h at 37°C in 5% CO₂. The medium was discarded and 200 μ l of the test extracts for each sample in different concentrations were loaded into the 96-well plates. After 24 h incubation, 20 μ l of the MTT solution was added to each well and reincubated for 4 h at 37°C before the medium was discarded and 100 μ l of DMSO added to dissolve the formazan crystals. The plate was shaken for 30 min to dissolve the crystals formed and the absorbance was measured at 570 nm with a microplate reader. Assays with each concentration were repeated three times. The MTT[3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide] (Sigma Chemical Co., St. Louis, MO) was dissolved in phosphate buffer saline (PBS) solution at concentration of 5 mg/ml and filtered through a 0.22 μ m filter to sterilize and remove insoluble residues.

Statistical Analysis

ANOVA was used to measure significance differences between the means.

RESULTS

In search for natural products with cytotoxic activity, 70% methanol and aqueous crude extracts were prepared from 3 medicinal mushrooms. The relationship between concentration of extracts and their cell viability inhibiting effect on V79 cells was investigated by MTT assay. MTT is a yellow water-soluble tetrazolium salt. Metabolically active cells are able to convert the dye to water-insoluble dark blue

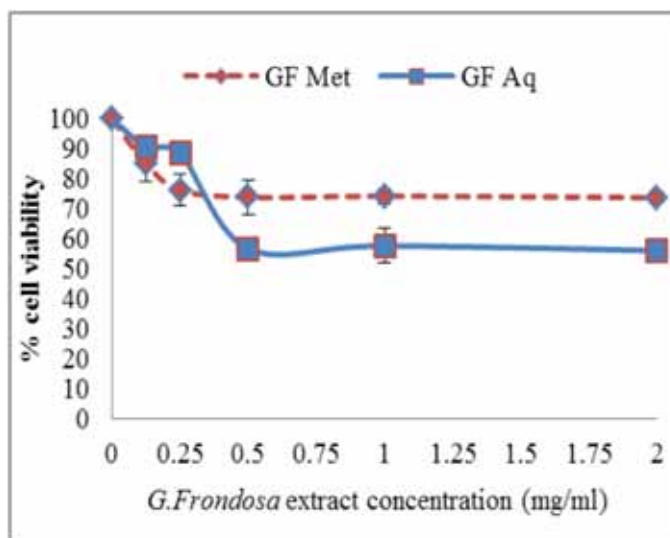


Fig. (2). The cells treated with different concentrations of *Grifola Frondosa* (GF) in aqueous and methanol for 24hour. Data are Means \pm SEM, N = 3. *P < 0.05 vs. control.

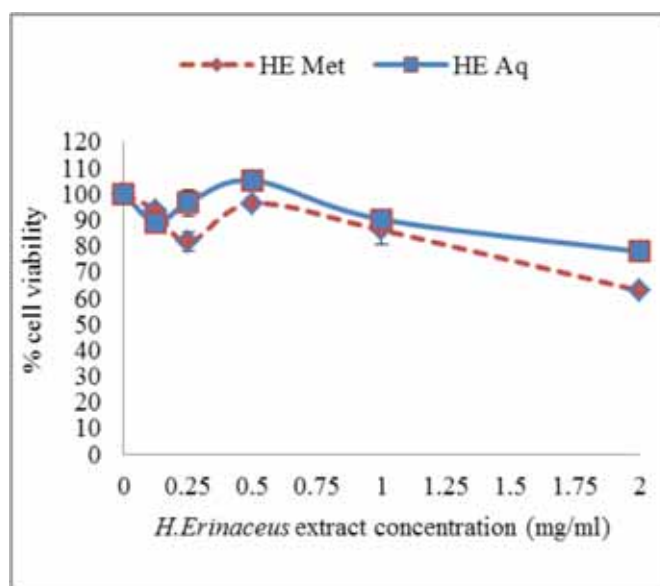


Fig. (3). The cells treated with different concentrations of *Heridium erinaceus* (HE) in aqueous and methanol for 24hour. Data are Means \pm SEM, N = 3. *P < 0.05 vs. control.

formazan by reductive cleavage of the tetrazolium ring. Cells were treated with three samples of mushroom extracts (*Agaricus blazei*, *Grifola frondosa* and *Heridium erinaceus*) in aqueous and 70% of methanol at concentrations ranging from 2 to 0.125mg/ml for 24 h at 37°C, then IC₅₀ values were determined graphically and the inhibition percentages were calculated. The results, summarized in Figs. (1, 2 and 3) showed that the tested normal fibroblast (V79-4) showed a good response to the effect of those mushroom extracts. As shown in Fig. (1), only *Agaricus blazei* in aqueous extract showed the IC₅₀ values at dose of 1.7mg/ml. *Grifola frondosa* and *Heridium erinaceus* did not show the cytotoxic effects. In methanol extract, these three mushrooms showed antiproliferative effect, inhibiting at least 25% of normal cell proliferation at 2mg/mL.

To allow sufficient time for sample to induce cell death, the duration of the experiment upon dose-response

relationships of the cell lines was determined for 24 h for all samples due to their low acute toxicity. The strongest cytotoxic activity was detected for the aqueous extract of *Agaricus blazei* with all presenting IC₅₀ values at 1.7mg/mL.

Many fungi are capable of producing secondary metabolites, some of which are pigments, antimicrobials or toxins for plants and animals. Fungal toxins can disrupt cellular energy production, inhibit glucose transport, block protein synthesis and cause the formation of DNA adducts, the latter being responsible for the carcinogenic and mutagenic properties of some fungi [8].

Previous studies have reported chemo protective activity for mushroom extracts, but it is proven that the protective effect depends on type of extract and which components (aqueous or organic) are present in its composition [2,6,7]. They also reported that *Agaricus blazei* aqueous extracts from different geographic locations varied in antigenotoxic

activity, some being more efficient while others had no protective effect [5]. Moreover, the protective effect provided by such extracts is variable depending on the type of damage caused by the damage-inducing agent, with a specific extract being protective against one agent but not necessarily against others. However, manifestation of these effects depends on the cell system, type of assay and protocols used, all of which could be involved in effects attributed to mushroom extracts. Working with crude extracts, also means working with complex mixtures of biologically active compounds. Some of the compounds in such mixture can be cytotoxic and/or genotoxic, others can be cytoprotective and/or anti [6]. It is possible that the protective effect of *A. blazei* aqueous extracts may be due to a mixture or complex of compounds and not just a single component, which would account for loss of activity when more purified extracts are studied [4, 11]. Summarizing, our results suggest that the aqueous extract of *Agaricus blazei* tested does not provide chemoprotection at 1.7mg/ml, and that all fractions are potentially cytotoxic as determined by the MTT assay. For *Grifola frondosa* and *Hericium erinaceus* in aqueous and methanol extract did not contain substances with cytotoxic, mutagenic and/or antimutagenic effects. Furthermore, it is proven that more tests are necessary for the investigation of the biological effects of both methanolic and aqueous extracts and their interactions with cell metabolism before recommending their large-scale use by the general public, something which is already occurring in various countries.

These findings indicate that *Agaricus blazei* aqueous extract should not be used due to their genotoxicity and care should be taken in the use of *Agaricus blazei*, *Grifola frondosa* and *Hericium erinaceus* by public until further bioassay characterization of these fungi is completed.

CONCLUSION

All data suggest that extracts from the medicinal mushroom studied deserve further investigations in order to isolate bioactive secondary metabolites with genotoxic properties. Experiments to analyze DNA may damage the extracts and mode of cell action, in order to determine the potential of genotoxicity of those compounds and to perform more extensive biological evaluations.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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