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Cytotoxicity and genotoxicity of selected medicinal mushroom used in traditional medicine

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ABSTRACT

Agaricus blazei, Grifola frondosa and *Hericium erinaceus* are popular medicinal mushroom. They have been widely consumed in many countries, including Malaysia, due to its therapeutic properties and commonly used as traditional medicine. This study was carried out to determine the *in vitro* toxicity of these medicinal mushrooms that were cultivated in Malaysia and their possible risk to human health. The cytotoxic and genotoxic activity were evaluated against Chinese hamster fibroblast cell line (V79-4) using MTT assay and alkaline comet assay. Five different concentrations of the extracts (2mg/ml, 1mg/ml, 0.5mg/ml, 0.25mg/ml and 0.125mg/ml) in 70% methanol and aqueous solvent were used. The cytotoxicity was determined following 24 hours treatment. Our results demonstrated that all extracts show decreased in cell viability at the maximum concentration of 2mg/ml. On the other hand, none of the extracts caused DNA damage to the V79-4 cells at 2mg/ml following 24 hour incubation, as compared to the positive control, menadione (tail moment-13.135±2.273). In conclusion, several mushroom extracts showed weak cytotoxic effect but did not cause DNA damage against Chinese hamster fibroblast cell line (V79-4), suggesting that these mushrooms are safe to consume.

KEYWORDS

Medicinal mushrooms;
MTT assay;
Comet assay.

INTRODUCTION

Alongside the mushrooms' long history as a food source is an equally long history of beliefs about their curative abilities in traditional medicine systems. Although there are limited direct human intervention trials, there is a rapidly growing volume of *in vitro* and *in vivo* animal trials describing a range of possible health benefits. Lately, attempt has been made by crop development in Malaysia to produce its own organic medicinal fungus such as *Agaricus blazei, Grifola frondosa* and *Hericium erinaceus* to develop a wealth of expertise in the use of medicinal mushrooms for conjunctive therapy and as wellness product.

Previous study have reported that some of the mushroom extracts from geographic locations varied in genotoxic activity, some being more efficient while others had no protective effect[1]. It is necessary to obtain

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epidemiological and experimental data on the beneficial effects of the extracts of the species studied. 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.

In vitro cytotoxicity tests such as MTT assay are useful to measure viability of cell and are considered as the initial step in toxicity study. MTT is a yellow water-soluble tetrazolium salt. Metabolically active cells are able to convert the dye to water-insoluble dark blue formazan by reductive cleavage of the tetrazolium ring. On the other hand, the comet assay or the single cell gel electrophoresis (SCGE) introduced by Ostling and Johanson in 1984[2] has been widely used for the assessment of the genotoxicity. The alkaline version of the comet assay developed by Singh and colleagues in 1988[3] detects DNA single strand breaks and alkali-labile sites. It has been recognized as one of the most sensitive methods available for detecting DNA strand breaks[4]. This method is a simple and rapid method, which can detect extremely small samples and observation can be made on single cells. The objectives of the study were to investigate the aqueous and methanol extracts of the selected medicinal mushroom for possible cytotoxic and genotoxic on Chinese hamster fibroblast cell line (V79-4). It is important to determine the safety and efficacy of these mushrooms before recommending their large-scale use by public.

EXPERIMENTAL

Samples collection

Selected species of mushrooms (*A.blazei*, *G.frondosa* and *H.erinaceus*) were obtained from BIORESIS, Perak (certification reference is JF0408255PR-744-0). 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 <4ºC within 24 hour prior to sample preparation.

Culture of Chinese hamster fibroblast cell lines (V79-4)

Chinese hamster fibroblast cell lines (V79-4) were obtained from Toxicology Laboratory UKM and cultured (ATCC Catalogue Details No CCL-93). Cells were grown as monolayer in a T-25 cm^2 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% CO_2 at 37°C and were harvested when they reached 80% confluence, ie. in their exponential growth phase. For bioassay activity, 70% methanol and aqueous extracts of each sample were dissolved to a final concentration of 200 mg/ml. These solutions were then filtered using sterile 0.45 µm syringe filter. Menadione at 50µM was used as positive control for MTT assay and alkaline comet assay.

Plant material and extraction procedures

The methanol extraction method is the modification from the method by Ugochukwu and Babady[5]. *G. frondosa, H. erinacium* and *A. blazei* were dried at 45°C in the oven for 21 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 40ºC 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 4ºC for further use. Prior to use, the crude extract was dissolved in distilled water[6]. Whilst the aqueous extraction method was a modification from the method by Sakanaka[7]. For aqueous extraction, the ground powder (200 g) of the mushroom was soaked in 500 ml of distilled water for 24h and stored at 4ºC in the dark to prevent microbial activity. The mixture was then filtered freeze-dried and the dry extract was kept at 4ºC in an air-tight jar prior to 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 by Mossman[8]. The cell monolayers in exponential growth were harvested and 5×10^4 cells in 100 µl were placed into each well of the 96-well plates (NunclonTM, VWR International Inc., MD). The plates were incubated for 24 h at 37ºC in

5%CO_2. The medium was discarded and 200 µl of the test extracts for each sample in different concentrations were loaded into the 96-well plates. After 24 h incubation, 20 µl of the MTT solution was added to each well and reincubated for 4 h at 37ºC. Then the medium was discarded and 100 µl 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 by using 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 µm filter to sterilize and remove insoluble residues.

Alkaline comet assay

Genotoxicity was evaluated via alkaline comet assay. Seeded cells in 6-well plate were treated with test compound at 2mg/ml and 1mg/ml, respectively for 24h. Menadione at 2.5µM was used as positive control. After 24 h incubation, cells were washed with PBS and trypsinized to detach the cells. The cells were transferred to eppendorf tubes and centrifuged at 2500 rpm for 5 min. The pellet was resuspended in PBS and recentrifuged. This process was repeated 3 times. Frosted slides were prepared with a layer of normal melting agarose (Sigma Chemical Co., St. Louis, MO, USA). Cells were then suspended in low-melting point agarose (Sigma Chemical Co., St. Louis, MO, USA), maintained at 37 ºC and placed on the slides coated with normal melting agarose. After the agarose gel had solidified, the slides were placed for at least 1 h in a lysing solution consisting of high salts and detergents (100 mM ethylenediaminetetraacetic acid [EDTA], 2.5 M sodium chloride, 10 mM Trizma base, adjusted to pH 10) with 1% Triton X-100 prior to use. The slides were then incubated in alkaline (pH > 13) electrophoresis buffer (1mM EDTA and 300mM sodium hydroxide) for 20 min to produce single stranded DNA. After unwinding, the DNA in the gel was electrophoresed under alkaline conditions at 25 V and 300 mA for 20 min to produce comets. The alkali in the gels was neutralized by rinsing the slides with Trizma buffer at pH 7.5 for every 5 minutes. Finally, slides were stained with ethidium bromide. The slides were analyzed via fluorescent microscope (Leitz Laborlux Epifluoresence Microscope, Germany) equipped with 515 barrier filter and 560 emission filter. Fifty cells per slide were scored and the percentage tail moment (TM) of DNA were analyzed.

Statistical analysis

The data were presented as mean±standard error of mean (SEM). Statistical analysis was performed by employing ANOVA test. A p value of <0.05 was considered significant.

RESULTS AND DISCUSSION

Cytotoxicity study on fibroblast cells

Following 24h treatment, the cytotoxic effects of aqueous and methanol for *A. blazei, G. frondosa* and *H. erinaceus* extract against V79-4 fibroblast cells were assessed by MTT assay as shown in Figure 1. V79-4 cells showed decrease in viability in a concentration-dependent manner following 24h treatment with both aqueous and methanol extracts. The results show that at highest concentration of *A. blazei* methanol extract, (58.3±4.3) % of viable cells were observed and it was comparable with *A. blazei* aqueous extract (38.0±8.1)%. IC_{50} values were observed for *A. blazei* aqueous at concentration of 1.75mg/ml. On the other hand, at highest concentration of methanol and aqueous extract for both *G. frondosa* treated on V79-4 cells, (73.7±1.9) % and (56.0±2.3)% of viable cells were observed respectively following 24h treatment. Viability of V79-4 cells observed on both methanol and aqueous extract of *H.erinaceus* at the highest concentration, were 62.8±0.4% and 77.8±2.1% respectively. *G. frondosa* and *H.erinaceus* did not give any IC_{50} value as compared to positive control, where a concentration response effect was seen following treatment with menadione, with an IC_{50} value of 4.5µg/ml. Our results therefore demonstrated that methanol extract for *A. blazei* and *G. frondosa* has better viability profile compared to the aqueous extract. Contrast with the aqueous extract of *H.erinaceus*, it gave higher viability profile than methanol extract.

Alkaline comet assay

Alkaline comet assay was employed to detect primary DNA damage induced by the extracts used in this study on Chinese hamster fibroblast cell line (V79-4). Low level of background damage was seen in the negative control of V79-4. For comparison, menadione at

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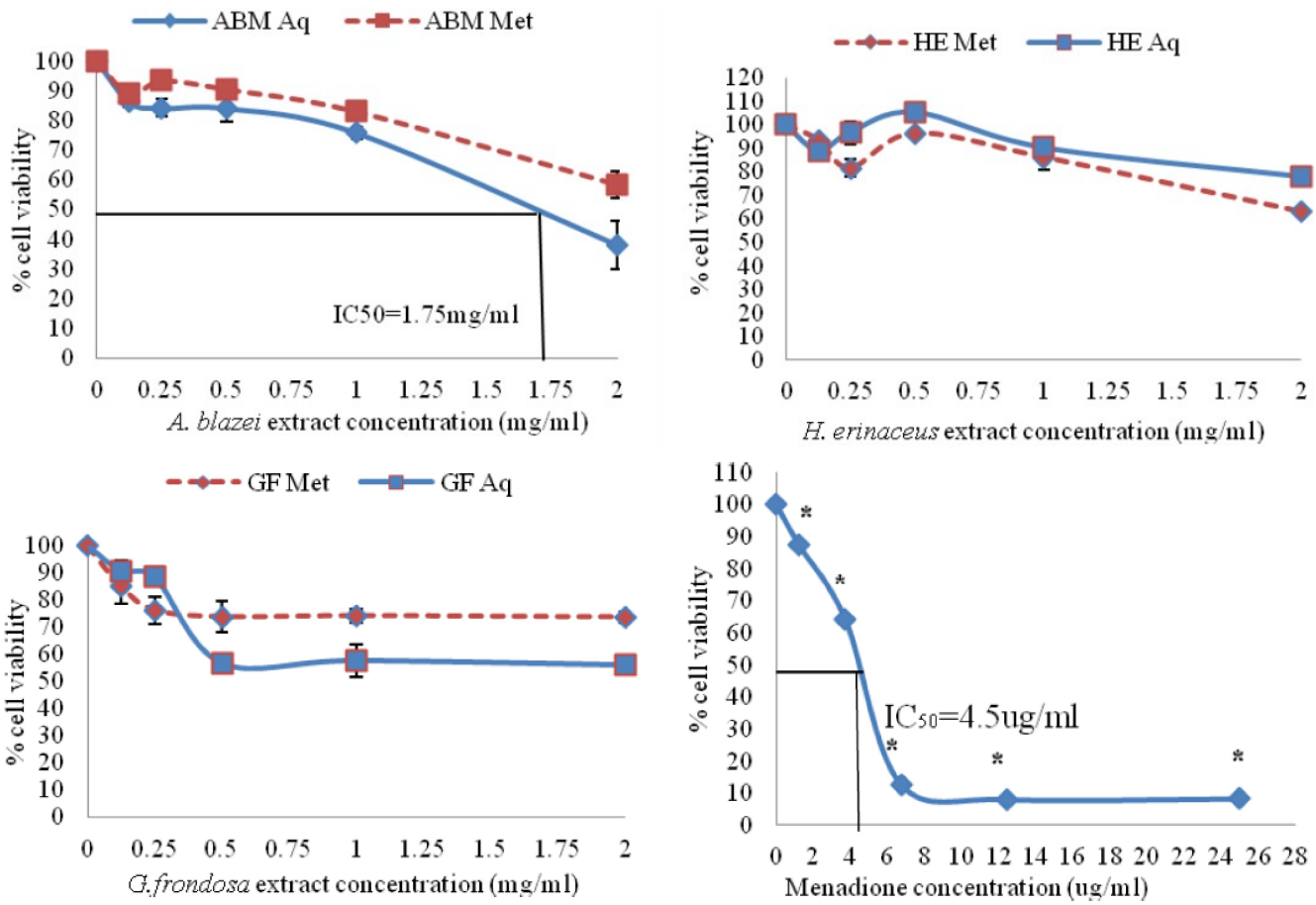

Figure 1 : Percentage of cell viability (%) with aqueous and methanol extract at different concentrations. Each point represents the mean ± SEM of three different independent experiments. (ABM)-*A. blazei*, (GF)-*G.frondosa*, (HE)-*H.erinaceus* and Menadione-Positive control.

concentration of 2.5µg/ml was employed. As shown in Figure 2, treatment with menadione in V79-4 cell lines demonstrated significant DNA damaged as compared to treatment with aqueous and methanol for *A. blazei*, *G. frondosa* and *H. erinaceus* extract. In this assay, scores were given according to the DNA damage of the cell using Comet Assay Analysis System software, Kinetics, USA. Tail moment (TM) was used to determine the DNA damage. TM is defined as the product of the distance between the head and the tail by the proportion of DNA in the tail, and used to evaluate the extent of DNA migration[9]. The TM provides the most stable estimates for DNA damage because it has a larger degree of uniformity in quartile dispersions. Furthermore, TM shows the smallest variability in the standardized extents of DNA damage among the tail parameters.

None of the extracts showed any severe DNA damage compared to the negative control. TABLE 2 tabulates tail moment for each samples used in this study. Methanol extract of *A. blazei* and *G. frondosa* exhibited higher DNA damage (TM of 3.221±1.078, TM of 2.116±4.893, respectively) than the aqueous extract. On the other hand, *H. erinaceus* caused the least damage to the DNA of the cell with methanol extract gave TM of 0.570±0.315, whilst aqueous extract gave a TM value of 1.821±3.626. Cells treated with menadione demonstrated significant DNA damage as compared to treatment with other extracts.

The captured image of cells in Figure 3 show that there was no DNA damage in the negative control, aqueous and methanol extracts of *A. blazei*, *G. frondosa* and *H. erinaceus*. Cells were intact and the comet tail was not observed in the slides. However, image in Figure 3 (B) shows cells with DNA damage following treatment of menadione (positive control) at 2.5µg/ml for 24 hour. The comet tail and DNA of the lysed cells were observed.

Natural products have been traditionally accepted as remedies due to popular belief that they present mi-

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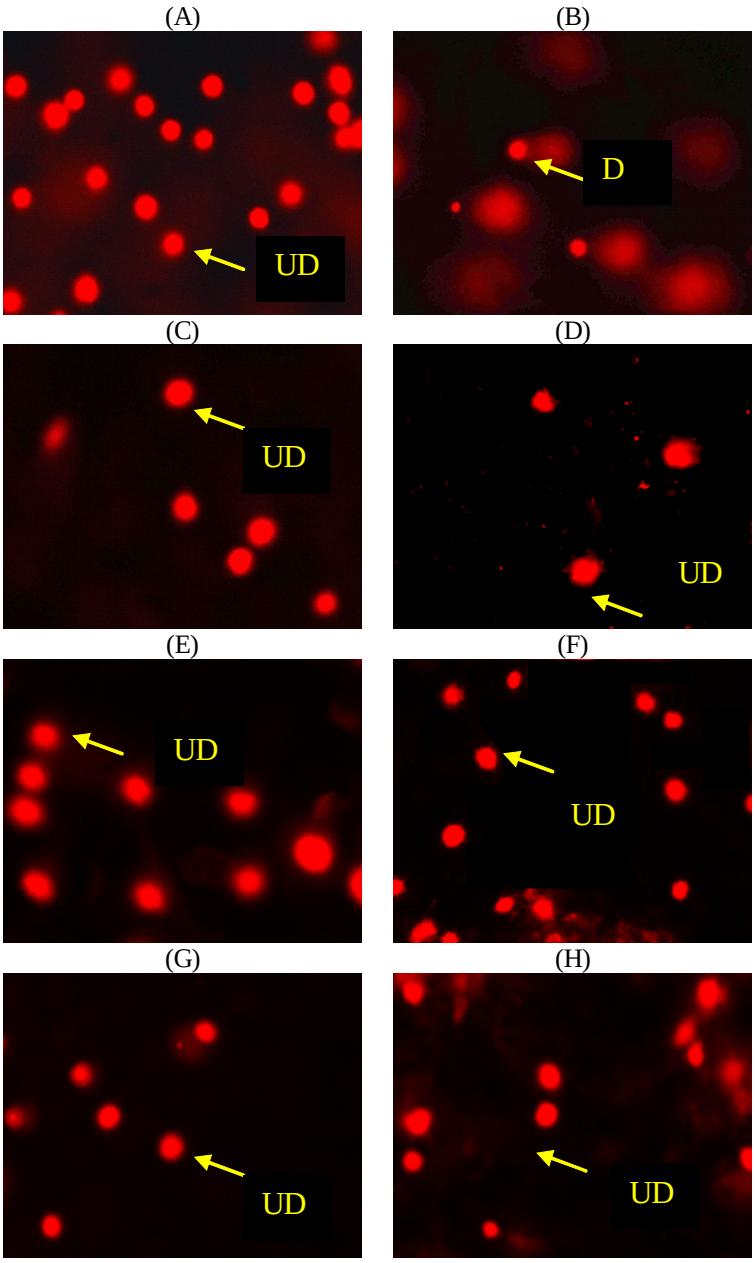

Note: UD: Undamaged DNA, D: Damaged DNA.

Figure 2 : Representative comet images of V79 cell lines; (A) Negative control (untreated V79-4), (B) Positive control (treated with 2.5µg/ml menadione) (C) Treated with 2mg/ml *A. blazei* aqueous extract (D) Treated with 2mg/ml *A. blazei* methanol extract (E) Treated with 2mg/ml *G. frondosa* aqueous extract- (F) Treated with 2mg/ml *G. frondosa* methanol extract (G) Treated with 2mg/ml *H. erinacues* aqueous extract (H) Treated with 2mg/ml *H. erinacues* methanol extract. Magnification: 200x

nor adverse effects. Therefore, understanding the natural products beneficial potential or adverse influence of used by human populations in implementing safety measures for public health. 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 abnormalities, the latter being responsible for the carcinogenic and mutagenic properties of some fungi[10].

Previous studies have reported 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[11-13]. Furthermore, Elgorashi and colleagues in 2003[1] have reported that some of mushroom extracts from geographic locations varied in genotoxic activity,

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TABLE 1 : Mean replicate±SEM, n=3. TM- Tail Moment. Menadione (Positive control)TM- 13.135±2.273, Negative control TM- 0.304±0.418.

Sample	Concentration	Methanol	Aqueous
Agaricus blazei	1000ug/ml	1.298±2.872	0.204±0.119
	2000ug/ml	3.221±1.078	0.411±0.559
Grifola frondosa	1000ug/ml	2.258±3.176	1.623±0.251
	2000ug/ml	2.116±4.893	1.725±3.017
Hericium erinaceus	1000ug/ml	1.242±3.091	2.788±4.699
	2000ug/ml	0.570±0.315	1.821±3.626

some being more efficient while others had no protective effect. In this respect, cytotoxic and genotoxic effects of selected medicinal mushrooms namely *A. blazei, G. frondosa* and *H. erinaceus* extracts were determined using MTT cytotoxicity test and alkaline comet assay. The negative control used was culture medium whilst menadione was used as positive control to ensure the validity of the experimental procedure.

MTT assay was carried out based on the reduction of tetrazolium salt to purple formazan by dehydrogenase enzyme activity that found in intact mitochondria of active living or viable cells. As shown in Figure 1, treatment with methanol *A. blazei, G. frondosa* and *H. erinaceus* in Chinese hamster fibroblast cell line (V79-4) resulted with no IC_{50} values. However, the mild cytotoxic activity was detected for the aqueous extract of *A. blazei* which demonstrated IC_{50} values at 1.7mg/mL compared to *G. frondosa* and *H. erinaceus* aqueous extract which did not show any cytotoxic effect at higher concentration.

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[14,15]. Working with crude extracts, also means working with complex mixtures of biologically active compounds. Some of the compounds in such mixture could be cytotoxic and/or genotoxic, others could be cytoprotective and/or anticytoprotective[12]. However, based on the classification of cytotoxicity by Abbas and friends[16], at 2mg/ml, *A. blazei* aqueous extracts showed weak cytotoxic effect. All other extracts showed no cytotoxicity at all.

We have further investigated the potential genotoxicity of the selected mushroom. Alkaline comet assay was used to assess the genotoxicity by measuring single-strand and double-strand breaks in DNA. The assay works upon the principle that strand breakage of the supercoiled duplex DNA, and expression of alkali labile sites as single strand breaks. Comets formed as the broken ends of the negatively charged DNA molecule become free to migrate in the electric field toward the anode[17].

Previous report found some edible mushrooms represent a valuable source of biologically active compounds with potential for protecting cellular DNA from oxidative damage, while other mushroom varieties do not[18]. In present study, no direct DNA damage effect was observed in aqueous and methanol extract for *A. blazei, G. frondosa* and *H. erinaceus*.

The main active compounds in *A. blazei, G. frondosa* and *H. erinaceus* are polysaccharide[11,19,20]. Polysaccharides from mushrooms, generally belong to beta-glucan family appear to inhibit tumour growth by stimulating the immune system[18]. It may cause a possible protective effect of component of beta-glucan extracted from selected mushroom. Furthermore, previous reports discovered a possible protective effect of beta-glucan extracted of *A. blazei* against DNA damage induced by benzo[a]pyrene, using comet assay (genotoxicity) in a Chinese hamster fibroblast cell line (V79-4). This has suggested that beta-glucan did not exert a genotoxic or mutagenic effect, but that it did protect against DNA damage via binding to benzo[a]pyrene or by the capture of free radicals produced during its activation[21]. However, Guterrez and colleagues in 2004[12], although using comet assay, did not find a protective effect for *A. blazei* aqueous extracts in V79 cells. These findings suggested that differences in the way of planting and cultivation, covering protection during storage and extracts preparation could influence in its effectiveness as a nutritional supplement protecting against DNA damage as proposed by Chang[22].

To validate the experiment, genotoxic effects of menadione (positive control) on cell line (V79-4) was evaluated following 24-h treatment at concentration of IC_{25} (2.5µg/ml) obtained from the MTT assay. These concentrations were chosen because higher concentrations such as IC_{50} will lead to false positive results as DNA strand breaks could result from dead or dying cells[23]. Additionally, the comet assay guideline recommends the use of doses that only decrease not more than 30% cell viability[17].

Our study revealed that *A. blazei*, *G. frondosa* and *H. erinaceus* in aqueous and methanol extract in Chinese hamster fibroblast cell line (V79-4) resulted in low level DNA damage. Although at highest concentration used, only *A. blazei* aqueous extract showed cytotoxic effect in MTT assay. There was no increase of DNA damage as compared to control. Furthermore, more tests are necessary for the investigation of the biological effects and their interactions with cell metabolism such as study on cell death in these selected medicinal mushrooms.

CONCLUSIONS

Our study found some selected medicinal mushroom extract to be cytotoxic to V79-4 cell but was negative in the alkaline comet assay. The results suggested that more toxicity test should be carried out before recommending their large-scale use by public. To date, there is no official recommendation on consumption or allowable daily intake for the edible mushroom that was used in traditional medicine in Malaysia.

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