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Water extracts of dietary mushrooms, *Agrocybe aegerita* and *Hypsizigus mamoreus,* inhibit antigen expression of human hepatitis B virus

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Hepatitis B virus (HBV) infection is prevalent but the current treatments remain to be improved. This study evaluated the effect of dietary mushrooms on expression of surface (HBsAg) and e (HBeAg) antigens of HBV. Hot-water extracts (HWE) of dietary mushrooms, *Agrocybe aegerita* (AA), *Hypsizigus mamoreus* (HM) and *Flammulina velutipes* (FV), were prepared and subjected to gel permeation chromatograph for molecular weight separation. Human hepatoma cell lines Hep3B and Huh7 harboring HBV genome were used as an *in vitro* model for expression of HBsAg and HBeAg. Primary cultured mouse hepatocytes were used as a normal counterpart. HWE of AA and HM, but not of FV, possessed significant inhibitory activity against the expression of HBsAg in Hep3B and Huh7 cells. The activity of high molecular weight fraction of AA and HM was greater than the lower ones. Furthermore, HWE of AA and HM inhibited the expression of HBeAg in Hep3B cells. The HWE of AA and HM did not affect cell viability of neither hepatoma cell lines nor primary normal hepatocytes. In conclusion, the hot-water extracts of dietary mushrooms *A. aegerita* and *H. mamoreus* could inhibit the antigen expression of human HBV in host cells without toxicity to normal primary hepatocytes.

Key words: Mushrooms, hepatitis B virus, HBsAg, HBeAg.

INTRODUCTION

Hepatitis B virus (HBV) infection is regarded as a critical health issue with increasing prevalence. HBV infection is highly correlative to the development of liver cirrhosis and carcinogenesis of hepatocellular carcinoma, a malignancy with extremely poor prognosis. The current antiviral agents for HBV infection include lamivudine, adefovir, entecavir, interferon (IFN)-alpha, etc (Cooke et al., 2010). However, the effectiveness of anti-viral agents, especially nucleoside analogs, is limited by drug resistance resulted from gene mutation of HBV after long-term administration (Papatheodoridis et al., 2008; Shaw et al., 2006). For example, administration of lamivudine results in high rates

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of resistance due to emergence of HBV strains with tyrosine-methionine-aspartate-aspartate (YMDD) mutation (Allen et al., 1998). Therapy of finite duration using IFN-α administration is frequently interrupted by its toxicity including pyrexia, fatigue, headache, myalgia, depression, and myelosuppression (Lau et al., 2005). These limitations compromise the therapeutic efficacy of current practice against HBV infection. Clearly, it is important to develop a novel category of therapeutics against HBV infection through different mechanisms of action. Since normal liver is the main organ responsible for detoxification, the development of anti-HBV agents from non-toxic food, such as dietary mushrooms, might be a legitimate strategy.

Cold-water extracts of dietary mushrooms, *Hypsizigus mamoreus* (HM), *Agrocybe aegerita* (AA) and *Flammulina velutipes* (FV), have been reported capable of stimulating cytokine secretion of mononuclear cells to inhibit growth of leukemia cells (Ou et al., 2005). The cytokines augmented in secretion from mononuclear cells included tumor necrosis factor-alpha and interlukin-1 beta. Of these cold-water extracts, the high molecular weight fractions possessed this activity greater than the low molecular weight counterparts. However, whether these bioactive dietary mushrooms can inhibit expression of viral proteins, including the HBsAg, remains to be determined.

The purpose of this study was to examine the anti-HBV effect of the commonly used dietary mushrooms. Expression of surface and e antigens of HBV in host cells was served as the major endpoint. Primary hepatocytes isolated from mice were used as normal counterpart for evaluation of hepatic toxicity.

MATERIALS AND METHODS

Preparation of extracts from mushrooms

Fresh dietary mushrooms of *A. aegerita* (AA), *H. marmoreus* (HM), or *F. Velutipes* (FV) were purchased from a supermarket with characterization by a qualified fungologist in the National Research Institute of Chinese Medicine, Taiwan. The edible portion was cleaned by washing 3 times in distilled water and was cut into pieces about 5 mm × 5 mm in size. In our preliminary work, we used solvents with various polarities, including water, ethyl acetate, methanol and n-hexane. It showed that water extract was the most active one on inhibiting expression of HBsAg. After initial screening of bioactivity against HBsAg expression, hot-water (65°C for 30 min) extracts (HWE) were determined more effective than cold-water (4°C for overnight) extracts. After centrifugation (12,000 *g*, 4°C, 30 min), the obtained supernatant was lyophilized for quantification for further experiments. The dissolved HWE was then fractionated by a Sephadex G-50 (molecular weight range 1.5 to 30 kDa) gel permeation chromatography (column size, 2.6 cm × 65 cm; eluent, distilled water; fractionation, 5 ml/tube) to pool the high (HF) and low (LF) molecular weight fractions under monitored at 280 mm. Lyophilized powders of HWE, HF and LF were dissolved and dialyzed against phosphate-buffered saline prior to use.

Hepatoma cell culture

Human hepatocellular carcinoma cell lines Hep3B and Huh7 harboring endogenous and integrated HBV genome, respectively, with stable production of HBsAg, were used as the *in vitro* model for HBV replication (Knowles et al., 1980; Twist et al., 1981). These hepatoma cells were cultured in Dulbecco's modified eagle's medium (DMEM) containing 10% fetal calf serum, 10^5 IU/L penicillin, 100 mg/L streptomycin, and 1 mmole/L L-glutamine, in a humidified 5% CO_2 incubator at 37°C.

Preparation and culture of primary mouse hepatocytes

Primary hepatocytes were isolated from 6 to 8 week-old male Balb/c mice according to the procedures established by Klaunig et al. (1981), with modification. In brief, after anaesthetized and laparotomy, the portal vein was perfused with calcium-free Hanks balanced salt solution containing ethylene glycol-bis-(b-aminoethyl) N, N9-tetraacetic acid and N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid. This was followed by perfusion with the same solution containing collagenase for isolation of hepatocytes. After removal of liver, hepatocytes were mechanically dissociated and filtered through mesh. Hepatocytes were isolated by gradient centrifugation with Percoll (GE Healthcare, Little Chalfont, UK) and then washed. Cells were further cultured and plated with complete medium and adequate hepatocyte growth factor. The medium was changed regularly as necessary.

Growth Inhibition and cell viability by MTT assay

The cell viability of hepatoma cells was assessed by a tetrazolium dye colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide) test (Mosmann, 1983) and expressed as - MTT value of experimental group/MTT value of untreated control group.

Assay for relative HBsAg and HBeAg expression

Hepatoma cells cultured in DMEM with 10% fetal bovine serum for 24 h were transferred to serum-free DMEM with or without fungal extractions and incubated thereafter. The secreted HBsAg and HBeAg in the culture medium were measured by commercial enzyme-linked immunosorbent assay (ELISA) kits (General Biological, Taipei, Taiwan). The detection of HBsAg in serum of patients indicated a current HBV infection and the risk for developing compensated cirrhosis and hepatocellular carcinoma. Thus, the HBsAg is a useful index to evaluate the viral activity (Czaja, 1979). The relative HBsAg expression was determined by the following formula: (HBsAg / MTT) from treated cells / (HBsAg / MTT) from the untreated cells. The measurement of secreted viral antigens from HBV host cells, such as HBsAg secretion from Hep3B cells has been applied in various investigations to evaluate the effect of drugs on HBV activity. To further validate the anti-HBV effect of active fractions, the relative HBeAg expression was determined with similar methods.

Statistical analysis

Results are presented as mean ± standard error of mean (SEM). Differences between the treatment groups was assessed by Student's t-test. A confidence level of 5% ($p < 0.05$) was considered significant.

Table 1. Bioactivity of high and low molecular weight fractions of mushrooms against expression of HBsAg.

Parameter	Relative expression of HBsAg (%)	
	Hep3B cell	Huh7 cell
AA		
HWE	36.6±3.2	52.9±5.4
HF	25.1±2.3*	33.5±3.6*
LF	39.4±2.9	57.3±4.3
HM		
HWE	41.3±5.8	63.1±3.8
HF	32.7±3.3*	44.8±4.5*
LF	48.6±3.1	60.7±2.7

Hep3B and Huh7 cells were treated with hot water extracts (HWE), their high molecular weight fraction (HF) or low molecular weight fraction (LF) of mushrooms at concentration of 50 µg/ml for 48 h and then subjected to ELISA. Triplicated data from separate experiments are expressed as mean ± SEM. *p < 0.05 for HF versus LF. AA: *Agrocybe aegerita*; HM, *Hypsizigus mamoreus*.

RESULTS

Effect of mushroom extracts on viability of hepatoma cells

HWE of AA, HM and FV at concentrations from 0 to 400 µg/ml did not significantly affect the cell viability of Hep3B and Huh7 cells (Figure 1A and B). Upon this basis, the biological activity of mushroom extracts in these cells could be considered not owing to cellular toxicity.

Reduction of relative HBsAg and HBeAg expression in hepatoma cells by mushroom extracts

Lamivudine, as a positive control, caused 83.9 ± 5.6% reduction in HBsAg expression at 25 µM. As shown in Figure 2A and B, HWE of AA, HM, but not FV, suppressed both the endogenously expressed HBsAg in Hep3B cells and the HBsAg produced from the stable clone of HBV DNA-integrated in human hepatoma Huh7 cells. This inhibitory effect was concentration-dependent up to 78.2 ± 4.1 and 71.3 ± 3.7% reduction in Hep3B and Huh7 cells, respectively. The estimated 50% HBsAg-inhibitory concentrations (IC_{50}) of AA and HM on Hep3B cells was 22.5 and 37.4 µg/ml, respectively. Further isolation and assessment of higher and lower molecular weight fractions from HWE of AA and HM demonstrated a greater HBsAg-inhibitory activity by the higher ones (Table 1). Furthermore, the HWE (50µg/ml) of AA and HM inhibited the expression of HBeAg by 70.3 ± 4.7 and 61.8 ± 5.1% in Hep3B cells, respectively (Figure 3).

Effect of mushrooms extracts on viability of normal hepatocytes

Primarily cultured murine hepatocytes were served as a normal counterpart to assess the normal liver toxicity of mushroom extracts. As demonstrated in Figure 4, HWE from AA and HM, at the concentrations given for inhibiting HBsAg expression in hepatoma cells, did not significantly reduce the viability of normal hepatocytes.

DISCUSSION

We found that hot water extracts of dietary mushrooms AA and HM inhibited the expression of human HBsAg and HBeAg in host cells without toxicity to normal primary hepatocytes. As mentioned before, the current treatments against HBV are either toxic or prone to be resisted by gene mutation. Our finding that the hot water extracts resembling cooking processes of two dietary mushrooms could inhibit the expression of HBsAg and HBeAg without toxicity to hepatocytes. This may shed a light for development of new therapeutic agents against HBV from food. Whether the bioactivity of mushroom extracts could be preserved or not in gastrointestinal tract after digestion remains unclear. Further *in vivo* tests are definitively necessary.

The experimental model used in this study includes Hep3B cells harboring endogenous HBsAg and the stable clone of HBV DNA-integrated Huh7 cells. HWE of AA and HM inhibited HBsAg expression in both cell lines, indicating a universal activity against HBsAg expression. However, this experimental model only provides a screening platform for anti-HBV agents (Chen et al 1997). To draw the conclusion that AA and HM can inhibit HBV. Further molecular studies including that for viral replication need to be examined. The other endpoints for assessment of HBV activity, including mRNA expression of HBsAg and expression of HBV DNA, could also be used as indicator of HBV activity.

Normal liver toxicity assessed by *in vitro* hepatocytes viability shows no evident hepatotoxicity of mushroom extracts. Compared with the current anti-HBV therapeutics with various kinds of toxicity (Lau et al 2005), this further augments the potential of mushroom extracts to be developed as novel anti-HBV agents.

In clinical practice, chemotherapy-related HBV reactivation and resultant fulminate failure is not rare in HBV-infected patients (Hoofnagle, 2009; Yeo et al., 2000). Prophylactic administration of anti-viral agents such as lamivudine (Hsu et al., 2008) has been proved effective. Given that dietary mushrooms are safe and commonly used food, it is expected that AA and HM could be another option for prophylaxis of HBV reactivation in cancer patients whom already are heavily treated by chemotherapeutics.

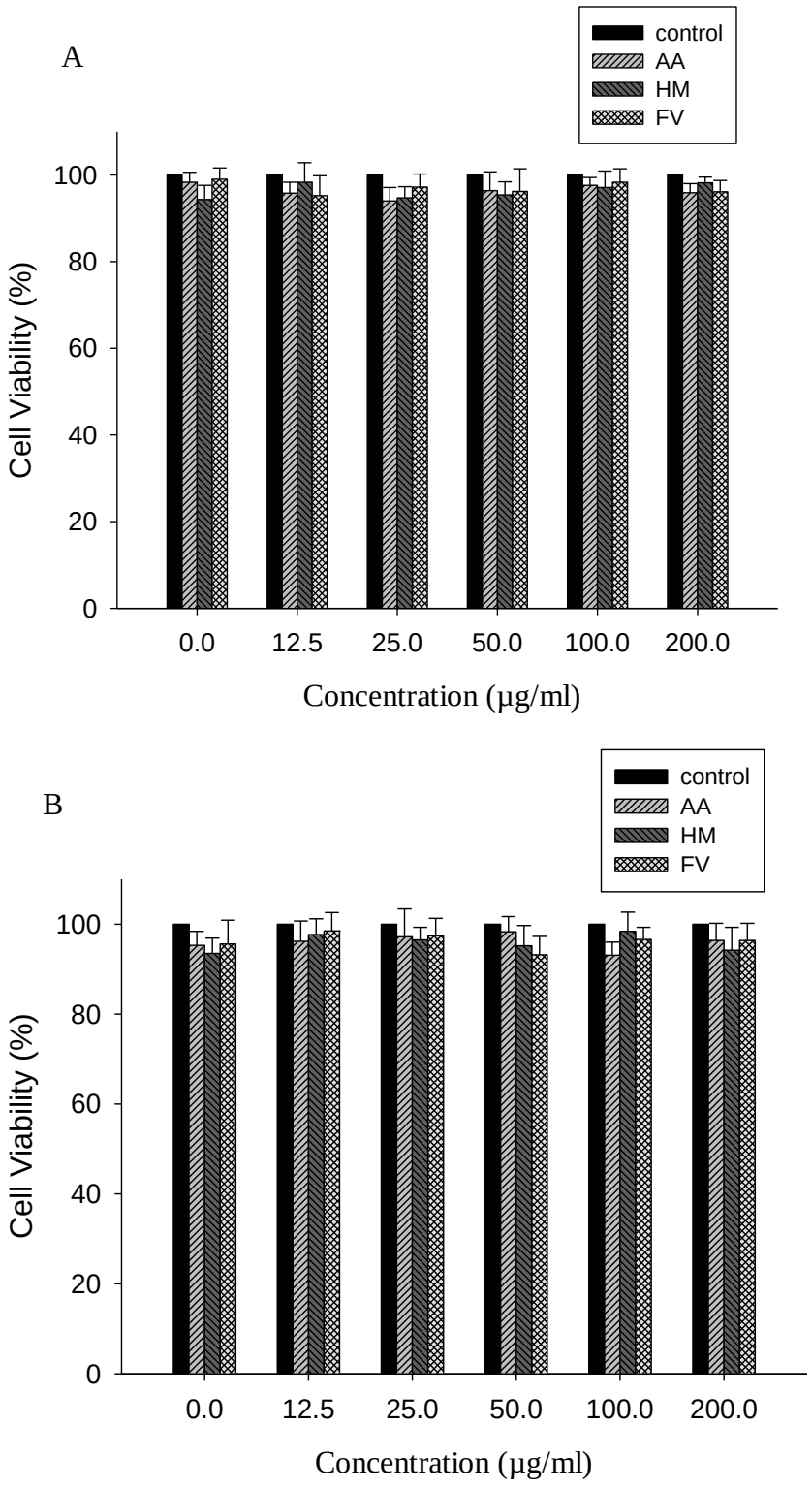

Figure 1. The viability of Hep3B and Huh7 hepatoma cells with treatment by extracts of mushrooms. A, Hep3B; B, Huh7 cells. Cells were treated with various concentrations of hot water extracts of mushrooms for 48 h and then subjected to MTT assay. Triplicated data from separate experiments are expressed as mean ± SEM.

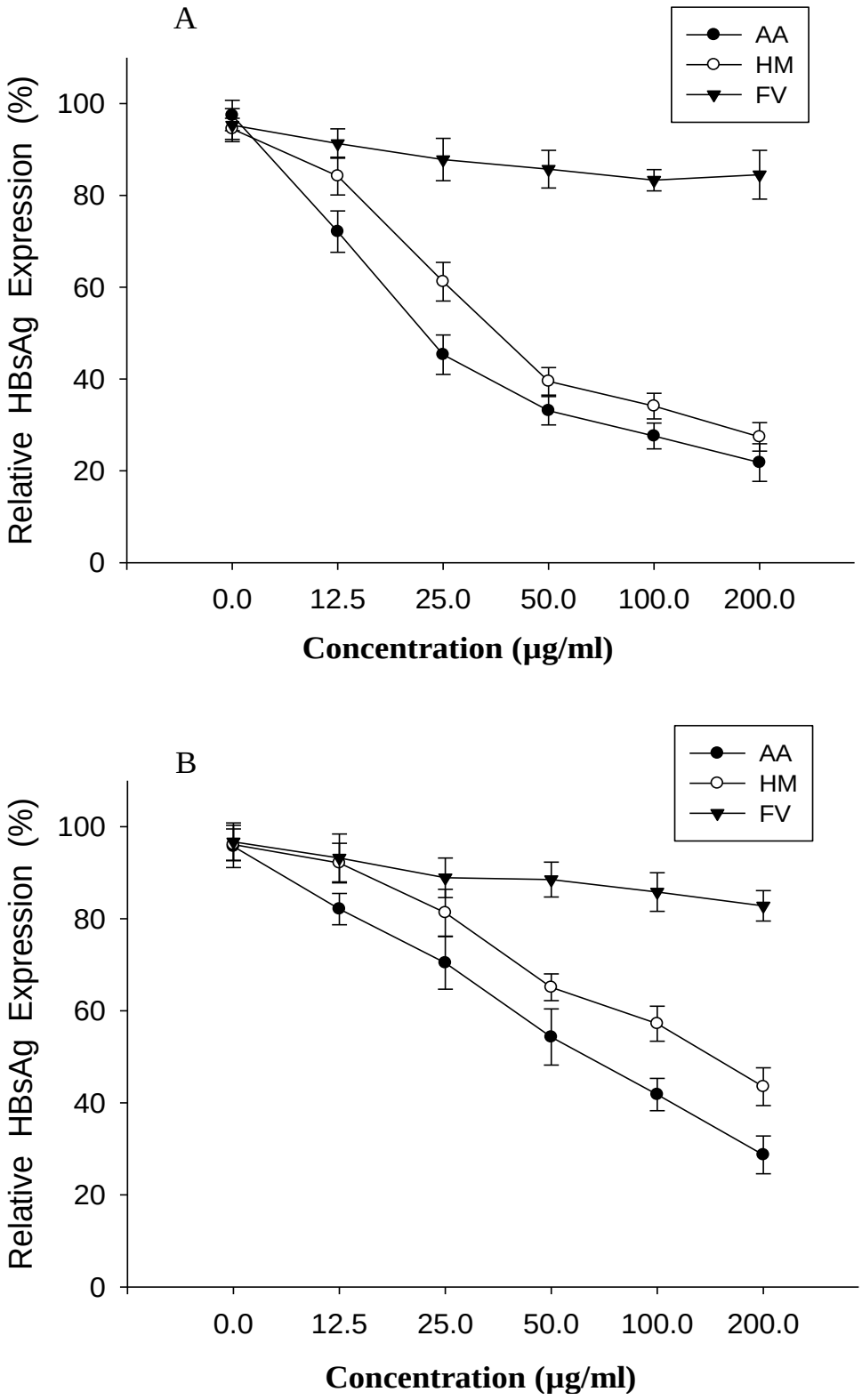

Figure 2. The relative HBsAg expression in Hep3B and Huh7 hepatoma cells.
(A) Hep3B; (B) Huh7 cells. Cells were treated with various concentrations of hot water extracts of mushrooms for 48 h and then subjected to ELISA. Triplicated data from separate experiments are expressed as mean ± SEM.

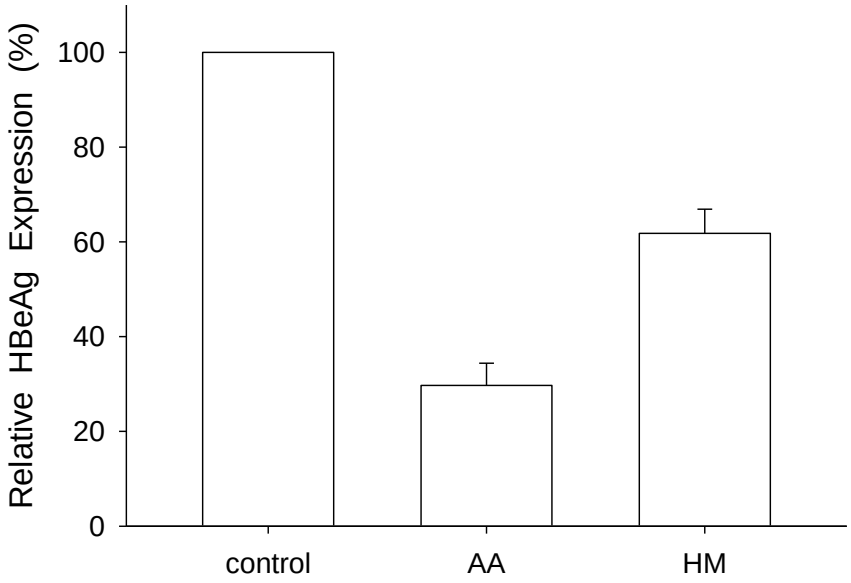

Figure 3. The relative HBeAg expression in Hep3B hepatoma cells. Cells were treated with various concentrations of hot water extracts of mushrooms for 48 h and then subjected to ELISA. Triplicated data from separate experiments are expressed as mean ± SEM.

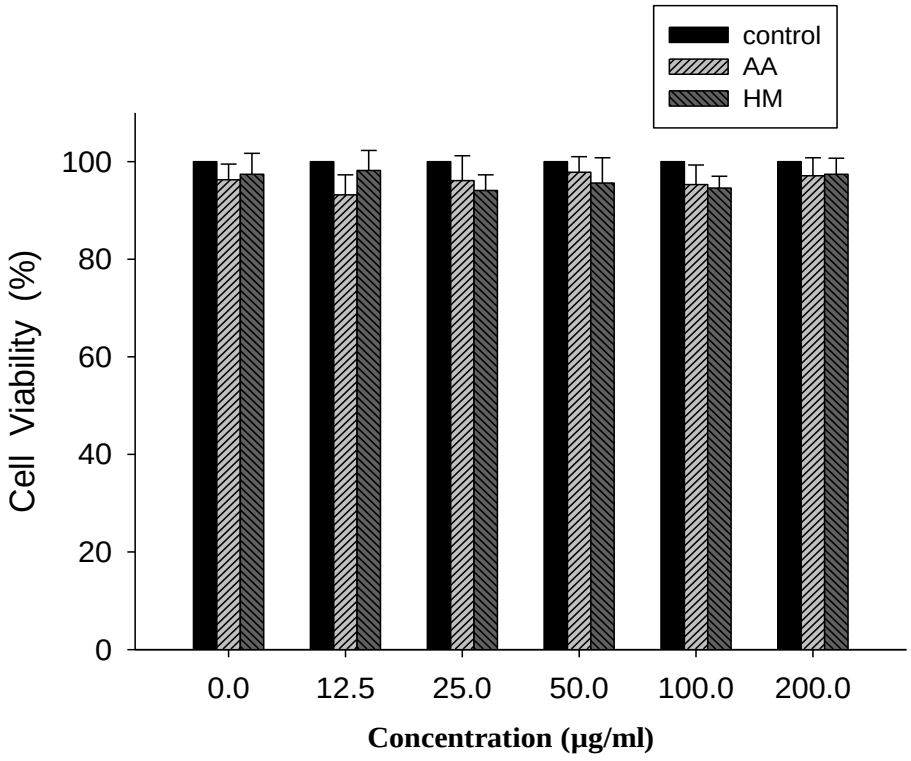

Figure 4. The viability of primary cultured hepatocyts with treatment by extracts of mushrooms. Cells were treated with various concentrations of hot water extracts of mushrooms for 48 h and then subjected to MTT assay. Triplicated data from separate experiments are expressed as mean ± SEM.

Conclusion

Hot water extracts of dietary mushrooms, *A. aegerita* (AA) and *H. mamoreus* (HM), could inhibit the antigen expression of HBV in host cells without toxicity to normal hepatocytes.

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