

원 저

## 알파 아مانิต인에 의한 간독성에 대한 갯방풍의 보호 효과

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### *In vitro* Protective Effects of *Glehnia Littoralis* on Alpha-amanitin Induced Hepatotoxicity

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**Purpose:** *Glehnia littoralis* has been used to treat ischemic stroke, phlegm, cough, systemic paralysis, antipyretics and neuralgia. The pharmacological mechanisms of *Glehnia littoralis* include calcium channel block, coumarin derivatives, anticoagulation, anti-convulsive effect, as well as anti-oxidant and anti-inflammatory effects. Alpha-amanitin ( $\alpha$ -amanitin) is a major toxin from extremely poisonous *Amanita* fungi. Oxidative stress, which may contribute to severe hepatotoxicity was induced by  $\alpha$ -amanitin. The aim of this study was to investigate whether *Glehnia littoralis* ethyl acetate extract (GLEA) has the protective antioxidant effects on  $\alpha$ -amanitin -induced hepatotoxicity.

**Methods:** Human hepatoma cell line HepG2 cells were pretreated in the presence or absence of GLEA (50, 100 and 200  $\mu$ g/ml) for 4 hours, then exposed to 60  $\mu$ mol/L of  $\alpha$ -amanitin for an additional 4 hours. Cell viability was evaluated using the MTT method. AST, ALT, and LDH production in a culture medium and intracellular MDA, GSH, and SOD levels were determined.

**Results:** GLEA (50, 100 and 200  $\mu$ g/ml) significantly increased the relative cell viability by 7.11, 9.87, and 14.39%, respectively, and reduced the level of ALT by 10.39%, 34.27%, and 52.14%, AST by 9.89%, 15.16%, and 32.84%, as well as LDH by 15.86%, 22.98%, and 24.32% in culture medium, respectively. GLEA could also remarkably decrease the level of MDA and increase the content of GSH and SOD in the HepG2 cells.

**Conclusion:** In the *in vitro* model, *Glehnia littoralis* was effective in limiting hepatic injury after  $\alpha$ -amanitin poisoning. Its antioxidant effect is attenuated by antidotal therapy.

**Key Words:** Alpha-amanitin, Antioxidant, *Glehnia littoralis*

## Introduction

*Glehnia* is a genus in the carrot family, Apiaceae, with one species, *Glehnia littoralis*, known by several common names including beach silvertop and American silvertop in English. It is native to eastern Asia, particularly eastern China, Korea, Japan, and far-eastern Russia. Its habitat is a coastal sand dune that is strongly influenced by the ocean. It is a unique ecosystem

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in which salinity is directly or indirectly introduced from seawater and is generally difficult to survive in other land plants. It was therefore expected that there would be secondary metabolites different from existing terrestrial plants.

The dried roots and rhizomes of this plant are listed in the Korean, Japanese, and Chinese pharmacopoeia and have been used in traditional medicine as diaphoretic, antipyretic, and analgesic agents. Previous studies reported that *Glehnia littoralis* has anti-oxidant, anti-tumor, anti-amnesic, blood circulation-promoting, immunomodulatory, and anti-microbial activities<sup>1-8)</sup>.

Powerful natural hepatotoxin alpha-amanitin ( $\alpha$ -amanitin) belongs to the bicyclic octapeptides named amatoxins isolated from deadly poisonous *Amanita phalloides* mushroom. Alpha-amanitin is readily absorbed from the gastrointestinal tract and carried to the liver via portal vein. Alpha-amanitin poisoning is characterized by liver necrosis, in many cases with acute hepatic insufficiency with subsequent complications including hepatic coma, coagulation disorders<sup>9)</sup>. The experimental evidence suggests that amanitin inhibits RNA polymerase II by enzymatic inhibition. But, recent in vitro and in vivo studies have suggested that oxidative stress is probably responsible for  $\alpha$ -amanitin induced hepatotoxicity in studies<sup>10-13)</sup>.

Recently, much attention has been focused on investigating the hepatoprotective function of naturally occurring compounds and their mechanisms of action. In this study, we investigated the effect of *Glehnia littoralis* against  $\alpha$ -amanitin induced hepatotoxicity in vitro model.

## Methods

### 1. Materials

3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT), phosphate buffer saline (PBS), dimethyl sulfoxide (DMSO) and  $\alpha$ -amanitin were purchased from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS), Dulbecco's Modified Eagle Medium (DMEM), trypsin and penicillin-streptomycin were obtained from Hyclone Laboratories, INC. (Logan, UT). Diagnostic kits

for aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), malondialdehyde (MDA), glutathione (GSH) and superoxide dismutase (SOD) were used for measuring its activity, following the commercial protocol (Abcam, Cambridge, UK). All other reagents used were of analytical grade.

### 2. Preparation of extract

*Glehnia littoralis* was collected by Professor Sung Dong Cho of Chosun University in South Jeolla Province (Republic of Korea) in October 2014. For the preparation of *Glehnia littoralis* ethyl acetate extracts (GLEA), roots and rhizomes of *Glehnia littoralis* were washed with distilled water, air-dried at 60°C, and ground into fine powder by a grinder. The powder was refluxed with 10 vol (v/w) of 70% ethanol at 70°C for 24 hours, and the extraction procedure was repeated three times. The extract was filtered through filter paper, concentrated with a vacuum evaporator, and it was completely dried with a freeze drier.

### 3. MTT assay for the cytotoxicity of $\alpha$ -amanitin and maximal noncytotoxic concentration of GLEA

The HepG2 (hepatocellular carcinoma) cell line was procured from American Type Culture Collection (ATCC, Rockville, MD). The cytotoxicity of  $\alpha$ -amanitin and the maximal noncytotoxic concentration (MNCC) were assessed according to a MTT method. In brief, exponentially growing HepG2 cells with a density of about  $2 \times 10^5$  cells / ml were plated into 96-well plates and allowed to adhere for 24 hours. The cells were then treated with various concentrations of GLEA (0, 50, 100, 200, 400, 800 and 1200  $\mu$ g/ml) or  $\alpha$ -amanitin (0, 10, 20, 40, 60, 80, 100 and 150  $\mu$ mol/L) in separate wells for 4 hours. The cells in the control wells received medium containing the same volume of DMSO (0.1%). After the incubation, 20  $\mu$ l of MTT reagent (5 mg/mL in PBS) was added and cells were incubated for an additional 4 hours. The formazan produced by the viable cells was solubilized by the addition of 100  $\mu$ l DMSO. The suspension was placed on a micro-vibrator for 10-15 minutes and the absorbance was recorded at

490 nm by the ELISA reader (BioTek EL×800, Winooski, VT). The experiment was performed in triplicate. The relative cell viability was calculated with respect to the untreated control using the following formula:

$$\% \text{ Relative cell viability} = (A_{\text{Test}} - A_{\text{Blank}}) / (A_{\text{Control}} - A_{\text{Blank}}) \times 100$$

#### 4. The cytoprotective effect of GLEA

The above-mentioned MTT method was used to evaluate the cytoprotective effect of GLEA. Briefly, HepG2 cells were preincubated in the presence or absence of GLEA at various concentrations (50, 100 and 200  $\mu\text{g}/\text{ml}$ ) for 4 hours, then the cells were stimulated with  $\alpha$ -amanitin (60  $\mu\text{mol}/\text{L}$ ) for an additional 4 hours. 20  $\mu\text{l}$  of MTT reagent (5 mg/mL in PBS) was added and cells were incubated for an additional 4 hours. The formazan produced by the viable cells was solubilized by the addition of 100  $\mu\text{l}$  DMSO. The suspension was placed on a micro-vibrator for 10-15 min and the absorbance was recorded at 490 nm by the ELISA reader (BioTek EL×800, Winooski, VT). The experiment was performed in triplicate. Cell viability was expressed as a percentage relative to the untreated controls.

#### 5. Determination of ALT, AST and LDH production in culture medium

The activities of ALT, AST and LDH in cell culture medium were measured by commercially available assay kits. Briefly, HepG2 cells ( $2 \times 10^6$  cells/dish) were plated into 100 mm-diameter culture dish. After incubation for 24 hours, HepG2 cells were preincubated in the presence or absence of GLEA at different concentrations (50, 100 and 200  $\mu\text{g}/\text{ml}$ ) for 4 hours, then the cells were stimulated with  $\alpha$ -amanitin (60  $\mu\text{mol}/\text{L}$ ) for a further 4 hours. Control cells received 0.1% of DMSO instead of GLEA. The cell culture medium was harvested and centrifuged at 4°C with a speed of 1500 rpm for 5 minutes to remove floating cell, the supernatant was collected for enzyme activity assay. Results were expressed as unit per liter (U/L).

#### 6. Determination of MDA, GSH and SOD levels in HepG2 cells

Accumulated intracellular MDA, GSH and SOD levels were measured following the manufacturer's instructions. Briefly, HepG2 cells ( $2 \times 10^6$  cells/dish) were plated into 100 mm diameter culture dish. After incubation for 24 hours, HepG2 cells were preincubated in the presence or absence of GLEA at different concentrations (50, 100 and 200  $\mu\text{g}/\text{ml}$ ) for 4 hours, then the cells were stimulated with  $\alpha$ -amanitin (60  $\mu\text{mol}/\text{L}$ ) for a further 4 hours. Control cells received 0.1% of DMSO instead of GLEA. After removing the culture medium, HepG2 cells were rinsed with PBS, and dislodged carefully from the culture dish into a 15 ml centrifuge tube by using a cell scraper, and centrifuged at 4°C with a speed of 1000 rpm for 10 minutes, the collected cells were resuspended in PBS and sonicated for 15 seconds in an ice bath. The intracellular MDA, GSH and SOD levels were then determined, and the total protein content was determined by the Bradford method according to the manufacturer's specification. Results were expressed per milligram or gram of the total protein.

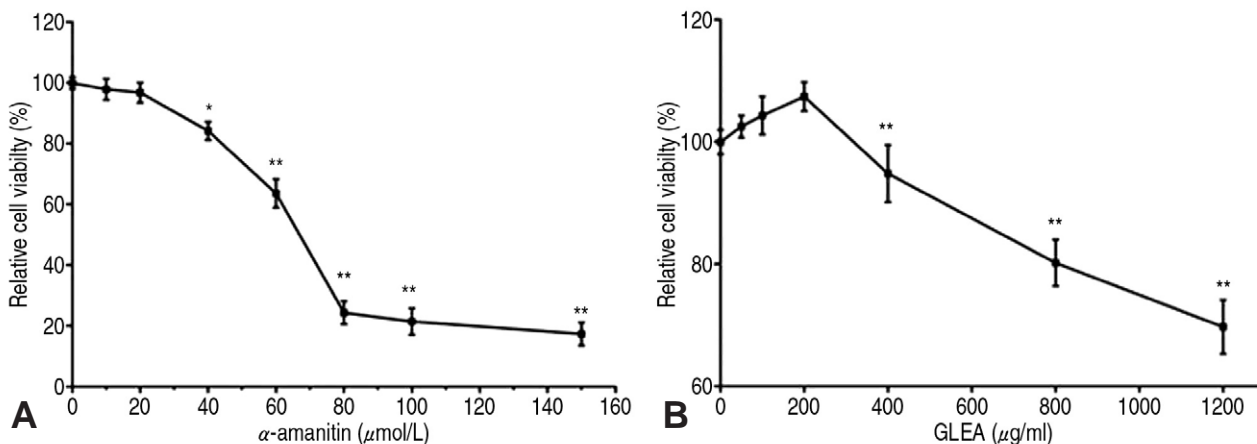
#### 7. Statistical analysis

All data are presented as mean  $\pm$  standard deviation of three determinations. Statistical comparison was performed via a one-way analysis of variance followed by Dunnett's test by using SPSS software version 19.0 (SPSS, Chicago, IL). A value of  $p < 0.05$  and  $p < 0.01$  was considered to be statistically significant.

## Results

#### 1. Determination for the cytotoxicity of $\alpha$ -amanitin and MNCC of GLEA

In vitro hepatoprotective studies, the cytotoxicity of  $\alpha$ -amanitin and the MNCC of GLEA was evaluated by the MTT assay in HepG2 cells. As shown in Fig. 1A, the relative cell viability decreased remarkably ( $p < 0.05$ ), after incubation with increasing concentra-

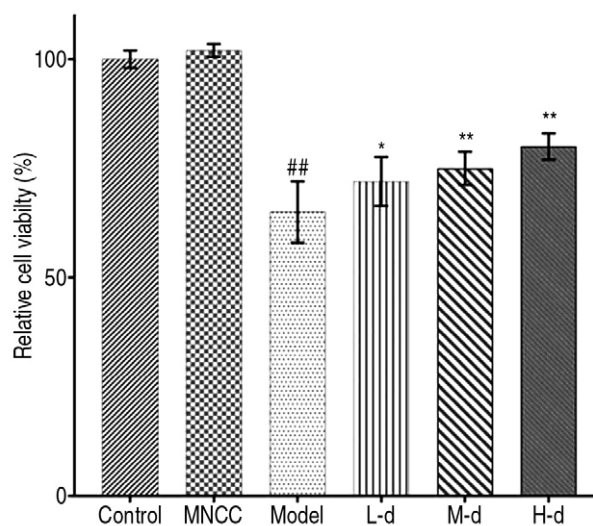


**Fig. 1.** Cytotoxicity of  $\alpha$ -amanitin and GLEA on HepG2 cells. (A) HepG2 cells were treated with different concentrations of  $\alpha$ -amanitin (0, 10, 20, 40, 60, 80, 100 and 150  $\mu$ mol/L) for 4 hours. (B) HepG2 cells were treated with various concentrations of GLEA (0, 50, 100, 200, 400, 800 and 1200  $\mu$ g/ml) for 4 hours. Relative cell viability was calculated according to the formula. All data are expressed as the means  $\pm$  SD (error bars) of values from three independent experiments. \* $p$ <0.05 and \*\* $p$ <0.01 were considered significant versus the control group. GLEA, *Glehnia littoralis* ethyl acetate extracts.

tion of  $\alpha$ -amanitin (> 40  $\mu$ mol/L) for 4 hours. When exposed with 60  $\mu$ mol/L of  $\alpha$ -amanitin, the relative cell viability was 68.22%, leading to 31.78% reduction in the relative cell viability ( $p$ <0.01). However, when the concentration used was 80  $\mu$ mol/L, the relative cell viability was only 24.98%, leading to 75.11% reduction in the relative cell viability ( $p$ <0.01). Considering these  $p$  value results, we determined 60  $\mu$ mol/L as the cytotoxic concentration of  $\alpha$ -amanitin. Furthermore, as given in Fig. 1B, the relative cell viability was 102.97% in HepG2 after treatment with 200  $\mu$ g/ml of GLEA ( $p$ >0.05), indicating that HepG2 cells were well tolerated to the treatment of GLEA ( $\leq$ 200  $\mu$ g/ml) without showing any cytotoxicity. However, the relative cell viability decreased evidently ( $p$ <0.01) with increasing concentrations ( $\geq$ 400  $\mu$ g/ml) of GLEA. Therefore, the MNCC of GLEA was determined as 200  $\mu$ g/ml, the cytotoxic concentration of  $\alpha$ -amanitin (60  $\mu$ mol/L) and noncytotoxic concentrations of GLEA (50  $\mu$ g/ml; low dose, 100  $\mu$ g/ml; medium dose and 200  $\mu$ g/ml; high dose) were selected for the subsequent assessment of whether GLEA were able to protect HepG2 cells against  $\alpha$ -amanitin-induced toxicity.

## 2. Evaluation for protective effect of GLEA

The cytoprotective effect of GLEA was evaluated by using the MTT assay. As presented in Fig. 2, it was



**Fig. 2.** Evaluation of the cytoprotective effect of GLEA by MTT assay. HepG2 cells were pretreated with indicated concentrations of GLEA for 4 hours, and then incubated with or without 60  $\mu$ mol/L of  $\alpha$ -amanitin for 4 hours. Relative cell viability was calculated according to the formula. The cell viability was significantly increased in a dose-dependent manner, when HepG2 cells were pretreated with 50 (L-d, low-dose group), 100 (M-d, medium-dose group) and 200  $\mu$ g/ml (H-d, high-dose group) of GLEA compared with 0  $\mu$ g/ml (Model, model group) of GLEA. All data are expressed as the means  $\pm$  SD (error bars) of values from three independent experiments. \* $p$ <0.05 and \*\* $p$ <0.01 were considered significant versus the model group. ## $p$ <0.05 and ### $p$ <0.01 were considered significant versus the control group.

found that pretreatment with GLEA (50  $\mu$ g/ml; low dose, 100  $\mu$ g/ml; medium dose and 200  $\mu$ g/ml; high

dose) significantly improved the relative cell viability by 7.11, 9.87 and 14.39%, respectively, compared with that in the model group ( $p < 0.05$ ).

### 3. Determination of ALT, AST and LDH production in culture medium

The leakage of ALT, AST and LDH in culture medium was measured in order to further evaluate the hepatoprotective activity of GLEA. As presented in Fig. 3A-C, compared with control cells,  $\alpha$ -amanitin challenge caused significant increase of ALT, AST and LDH leakage from the cytoplasm into culture medium in the model group ( $p < 0.01$ ). However, the activities of these three biochemical enzymes did not change obviously in the MNCC group ( $p < 0.05$ ). Additionally, pretreatment of the cells with various concentrations (50  $\mu\text{g/ml}$ ; low dose, 100  $\mu\text{g/ml}$ ; medium dose and 200  $\mu\text{g/ml}$ ; high dose) of GLEA significantly inhibited the leakage of ALT by 10.39, 34.27 and 52.14%, AST by 9.89, 15.16 and 32.84%, and LDH by 15.86, 22.98 and 24.32% in culture medium, respectively ( $p < 0.05$  or  $p < 0.01$ ).

### 4. Determination of MDA, GSH and SOD levels in HepG2 cells

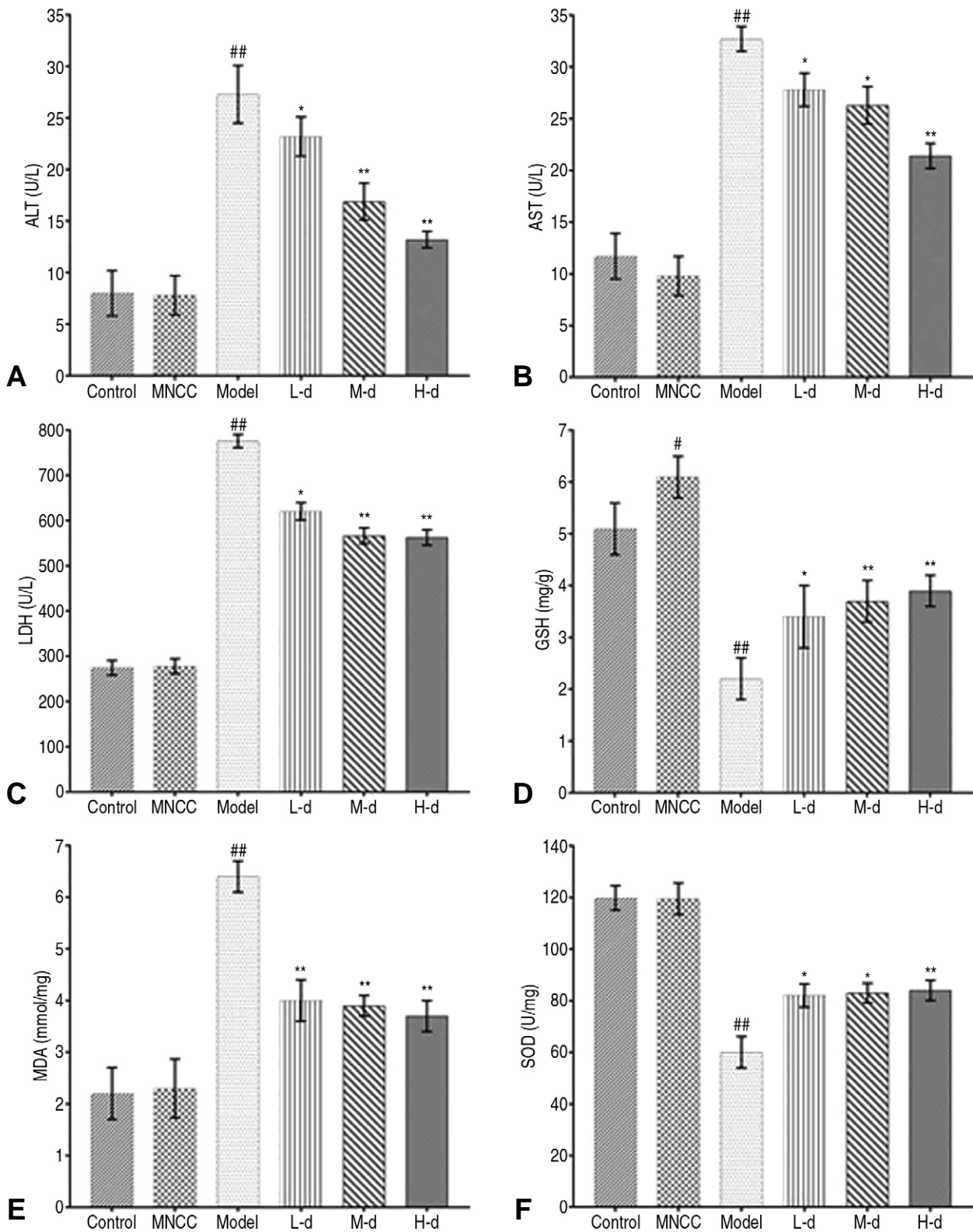
The intracellular level of MDA, GSH and SOD were quantified in this study. As shown in Fig. 3D-F, compared with the control cells,  $\alpha$ -amanitin treatment resulted in a significant increase in MDA and decrease in GSH, and SOD in HepG2 cells in the model group ( $p < 0.01$ ). Nevertheless, no obvious change was observed in the MNCC group ( $p < 0.05$ ). Furthermore, it was found that GLEA (50  $\mu\text{g/ml}$ ; low dose, 100  $\mu\text{g/ml}$ ; medium dose and 200  $\mu\text{g/ml}$ ; high dose) pretreatment caused remarkable increase in the intracellular content of SOD by 38.88, 44.67, and 52.95% and GSH by 49.91, 61.16 and 70.97%, respectively, with a concurrent decrease in the intracellular level of MDA by 35.75, 39.42 and 44.69%, respectively ( $p < 0.05$  or  $p < 0.01$ ).

## Discussion

$\alpha$ -Amanitin is a cyclic peptide isolated from *Amanita*

mushrooms and responsible for their extreme toxicity. The liver is the critical organ affected in  $\alpha$ -amanitin poisoning. However, the exact mechanism of poisoning has yet to be definitively elucidated. The experimental evidence suggests that  $\alpha$ -amanitin inhibits RNA polymerase II by enzymatic inhibition. Transport of  $\alpha$ -amanitin into hepatocytes likely occurs through a  $\text{Na}^+$ -mediated transporter<sup>14</sup>. But, recently increasing evidence has indicated that hepatic cellular damage mediated by oxidative stress contributed to the initiation and progression of  $\alpha$ -amanitin intoxication<sup>10-13</sup>. The free radical intermediates that are generated in hepatocytes due to  $\alpha$ -amanitin are associated with the production of increased reactive oxygen species (ROS). Subsequent intense oxidative stress would then lead to hepatocyte peroxidation, liver glutathione depletion, and death, contributing to severe hepatotoxicity. Damage to the liver is characterized by massive centrilobular necrosis, vacuolar degeneration, and a positive acid-phosphatase reaction<sup>11,15,16</sup>. Treatment of  $\alpha$ -amanitin poisonings involves gastrointestinal decontamination, supportive measures, antidotes and, if liver failure occurs, liver transplantation. In antidotal therapy, various substances (silibinin, steroids, cimetidine, thiocetic acid, benzylpenicillin, acetylcysteine) have been widely used in the past to treat  $\alpha$ -amanitin poisonings. In a 20-year retrospective analysis assessed the treatments in amanitin poisonings, benzylpenicillin (penicillin G) used alone and in combination with other agents was the most frequently utilized chemotherapy, but showed little efficacy<sup>17,18</sup>. No benefit was found in the use of thiocetic acid or steroids. Some clinical reports indicated that silibinin or acetylcysteine appear to be more effective in mushroom poisoning therapy than benzylpenicillin. But in experimental and clinical studies today, all these drugs are said to remain inadequate in treating  $\alpha$ -amanitin poisoning, they do not even have any noticeable advantage over one another<sup>16,19-21</sup>. Many cytoprotective agents still continue to be tried for this purpose.

*Glehnia littoralis*, as a traditional herbal medicine to heal various health ailments in East Asia, displays various therapeutic properties including antioxidant effects. Quercetin, isoquercetin, rutin, chlorogenic acid, and caf-



**Fig. 3.** GLEA decreased the up-regulation of ALT, AST, LDH and MDA and increased the down-regulation of GSH and SOD induced by  $\alpha$ -amanitin in HepG2 cells. The leakage of (A) ALT, (B) AST and (C) LDH released by HepG2 cells was measured in the culture supernatant. Hepatocellular (D) GSH, (E) MDA and (F) SOD were assessed from HepG2 cell lysates. All data are expressed as the means  $\pm$  SD (error bars) of values from three independent experiments. \* $p$ <0.05 and \*\* $p$ <0.01 were considered significant versus the model group; # $p$ <0.05 and ## $p$ <0.01 were considered significant versus the control group.

feic acid have been isolated as the major antioxidative constituents in the underground parts of *Glehnia littoralis*<sup>22</sup>. Our current work aimed to investigate the cytoprotective activity of GLEA in vitro against the oxidative damage induced by  $\alpha$ -amanitin in HepG2 cells, aiming to provide a better understanding of the hepatoprotective efficacy. HepG2 cell lines have been proposed as an alternative model to hepatocytes, which are used in various metabolic and drug toxicity studies, possessing the advantage of being available in plenty, easy maintenance, rapid cryopreservation and ability to retain drug metabolism and enzyme activities. Moreover, HepG2 cells possess many morphological and biochemical features of normal hepatocytes. Since it retains many of the phenotypic and genotypic characteristics of liver cells, this cell line has been used in various studies related to medicinal plants for their liver protecting property<sup>23</sup>.

Our study demonstrated that GLEA had a protective effect in  $\alpha$ -amanitin-induced hepatotoxicity by significantly improving the cell viability, functional and antioxidant parameters. 60  $\mu$ mol/L of  $\alpha$ -amanitin (inhibitory rate of 31.78%) and various concentrations ( $\leq 200$   $\mu$ g/ml, MNCC) of GLEA were employed to investigate the hepatoprotective effect of GLEA from the result of MTT assay (Fig. 1A, B). It was found that pretreatment with various concentrations (50, 100 and 200  $\mu$ g/ml) of GLEA could increase cell viability significantly (Fig. 2), when exposed to 60  $\mu$ mol/L of  $\alpha$ -amanitin.

The mechanism involved was ascertained by evaluation of the leakage of enzymes like ALT, AST and LDH, quantification of intracellular MDA and GSH levels and measurement of SOD activity. The liver cells contain high concentration of ALT and AST in cytoplasm. Damage to hepatic cells causes a leakage of liver-specific enzymes in plasma, causing increased serum enzyme levels like ALT and AST in serum. They are recognized as the notable signs of cellular damage and functional integrity of liver cell membrane. And hepatotoxicity in vitro can be directly determined by measuring levels of hepatic transaminase release into the culture medium<sup>24</sup>. LDH is localized in the cytoplasm of cells and thus extruded into the serum, when cells

are damaged or necrotic. The measurement of total lactate dehydrogenase can be useful when only a specific organ, such as the liver, is known to be involved. LDH is a sensitive intracellular enzyme, which can be used as an indication in liver cell damage<sup>25</sup>. Therefore, the extent of cellular damage was measured in terms of release of leakage enzymes including AST, ALT and LDH. Increased release of these intracellular enzymes indicated membrane damage and instability owing to oxidative injury induced by the hepatotoxin. The leakage of ALT, AST and LDH in culture medium was measured in order to demonstrate the hepatoprotective activity of GLEA. As shown in Fig. 3A-C,  $\alpha$ -amanitin challenge caused significant increase of ALT, AST and LDH leakage from the cytoplasm into culture medium in model group ( $p < 0.01$ ), which was agreement with the observation<sup>26</sup>. Pretreatment with various GLEA decreased the level of ALT, AST and LDH in culture medium significantly ( $p < 0.05$  or  $p < 0.01$ ).

Toxicity assays, beyond the use of classical markers including transaminases and lactate dehydrogenase, have commonly focused on the redox state of organs by antioxidant activity enzymes and measurements of defense molecule, macromolecule oxidation<sup>27</sup>. As given in Fig. 3D-F,  $\alpha$ -amanitin treatment led to a significant decrease in the level of SOD and GSH, along with an evident increase in the level of MDA in HepG2 cells in the model group ( $p < 0.01$ ). The changes induced by  $\alpha$ -amanitin were alleviated gradually with the increasing concentration of GLEA ( $p < 0.05$  or  $p < 0.01$ ). Taken together, it was suggested that the possible underlying mechanism for the hepatoprotective effect of GLEA in vitro was because of its ability to increase antioxidant activity, inhibit lipid peroxidation and maintenance of glutathione in reduced state.

## Conclusion

To summarise, GLEA offered significant hepatoprotection against the oxidative damage induced by  $\alpha$ -amanitin in HepG2 cells, as revealed by reduction of ALT, AST and LDH levels in the culture medium and remarkable decrease in intracellular MDA content,

together with elevation of SOD activity and GSH concentration. The mechanisms involved in this action may be the prevention of free radical damaging cascades, oxidant radical release, and its prevention from proinflammatory processes. But further *in vivo* experimental and clinical studies are required to confirm these findings and to reveal its mechanisms of action more clearly.

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