Italian Journal of Food Science, 2023; 35 (3): 90–98

Ganoderma lucidum extract reverses hepatocellular carcinoma multidrug resistance via inhibiting the function of P-glycoprotein *in vitro* and *in vivo*

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Received: 10 April 2023; Accepted: 12 July 2023; Published: 12 August 2023
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Abstract

Cancer is a leading cause of death globally. Chemotherapy still plays an indispensable role in the clinical treatment of cancer. However, the emergence of multidrug resistance (MDR) has greatly obstructed the further application of chemotherapy agents. *Ganoderma lucidum* (*G. lucidum*) is a traditional Chinese medicine as well as an edible mushroom. In this study, we first explored the effect of six extract samples derived from *G. lucidum* on the cell viability of adriamycin-resistant human hepatocellular carcinoma multidrug-resistant cell subline (HepG-2/ADM). All these samples showed no obvious toxicity to cells; however, only *G. lucidum* ethanol extract could reverse resistance to doxorubicin and paclitaxel. Then the P-glycoprotein (P-gp) function in hepatocellular carcinoma G2 (HepG-2) and HepG-2/ADM cells was determined after incubated with these samples, and we found that *G. lucidum* ethanol extract could inhibit P-gp function *in vitro*. Furthermore, *G. lucidum* ethanol extract could reverse resistance to paclitaxel in HepG-2/ADM tumor-bearing mice *in vivo*, while the protein expression level of P-gp was unchanged. Taken together, these results indicated the potential role of *G. lucidum* ethanol extract in reversing MDR in the clinical treatment of hepatocellular carcinoma.

Keywords: *Ganoderma lucidum*, hepatocellular carcinoma, multidrug resistance, paclitaxel, P-glycoprotein

Introduction

Cancer has become a leading cause of death globally, and has severely threatened the life expectancy of humans (Bray *et al.*, 2021). It is estimated that about 19.3 million new cases and 10 million cancer deaths occurred worldwide in 2020 (Sung *et al.*, 2021). Although different approach to treating cancer have developed successfully, the incidence and mortality are still increasing rapidly. The main cause of failure of anti-cancer therapy is the emergence of acquired resistance, among which multidrug resistance (MDR) has played a major role (Gottesman *et al.*, 2002). MDR is a phenomenon in which cancer cells develop resistance to multiple chemotherapy agents with distinct structures or mechanisms (Kumar and Jaitak, 2019). P-glycoprotein (P-gp), encoded by adenosine triphosphate (ATP)-binding cassette (ABC) subfamily B member 1 (ABCB1) is the extensively studied factor in the development of MDR. ABCB1 could utilize the energy of ATP to actively transport drugs out of cytoplasm, resulting in the survival of cancer cells (Kumar and Jaitak, 2019). P-gp has been confirmed to be involved in the resistance to several anti-cancer agents, such as paclitaxel, doxorubicin, cisplatin, etoposide, etc. (Gottesman *et al.*, 2002; Szakacs *et al.*, 2006; Zeino *et al.*, 2015). In spite of great efforts in exploring P-gp inhibitors, most of

 ISSN 1120-1770 online, DOI 10.15586/ijfs.v35i3.2354

them failed to show ideal efficacy while possessing major toxicities (Chen *et al.*, 2016). Therefore, discovering novel P-gp inhibitors with potent efficacy and little toxicity is required urgently.

Ganoderma lucidum (*G. lucidum*) is a traditional Chinese medicine widely used in China (named Ling Zhi) for hundreds of years (Ahmad, 2018). As a medicinal and edible mushroom, *G. lucidum* has proved to possess diverse pharmacological activities, including antioxidant (Zhang *et al.*, 2021), antidiabetic (Ma *et al.*, 2015), antimicrobial (Cor *et al.*, 2018), anti-inflammatory (Cai *et al.*, 2016), immunomodulatory (Li *et al.*, 2020), and anticancer effects (Jin *et al.*, 2016). Researchers have found that *G. lucidum* could exert its anticancer effects by directly killing cancer cells, inhibiting angiogenesis, inducing cell differentiation, and activating immune response of the host (Ahmad, 2018). However, whether constituents of *G. lucidum* could reverse P-gp-related MDR *in vivo* has not been studied amply.

In this study, we explored the effect of six extract samples derived from *G. lucidum* on the cell viability and MDR of adriamycin-resistant human hepatocellular carcinoma multidrug-resistant cell subline (HepG-2/ADM). Then the difference in P-gp function between hepatocellular carcinoma G2 (HepG-2) and HepG-2/ADM cells was compared, and we further investigated the effect of extract samples on P-gp function. Finally, the effect of *G. lucidum* ethanol extract alone and in combination with paclitaxel on tumor growth and expression level of P-gp was measured in HepG-2/ADM tumor-bearing mice *in vivo*.

Materials and Methods

Materials and chemicals

Samples 1–6 were obtained from Guangzhou Hanfang Pharmaceutical Co. Ltd. (Guangzhou, China). Sample 1 was the major product of *G. lucidum* spore oil using supercritical fluid CO_2. Sample 2 was the product of *G. lucidum* spore oil using supercritical fluid CO_2 with modifier ethanol. Sample 3 was ethanol extract of the remains after using supercritical fluid CO_2. Sample 4 was water extract of the remains after using supercritical fluid CO_2. Sample 5 was the water extract of *G. lucidum*. Sample 6 was the ethanol extract of *G. lucidum.* Verapamil, paclitaxel, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), and rhodamine-123 (Rh-123) were purchased from Sigma-Aldrich (Deisenhofer, Germany). Doxorubicin (Dox) was obtained from Zhejiang HISUN Pharmaceuticals Co. Ltd. (Zhejiang, China).

Cell lines and culture

Human hepatocellular carcinoma (HCC) cell line HepG-2 and the multidrug-resistant P-gp overexpressing HCC cell line HepG-2/ADM were provided by Cancer Institute & Hospital, Chinese Academy of Medical Sciences (Beijing, China). All cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Corning, New York, US) containing 10% fetal bovine serum (FBS; Life Technologies, New York, US) and 1% penicillin–streptomycin solution (Life Technologies). Cell lines were maintained at 37°C in a humidified atmosphere with 95% air and 5% CO_2.

Cell viability assay

Cells were seeded in 96-well plates at a density of 4×10^3 cells per well and cultured overnight. The cells were treated with various concentrations of samples 1–6 or compounds in the presence or absence of verapamil for 48 h. MTT, 20 μL, was added into each well and incubated for an additional 4 h. Finally, purple formazan crystals were dissolved in 150-μL dimethyl sulfoxide (DMSO) and the absorbance was observed at 490 nm by multimode microplate reader (Bio-Rad Laboratories, CA, US).

Rhodamine-123 intracellular accumulation assay

Cells were seeded in six-well plates at a density of 1×10^5 cells per well and cultured overnight. The cells were treated with 500 μg/mL of samples 1–6 for 48 h and incubated with 10-μM Rh-123 for an additional 4 h in dark at 37°C. Then the cells were collected, washed with phosphate-buffered saline (PBS) thrice, and analyzed by flow cytometer (BD Bioscience, San Jose, CA, US). The data were analyzed using the FlowJo 7.6.1 software.

Xenograft mouse model experiments

BALB/c nude mice were purchased from Guangdong Province Medical Animal Center and monitored under specific pathogen-free conditions. All animal experiments complied with the *National Institute of Health Guide for the Care and Use of Laboratory Animals*, 8th edition (National Institutes of Health, 2011). HepG-2/ADM cells (1×10^7 in 200 μL) were collected and injected subcutaneously into the right flank of mice. Treatments were initiated when tumors reached a mean volume of 100 mm^3. Mice were randomly divided into the following four groups (n = 5): (1) control group (mice were given normal saline [NS]); (2) paclitaxel group (mice were given 18-mg/kg paclitaxel); (3) sample 6 group (mice were given 400-mg/kg sample 6); and (4) sample 6 + paclitaxel

group (mice were given 400-mg/kg sample 6 + 18-mg/kg paclitaxel). Mice were daily administrated sample 6 orally. Mice were injected with paclitaxel intraperitoneally every 2 days. Body weight and tumor volume were determined every 2 days, and tumor volume was calculated using the following formula:

$$V = \pi \text{ (length} \times \text{width}^2)/6.$$

Animals were euthanized with CO_2 when the average tumor volume in the control group reached 1,300 mm^3, and tumors were collected and weighed for the following experiments.

Western blot analysis

Tumor tissues were homogenized and total protein was quantified by Bradford assay. Protein extract was separated in 4–12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred on polyvinylidene fluoride (PVDF) membranes. After blocking with 5% skimmed milk for 2 h, the membranes were incubated overnight at 4°C with primary antibodies against P-gp/ABCB1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Cell Signaling Technology, Beverly, MA, US). Next, the membranes were incubated with a rabbit secondary horseradish peroxidase conjugated antibody (CST) for 1 h at 37°C. Protein–antibody complexes were discovered using electrochemiluminescence (ECL) kit (Thermo Fisher, IL, US), and the intensity of protein bands was quantitated by the ImageJ software.

Hematoxylin and eosin (H&E) staining

Tumor tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned with a thickness of 5 μm. The sections were stained with H&E according to a standard protocol, and observed using Leica DM750 microscope (Leica, Heidelberg, Germany).

Assay for Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST)

The blood samples were collected and centrifuged at 3,500 rpm for 5 min at 4°C. The supernatant was collected and the levels of serum ALT and AST were analyzed using Drew Trilogy Analyzer (Diamond Diagnostics, Holliston, MA, US).

Statistical analysis

Data were expressed as mean ± SD. Comparison of differences between multiple groups was performed using one-way ANOVA. Two-tailed Student's *t*-test was used to compare difference between two groups. Statistical analysis was performed using the SPSS 22.0 software; $p < 0.05$ were considered statistically significant.

Results

G. lucidum samples showed no obvious toxicity to HepG-2/ADM cells

We measured the viability of HepG-2/ADM cells after incubation with various concentrations of samples 1–6 using MTT assay to evaluate whether the six samples derived from *G. lucidum* possessed toxicity to multidrug-resistant HCC cells. As shown in Figure 1, all samples showed little toxicity for HepG-2/ADM cells. Over 90% cells were alive even when the concentration reached 1,000 μg/mL. The results indicated that these samples derived from *G. lucidum* had no obvious toxicity to multidrug-resistant HCC cells, which could be candidates for investigating the effect of *G. lucidum* extract on MDR.

G. lucidum ethanol extract reversed multidrug resistance in HepG-2/ADM cells

In order to investigate the effect of *G. lucidum* extract on the MDR of HepG-2/ADM cells, we measured cell viability after incubation with doxorubicin or paclitaxel in combination with samples 1–6 or a classic inhibitor of P-gp (verapamil). As shown in Table 1, verapamil could potently suppress the function of P-gp and diminish cell viability at the 50% inhibitory concentration (IC_{50}) of doxorubicin and paclitaxel, which proved the MDR characteristic of HepG-2/ADM cells. To our surprise, we also observed that only sample 6 could effectively reverse resistance to doxorubicin and paclitaxel at IC_{50} and 20% inhibitory concentration (IC_{20}) and further reduced the viability of HepG-2/ADM cells. The fold reversal of resistance to doxorubicin and paclitaxel was almost three times at IC_{50}, while that was nearly five to seven times at IC_{20}. These data indicated that *G. lucidum* ethanol extract could reverse MDR in HCC cells.

G. lucidum ethanol extract inhibited the function of P-gp in HepG-2/ADM cells

Rh-123 is a specific fluorescent substrate of P-gp used to monitor the efflux function of P-gp. Therefore, we first treated HepG-2 cells and HepG-2/ADM cells with Rh-123 to validate their difference in P-gp function. As shown in Figure 2A, the fluorescent intensity of Rh-123 was significantly higher in HepG-2 cells than that in

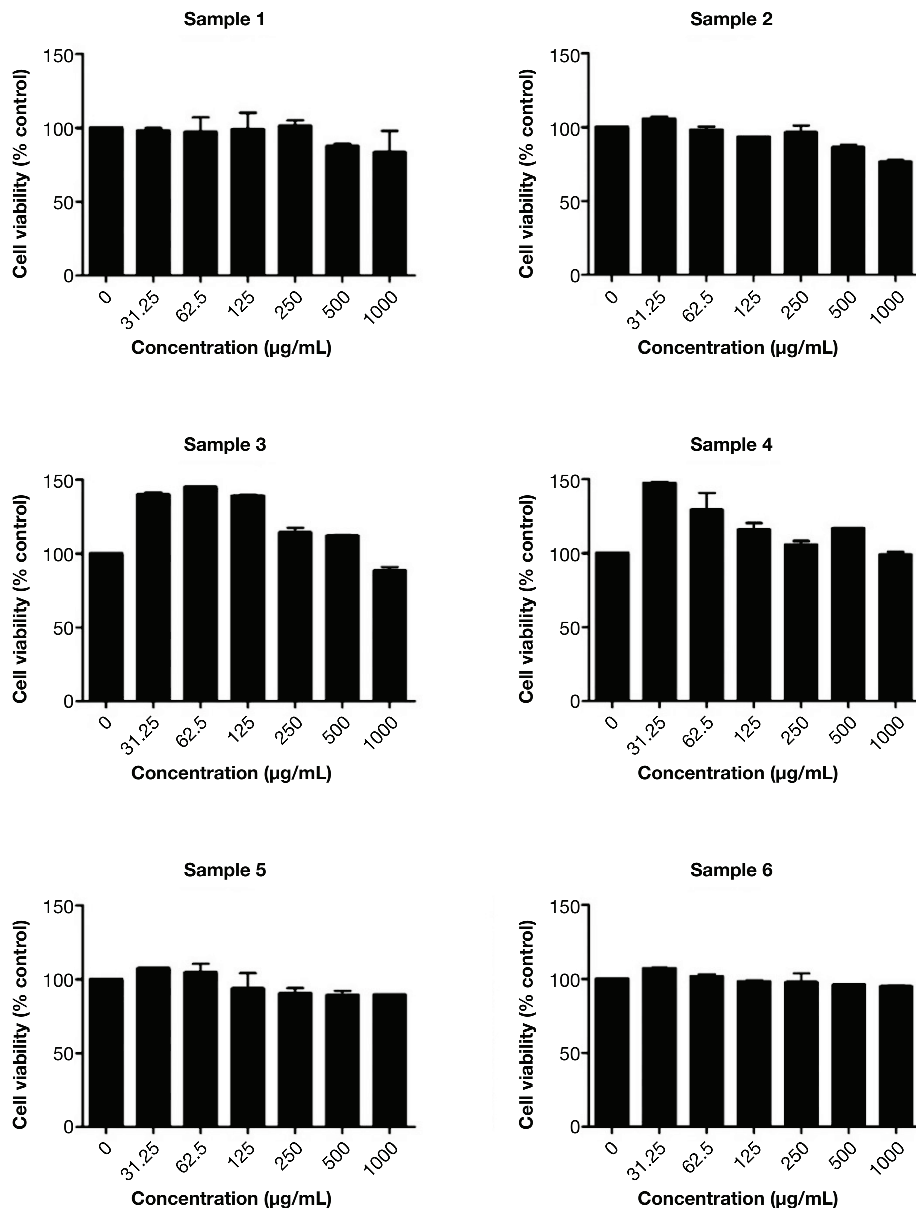

Figure 1. Samples derived from *G. lucidum* showed no obvious toxicity to HepG-2/ADM cells. HepG-2/ADM cells were treated with samples 1–6, and the cell viability was measured by MTT assay ($n = 5$). Data are presented as mean ± SD.

HepG-2/ADM cells, indicating that there was less Rh-123 accumulation in multidrug-resistant HCC cells. We further treated HepG-2/ADM cells with 500 μg/mL of samples 1–6 to investigate their effect on P-gp function. As shown in Figure 2B, samples 1–5 failed to change the accumulation of Rh-123 in HepG-2/ADM cells, while only sample 6 demonstrated a significant inhibitory effect on P-gp function and elevated fluorescent intensity, which was in consistent with the reversal effect assay. The results indicated that *G. lucidum* ethanol extract could inhibit the function of P-gp in multidrug-resistant HCC cells.

G. lucidum ethanol extract reversed resistance to paclitaxel in HepG-2/ADM tumor-bearing mice without additional toxicity

We established HepG-2/ADM tumor-bearing mice models and administrated with paclitaxel and sample 6. As shown in Figures 3A–3C, paclitaxel treatment alone failed to inhibit the growth of tumor, indicating that resistance to paclitaxel was maintained in the xenografted tumor model. Furthermore, although treatment with sample 6 alone did not inhibit the growth of HepG-2/ADM tumors *in vivo*, it was apparent that sample 6 could

Table 1. The reversal effect of *G. lucidum* ethanol extract on the MDR of HepG-2/ADM cells ($n = 5$).

	Cell viability ± SD (%) (fold-reversal)
Doxorubicin (IC_{50})	57.393 ± 4.889
+Sample 6 (500 μg/mL)	16.118 ± 4.093*** (3.561)
+Sample 6 (250 μg/mL)	21.346 ± 8.690*** (2.689)
+Verapamil (5 μmol)	4.646 ± 0.049*** (12.353)
Doxorubicin (IC_{20})	84.807 ± 3.378
+Sample 6 (500 μg/mL)	11.054 ± 3.366*** (7.672)
+Sample 6 (250 μg/mL)	13.922 ± 11.810*** (6.092)
Paclitaxel (IC_{50})	47.507 ± 10.849
+Sample 6 (500 μg/mL)	15.402 ± 3.538### (3.085)
+Sample 6 (250 μg/mL)	16.279 ± 4.660### (2.918)
+Verapamil (5 μmol)	0.942 ± 0.121### (50.43)
Paclitaxel (IC_{20})	83.588 ± 11.879
+Sample 6 (500 μg/mL)	14.311 ± 2.058### (5.841)
+Sample 6 (250 μg/mL)	16.989 ± 4.604### (4.920)

Data are presented as mean ± SD, and significant differences are indicated as ***$p < 0.001$ vs the doxorubicin group, ###$p < 0.001$ vs the paclitaxel group.

reverse resistance to paclitaxel and decrease tumor volume and weight combined with paclitaxel. H&E staining of tumor sections showed that after treatment with paclitaxel + sample 6, tumor tissues demonstrated large areas of nuclear consolidation, cell vacuolization, and necrosis, with lymphocyte-infiltrated hyperemia in Figure 3E. We also measured the body weight and serum levels of ALT and AST in mice, and the results showed that neither sample 6 nor paclitaxel was toxic to mice as shown in Figures 3D and 3F. The results showed that *G. lucidum* ethanol extract could reverse resistance to paclitaxel in HepG-2/ADM tumor-bearing mice without additional toxicity.

G. lucidum ethanol extract showed no effect on the protein expression level of P-gp

In order to investigate the underlying mechanism of the reversal effect of sample 6 on MDR of HCC tumor, we extracted the total protein of tumor tissues and carried out Western blot assay. As shown in Figure 4, administration of paclitaxel could slightly increase the expression level of P-gp; however, sample 6 showed no additional effect on P-gp expression level. The results indicated that the reversal effect of *G. lucidum* ethanol extract on MDR

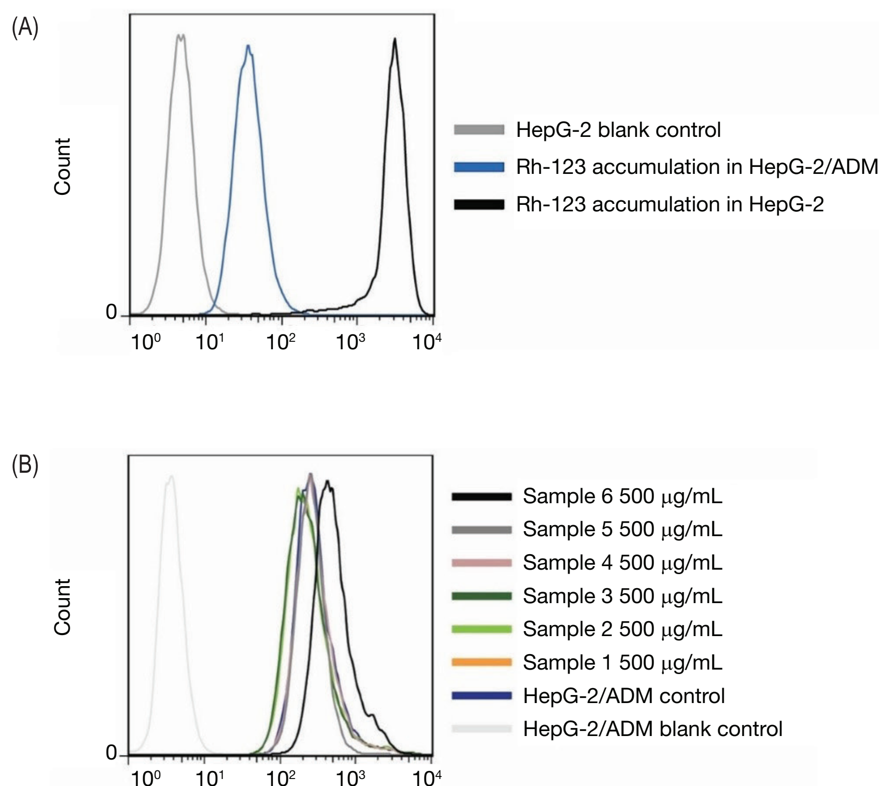

Figure 2. *G. lucidum* ethanol extract inhibited the function of P-gp in HepG-2/ADM cells. (A) HepG-2 and HepG-2/ADM cells were incubated with Rh-123, and the fluorescent intensity was measured by flow cytometry. (B) HepG-2/ADM cells were treated with samples 1–6 and incubated with Rh-123, and the fluorescent intensity was measured by flow cytometry.

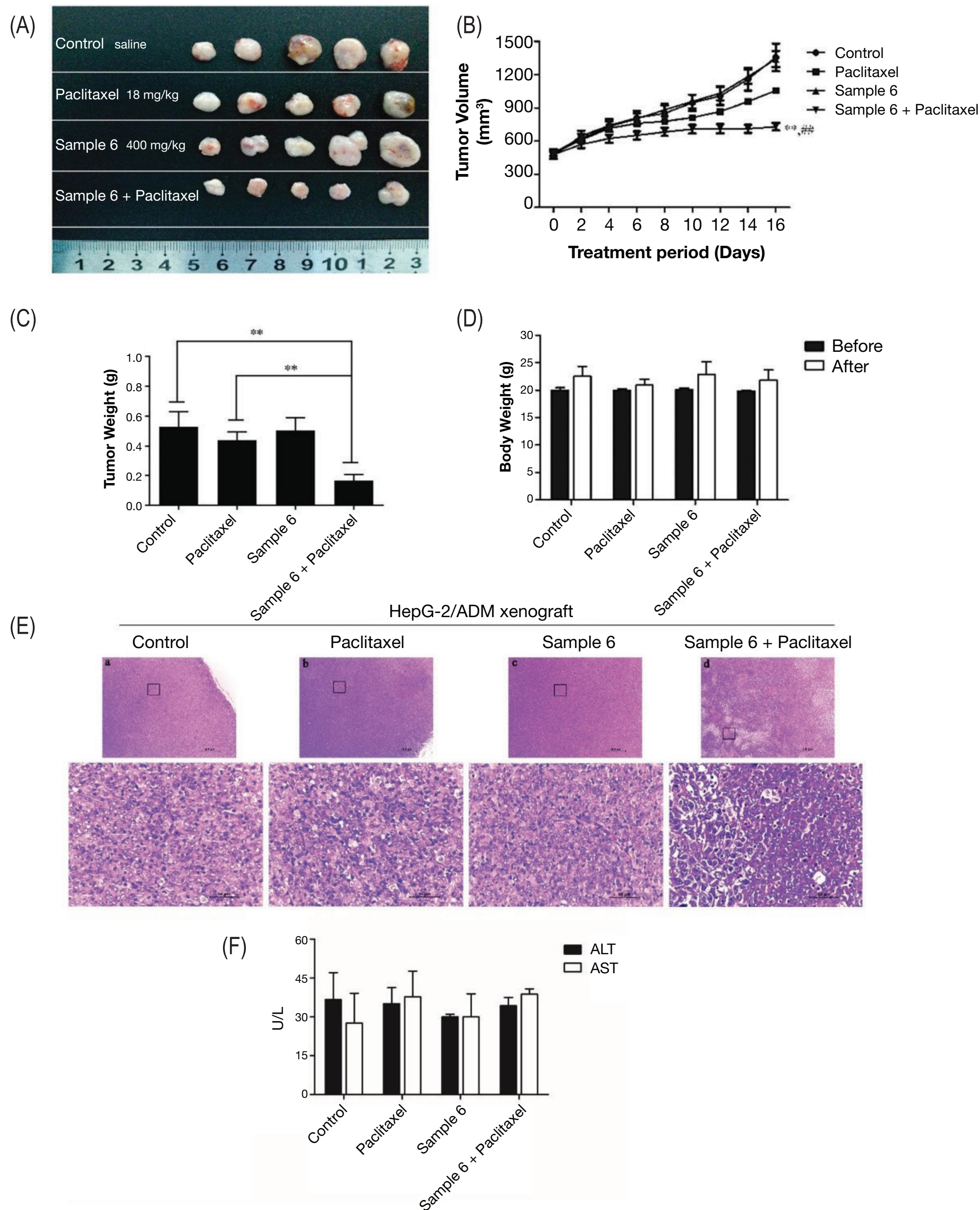

Figure 3. ***G. lucidum*** **ethanol extract reversed resistance to paclitaxel in HepG-2/ADM tumor-bearing mice without additional toxicity. (A) Tumors from nude mice in each treatment group. (B) Tumor volume and (C) tumor weight in each treatment group (*n* = 5). (D) Body weight of mice in each treatment group (*n* = 5). (E) H&E staining of paraffin-embedded tumor tissue sections in each treatment group (*n* = 5). (F) Serum levels of ALT and AST in each treatment group (*n* = 5). Data are presented as mean ± SD., and significant differences are indicated as $^{**}p < 0.01$ vs the control group, $^{\#\#}p < 0.01$ vs the sample 6 + paclitaxel group.**

could be related to the direct inhibition of P-gp function, rather than down-regulation of protein expression level.

Discussion

In the current study, the effect of six extract samples derived from *G. lucidum* on the viability of HepG-2/ADM human hepatocellular carcinoma multidrug-resistant cells was determined, and they exhibited no obvious toxicity. However, *G. lucidum* ethanol extract could reverse resistance to doxorubicin and paclitaxel. Then the P-gp function in HepG-2 and HepG-2/ADM cells was determined after incubated with these samples, and it was found that *G. lucidum* ethanol extract could inhibit P-gp function *in vitro*. Furthermore, *G. lucidum* ethanol extract could reverse resistance to paclitaxel in HepG-2/ADM tumor-bearing mice *in vivo*, without

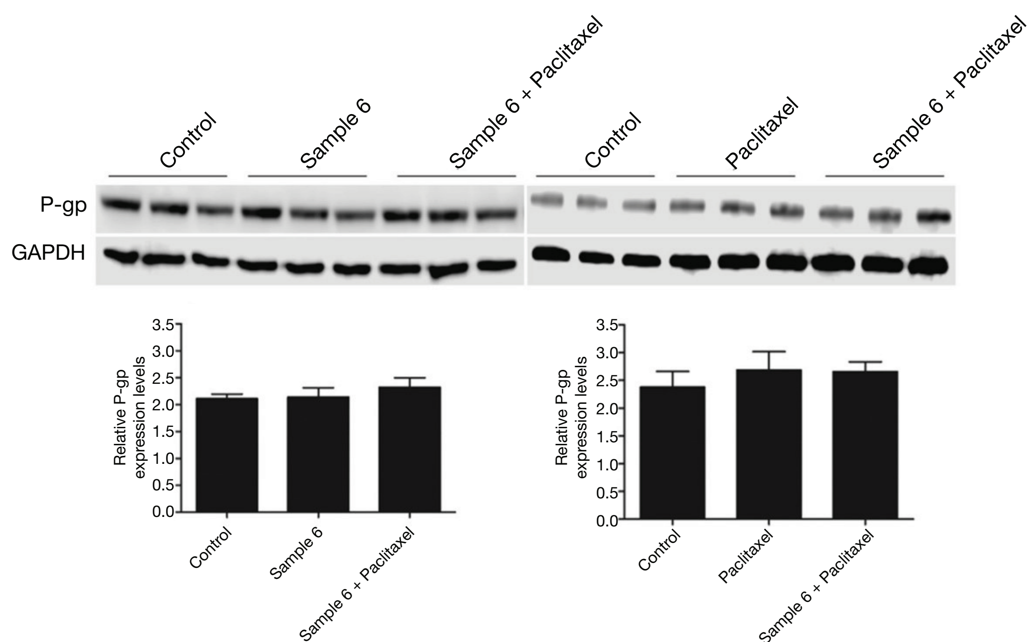

Figure 4. ***G. lucidum*** **ethanol extract showed no effect on the protein expression level of P-gp *in vivo*. The protein expression level of P-gp was assessed in HepG-2/ADM xenografts (*n* = 3). Data are presented as mean ± SD.**

affecting the protein expression level of P-gp, indicating that *G. lucidum* ethanol extract might directly inhibit the function of P-gp.

In the past decades, cancer has become one of the major threats to human health worldwide, with increased incidence and mortality (Sung *et al.*, 2021). Currently, in the clinical treatment of cancer, chemotherapy still plays an indispensable role because of good response and wide utility. Unfortunately, the development of MDR has severely limited the use of chemotherapeutic drugs, such as paclitaxel, doxorubicin, etoposide, and cisplatin (Robey *et al.*, 2018).

G. lucidum, a basidiomycete, has been recognized as a traditional Chinese medicine with a history of hundreds of years in ancient China. It was believed that *G. lucidum* could promote immunity and extend life expectancy (Chan *et al.*, 2021). Recently, researchers have discovered that *G. lucidum* exhibits multiple biological activities, including antioxidant, anticancer, anti-inflammatory, and immunomodulatory effects (Cor *et al.*, 2018).

In this study, to further investigate the anticancer characteristics of *G. lucidum*, we prepared six samples with different isolation methods, and measured their effect on the viability of HepG-2/ADM cancer cells. The results revealed that, within a range of experimental dose, none of the six samples showed any inhibitory effects on cell growth, indicating that they posed no direct toxicity to HepG-2/ADM cancer cells.

P-gp, a member of the ABC transporter family, has been identified as a major factor that leads to MDR via utilizing the energy of two ATP molecules. P-gp could fulfill one catalytic cycle and the following conformational change, and pump the substrate out of the cytoplasm, resulting in resistance to several cytotoxic chemotherapy agents (Zeino *et al.*, 2015). The overexpression of P-gp has been confirmed in different malignancies, such as hepatocellular carcinoma (Komori *et al.*, 2014), breast carcinoma (Ding *et al.*, 2021), colorectal carcinoma (Hu *et al.*, 2014), and chronic myeloid leukemia (Ammar *et al.*, 2020). Although great efforts have been invested in the research of P-gp inhibitors, most candidates failed because of inevitable toxicity and frustrating efficacy. Nowadays, natural products have demonstrated promising capabilities in regulating the function of P-gp while providing safer outcomes (Kumar and Jaitak, 2019).

In this study, we first applied a classic P-gp inhibitor, verapamil, and found that it could dramatically reverse the resistance of HepG-2/ADM cells to doxorubicin or paclitaxel, implying the role of P-gp in the MDR of HepG-2/ADM cells. Interestingly, we further measured the reversal effect of *G. lucidum* extract samples and discovered that only the ethanol extract sample 6 could significantly reverse resistance to doxorubicin and paclitaxel. Therefore, in order to investigate the effect of *G. lucidum* components on P-gp function, we applied Rh-123, a specific fluorescent substrate of P-gp, and performed flow cytometry experiments. Our results showed that, compared to HepG-2 cells, the multidrug-resistant HepG-2/

ADM cells accumulated less Rh-123 in cytoplasm, indicating that the elevated function of P-gp contributed to MDR in HepG-2/ADM cells. Moreover, *G. lucidum* ethanol extract sample 6 could inhibit P-gp and elevate the level of Rh-123 in HepG-2/ADM cells, which further validated our previous results in the assay of reversal effect.

Based on the above results, xenograft HepG-2/ADM tumor-bearing mouse models were created and administered with paclitaxel and sample 6 to investigate whether *G. lucidum* ethanol extract could reverse MDR in HepG-2/ADM cells *in vivo*. The results demonstrated that paclitaxel had little effect on the growth of HepG-2/ADM tumors, confirming the MDR characteristic of xenografted tumor model. Further, *G. lucidum* ethanol extract sample 6 could reverse the MDR of xenografted tumors in combination with paclitaxel, resulting in the suppression of tumor volume and weight, accompanied with deteriorated tumor pathologic conditions. The results indicated the reversal effect of *G. lucidum* ethanol extract on MDR *in vivo*. Meanwhile, we also measured the body weight and serum levels of ALT and AST, and the data showed that the administration of sample 6 did not alter body weight and liver functions of mice, implying that *G. lucidum* ethanol extract had no obvious toxicity *in vivo*. Further, we extracted the total protein of tumor tissues and determined the expression level of P-gp in each treatment group. However, the results showed that sample 6 did not change the protein expression level of P-gp, while paclitaxel could slightly increase the expression of P-gp *in vivo*. Based on these results, we deduced that sample 6 could exert the reversal effect of MDR by directly inhibiting the function of P-gp, rather than affecting protein expression level. Therefore, our results indicated that *G. lucidum* ethanol extract could exert the reversal effect on MDR in HCC cells by directly inhibiting the function of P-gp both *in vitro* and *in vivo*.

Conclusion

Our study explored the effects of *G. lucidum* extract on multidrug-resistant HCC cells, and established that *G. lucidum* ethanol extract could reverse MDR in HCC cells by inhibiting the function of P-gp both *in vitro* and *in vivo* without severe adverse reactions. These findings implied the promising role of *G. lucidum* ethanol extract in reversing MDR because of chemotherapy agents in the clinical treatment of HCC.

Acknowledgments

This work was supported by the Ministry of Science and Technology of China (No. 2017YFC1703104), the Key Laboratory Project of Pharmaceutical Lipids in Guangdong Province (No. 2020B1212070024), the Guangdong Province Key Areas R&D Program Project (No. 2020B1111120002), and the National Key R&D Program of China (No. 2022YFC3500302).

Authors of this research are in deep gratitude toward Professor Renwang Jiang of Jinan University for his guidance and support to this work.

Conflict of interest

The authors stated that they had no conflict of interest to declare.

Author Contributions

Renwang Jiang, Juyan Liu, and Jing Li designed the study. Lin Cao wrote the manuscript. Jing Li, Cheng Yuan, Wendong Xu, Yaming Han, and Juyan Liu revised the manuscript. Jing Li, Hongfei Cai, Liang Chen, and Qin Zhang carried out the experiments. Lin Cao, Zhaojian Jiang, and Hongfei Cai analyzed the data. All authors approved the final version of the paper.

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