Int J Clin Exp Pathol 2019;12(8):2875-2886
www.ijcep.com /ISSN:1936-2625/IJCEP0091475

Original Article

Paris saponin H suppresses human hepatocellular carcinoma (HCC) by inactivation of Wnt/β-catenin pathway *in vitro* and *in vivo*

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Received January 18, 2019; Accepted February 21, 2019; Epub August 1, 2019; Published August 15, 2019

Abstract: Background: Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death in the world. *Paris polyphylla*, also known as Chong-lou in China, is traditionally used as an anticancer medicine. Paris saponin H (Ps H) has been reported to be one potential antitumor active component from *Paris polyphylla* and shows cytotoxicity on tumor cells. However, the role of Ps H in HCC is not clear. Methods: PLC/PRF/5 and Huh7 cells were exposed to Ps H. Cell viability, migration, and invasion were measured with CCK-8 assay, EMT and Transwell assay, respectively. Western blot was employed to detect the expression of cleaved caspase 3, E-cadherin, vimentin, β-catenin, p-GSK-3β and GSK-3β. Apoptosis was assessed by flow cytometry, and caspase 3 activity assay. For in vivo experiments, xenograft tumors were induced with PLC/PRF/5 cells. Results: Ps H reduced cell viability and induced apoptosis in HCC cells in the dose-dependent manner; EMT and invasion were inhibited by Ps H. Ps H downregulated expression of β-catenin and p-GSK-3β; in addition, β-catenin silencing mediated Ps H-induced suppression of cell progression in PLC/PRF/5 cells. An administration of Ps H effectively suppressed the tumor growth in the HCC xenograft model *in vivo*. Conclusion: Ps H suppresses HCC cell progression through downregulation of β-catenin *in vitro*, and inhibits xenograft tumor growth, suggesting Ps H is an attractive candidate for clinical therapy for HCC.

Keywords: Paris saponin H (Ps H), hepatocellular carcinoma (HCC), β-catenin, cell progression

Introduction

Hepatocellular carcinoma (HCC) is the fifth most prevalent cancer and the third leading cause of cancer-related death in the world. 40-70% patients eventually suffer from postoperative recurrence within 5 years [1, 2]. HCC is considered a major health problem due to endemic hepatitis B and C and regional exposure to environmental pathogens, and its invasion and resistance to chemotherapeutic agents limit treatment options [3, 4]. At present, surgery, the major curative treatment for HCC, is effective for only 15% to 25% of early stage HCC patients and long-term survival is rare [5]. Therefore, it is imperative to search for and develop new agents, such as phytochemicals from plants, for the prevention and treatment of HCC.

Paris polyphylla var. *yunnanensis* (PPY) and *Paris polyphylla* var. *chinensis* (PPC), also known as Chong-lou in China, are famous traditional Chinese medicines included in the Chinese pharmacopoeia [6]. They are also the material basis of some Chinese patent anticancer medicines, such as Gan-Fu-Le capsules, Bo-Er-Ning capsules, Lou-Lian capsules, Ruan-Jian oral liquid, and Qi-Zhen capsules [7]. Extensive phytochemical and pharmacologic studies further identified Rhizoma Paridis saponins (Polyphyllin) to be the main antitumor active components in *Paris polyphylla* [8, 9]. Several studies show that Paris saponin H (Ps H) shows cytotoxic activities on several cancer cells. Ps H could inhibit lung adenocarcinoma growth and metastasis *in vitro* and *in vivo* [10]. Ps H, the predominant saponin of *Paris fargesii* var. *brevipetala* (PFB), was tested to show cytotoxic activities on HepG2, A549, RPE, and L929 cells with a positive control of Cisplatin [11]. Very recently, Ps H from *P. forrestii* [7], was claimed to show significant inhibitory activity against the growth of five cancer cell lines (HL-60, SMMC-7721, A-549,

MCF-7, and SW480). Moreover, Ps H took part in the suppressive effect of total saponins on mouse hepatocellular carcinoma H22 tumor growth through intraperitoneal administration in mice. However, the role of Ps H on tumors, especially hepatocellular carcinoma (HCC) is not fully uncovered yet.

The canonical Wnt/β-catenin pathway is a complex, evolutionarily conserved signaling mechanism that regulates fundamental physiological and pathological processes. Aberrant Wnt/β-catenin signaling has been shown to be common in HCC tumors and to have significant clinical impact on tumor behavior, prognosis, and response to treatment [12, 13]. β-catenin, an intracellular signal transducer and the core protein of the Wnt signaling cascade, has been identified to play a central role in the cadherin protein complex and is crucial for the activation of the Wnt/β-catenin signaling pathway during embryonic development and tumorigenesis [14]. In the activated Wnt/β-catenin pathway, Wnt proteins bind to membrane receptors belonging to the Frizzled (Fzd) family and low-density lipoprotein receptor-related protein 5/6 (LRP5/LRP6) leading to the recruitment of the cytoplasmic phosphoprotein of Disheveled (Dsh/Dvl). Dsh is the key intermediate in the process to activate and deliver signals from the formed Wnt/β-catenin receptor complex to the axin and glycogen synthase kinase 3β (GSK-3β) destruction complex to suppress the phosphorylation of β-catenin. A hallmark of Wnt/β-catenin signaling is the accumulation of unphosphorylated β-catenin in the cytoplasm and then nuclear translocation of β-catenin, which subsequently either activates other transcription factors or cooperates with TCF/LEF to activate target genes, such as c-Myc.

In this study, we aimed to further identify the effect of Ps H on cell viability, apoptosis, migration, and invasion of HCC *in vitro* and *in vivo* and the underlying mechanism was explored through the Wnt/β-catenin signaling pathway.

Materials and methods

Cells and cell culture

Human hepatocellular carcinoma (HCC) cell lines, PLC/PRF/5 were purchased from *American Type Culture Collection* (ATCC; Manassas, VA, USA) and Huh7 was provided from *Japanese Collection of Research Biologicals* (JCRB; Osaka, Japan). All cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin at 37°C in a humidified atmosphere of 5% CO_2.

Ps H (cas. 81917-50-2; cat. wkq-01627) was provided from *Weikeqi Biotech* (Sichuan, China) and dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA). Ps H was stored at -20°C after dissolution. PLC/PRF/5 and Huh7 cells were treated with 1.25, 2.5, 5, 10, and 20 μM of Ps H for indicated time (6, 12 and 24 h) when cells achieved 90% cell confluence. The control group was treated with 0 μM of Ps H (1‰ DMSO).

Cell transfection

For silent expression of β-catenin, small interfering RNA (siRNA) against β-catenin (si-β-catenin), and its scrambled siRNA (scrambled) were purchased from *RiboBio* (Guangzhou, China). Transient transfection of siRNA was performed by Lipofectamine 2000 reagent (Invitrogen, Shanghai, China) in PLC/PRF/5 cells according to the manufacturer's instructions. Transfected cells were exposed to 10 μM of Ps H or not for further studies.

Cell counting kit-8 (CCK-8) assay

The viability of cells was determined according to Cell Counting Kit-8 (CCK-8; Beyotime, Shanghai, China) manufacturers. In brief, PLC/PRF/5 and Huh7 cells were seeded into 96-well plate (Corning, NY, USA) at a density of 1×10^4 cells/well for 24 h. Different treated cells were cultured with 20 μl CCK-8 solution (5 g/l) in PBS for another 1-3 h, and the optical density was measured at 450 nm using SpectraMax M4 (Molecular devices, Shanghai, China. All experiments were performed in triplicate.

Colony formation

Clonogenic survival is the ability of cells to maintain clonogenic capacity and to form colonies. PLC/PRF/5 and Huh7 cells treated with Ps H for 24 h were immediately collected, followed by transplanting of 200 cells onto 10 cm dishes (Corning). The colony formation was visible and occurred after incubation for 15 days. Then,

the colonies formed were fixed with ethanol and stained with crystal violet. Colonies with over 10 cells were scored as surviving colonies. The control group was treated with 0 μM of Ps H. The colony forming efficiency (%) = 100% × number of colonies/number of inoculated cells.

Flow cytometry

Ps H-induced PLC/PRF/5 and Huh7 cells were analyzed by Annexin V-FITC/PI kit (Beyotime) on flow cytometry. Apoptotic cells were labelled complying with the protocol. In brief, the adherent and floating cells were harvested and washed with PBS twice. Then, 100 μl cells of each group was stained in the buffer containing FITC-Annexin V and PI for 30 min in the dark. Fluorescence was analyzed on cytoFLEX LX flow cytometer (Beckman-Coulter, CA, USA) using CytExpert software. Quadrants were positioned on Annexin V/PI plots to distinguish living cells (Annexin V-/PI-), and apoptotic cells (Annexin V+/PI-, Annexin V+/PI+, Annexin V-/PI+).

Caspase 3 activity assay

The caspase 3 activity was detected with Caspase 3 Activity Assay Kit (Beyotime) to assess cell apoptosis as per instructions. In short, PLC/PRF/5 and Huh7 cells were plated onto 96-well plate (Corning) at a density of 1×10^4 cells/well for 24 h, followed by Ps H exposure for another 24 h. The adherent cells were incubated in 100 μl of lysis buffer on ice for 20 min. 50 μl of samples were added into the mixture of 40 μl of detection buffer and 10 μl of Ac-DEVD-pNA (2 mM) with gentle shaking for 5 min. The reaction systems were cultured in 37°C for 1 h, and the OD405 values were read on SpectraMax M4 (Molecular devices). The control group was treated with 0 μM of Ps H. Every experiment was carried out in triplicate.

Western blot

For tumor tissues, protein samples were isolated by Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime) in accordance with the operation manual; for treated cells, protein samples were extracted in RIPA lysis buffer (Beyotime) containing 1% PMSF and the concentration of protein samples was determined using BCA™ Protein Assay Kit (Pierce, Rockford, IL, USA). Western blot assay was performed according to standard procedures with 20 μg sample, and GAPDH on the same membrane was an internal standard to normalize protein levels. The primary antibodies were purchased from *Abcam* (Cambridge, United Kingdom) and as follows: E-cadherin (#76055, 1:1000); vimentin (#20346, 1:1000); β-catenin (#6302, 1:4000); p-GSK-3β (#75745, 1:1000); GSK-3β (#93926, 1:2000); PCNA (#92552, 1:100); cleaved caspase-3 (#49822, 1:500); GAPDH (#9485, 1:2500). The protein bands were visualized using ECL procedure.

Transwell assay

For invasion assay, PLC/PRF/5 and Huh7 cells with administration of Ps H or not were supplemented in 200 μl of serum-free medium and plated in the upper chamber with matrigel-coated membrane (Corning). The medium containing 10% FBS (Gibco) was used as a chemoattractant and loaded in the low chamber. Transwell system was maintained at 37°C for 48 h. The invaded cells into the lower chambers were stained with crystal violet and quantitated under microscopy.

Xenograft model in vivo

4-wk-old nude mice were obtained from Model Animal Research Center of Nanjing University. The animals' experiments were approved by the Institutional Review Board of the West China School of Pharmacy, Sichuan University and were carried out in accordance with National Institutions of Health Guide for Care and Use of Laboratory Animals. PLC/PRF/5 cells were divided into four groups suffering for independent processing: inoculation of cells in saline with DMSO (control group), doxorubicin (DOX, 1 mg/kg) (positive control group) and 5 mg/kg of Ps H and 10 mg/kg of Ps H (experiment groups). Equal numbers (10^6) of treated PLC/PRF/5 cells in 0.2 mL of saline were injected in subcutaneous area of nude mouse (4 mice per group) for 30 days. The tumors and body weight was measured once per 3 d with caliper and electronic balance separately. The tumor volume was calculated using the formula: V (cm^3) = $1/2\ ab^2$ (a is the longest tumor axis and b is the shortest tumor axis). The mice were practiced with euthanasia on day 30 and the weight of tumors were evaluated with electronic balance. Immediately, the xenograft tumors were stored in -80°C for further isolation of total protein.

Statistical analysis

Data given were mean ± standard deviation (SD) from three separate experiments. The significance between two groups was determined using Student's *t* test on GraphPad Prism (Version 6.0; La Jolla, CA, USA). Results with values of $P < 0.05$ were considered significant.

Results

Paris saponin H (Ps H) reduces cell viability in dose- and time-dependent manners

To assess the effects of Ps H on cell viability, human HCC cells were cultured and exposed with Ps H. After 24 h-exposure in PLC/PRF/5 and Huh7 cells, 1.25-20 μM of Ps H significantly decreased cell viability in a dose-dependent manner (**Figure 1A** and **1B**); moreover, the cell viability was reduced by 10 μM of Ps H within 24 h in a time-dependent manner (**Figure 1C** and **1D**). To further investigate the anti-cell-growth activity of Ps H, a clonogenic assay was performed to determine the long-term effect of Ps H treatment on PLC/PRF/5 and Huh7 cells. As shown in **Figure 1E** and **1F**, the number of coloniesdeclined with 1.25, 2.5, 5, and 10 μM of Ps H treatment, indicating the colony-formation ability was inhibited by Ps H. These data showed Ps H could potently suppress cell viability in HCC cells.

Ps H induces apoptosis in HCC cells

In order to investigate the effects of Ps H on cell apoptosis, apoptosis rate and apoptosis-related protein, caspase 3, were measured following indicated dose of Ps H exposures for 24 h. As **Figure 2A** showed, PLC/PRF/5 and Huh7 cells suffered more apoptotic cells according to flow cytometry data and 1.25 μM of Ps H was enough to substantially induce apoptosis (**Figure 2B** and **2C**). Consistent with apoptosis rate, caspase 3 activity was elevated by Ps H (2.5, 5 and 10 μM) in comparing of PLC/PRF/5 or Huh7 without Ps H insult (**Figure 2D** and **2E**). These outcomes indicated Ps H could induce cell apoptosis in HCC cells.

Ps H inhibits EMT and invasion of HCC cells

The role of Ps H on HCC cell motility was studied. Epithelial-mesenchymal transition (EMT) and invasion were assessed. In PLC/PRF/5 cells, expression of EMT-related proteins was examined with western blot (**Figure 3A**) and E-cadherin expression was upregulated, vimentin was downregulated by 2.5, 5, and 10 μM of Ps H exposure for 24 h (**Figure 3B** and **3C**). Similar results were observed in Ps H-stimulated Huh7 cells, in which Ps H promoted E-cadherin expression and impaired vimentin expression (**Figure 3D-F**). Numbers of invasive cells were reduced depending on Transwell assay in PLC/PRF/5 and Huh7 cells by Ps H induction (**Figure 3G** and **3H**), suggesting the ability of HCC cell invasion was weakened by Ps H. These findings showed the inhibitory effect of Ps H on HCC cell motility.

Ps H inactivates Wnt/β-catenin signaling pathway and β-catenin silencing mediates Ps H-induced inhibition of HCC cell progression

We hypothesized an anti-tumor role of Ps H in HCC, and the underlying mechanism was further investigated. In consideration of the significance of Wnt/β-catenin pathway in tumor progression, effects of Ps H on Wnt/β-catenin signaling pathway-relevant genes were figured out in PLC/PRF/5 and Huh7 cells. As shown in **Figure 4A** and **4B**, expressions of β-catenin, p-GSK-3β and GSK-3β were monitored after Ps H exposure for 6, 12 and 24 h with western blot, and the results suggested that expressions of β-catenin and p-GSK-3β were sharply downregulated, and there was no difference in GSK-3β levels between Ps H-treated HCC cells and HCC cells without Ps H insult. Further, β-catenin was knocked down with siRNA (si-β-catenin) to mimic Ps H effect in PLC/PRF/5 cells treated with incubation of Ps H or not, that is, PLC/PRF/5 cells were divided into four groups: scrambled, si-β-catenin, Ps H, and si-β-catenin + Ps H. Expression of β-catenin was downregulated by si-β-catenin and Ps H independently, and was further decreased by treated of both simultaneously (**Figure 4C**). Besides, the cell viability, apoptosis, and invasion were measured. Cell viability and invasive cells were attenuated (**Figure 4D** and **4F**), and apoptosis rate was promoted (**Figure 4E**) by si-β-catenin and/or Ps H. These results showed Ps H could inactivate Wnt/β-catenin signaling pathway and β-catenin knockdown could mimic Ps H-mediated suppressive effect of Ps H on HCC cell progression.

Ps H inhibits tumor growth in vivo

We already acknowledged that Ps H suppresses HCC cell viability, migration, and invasion *in*

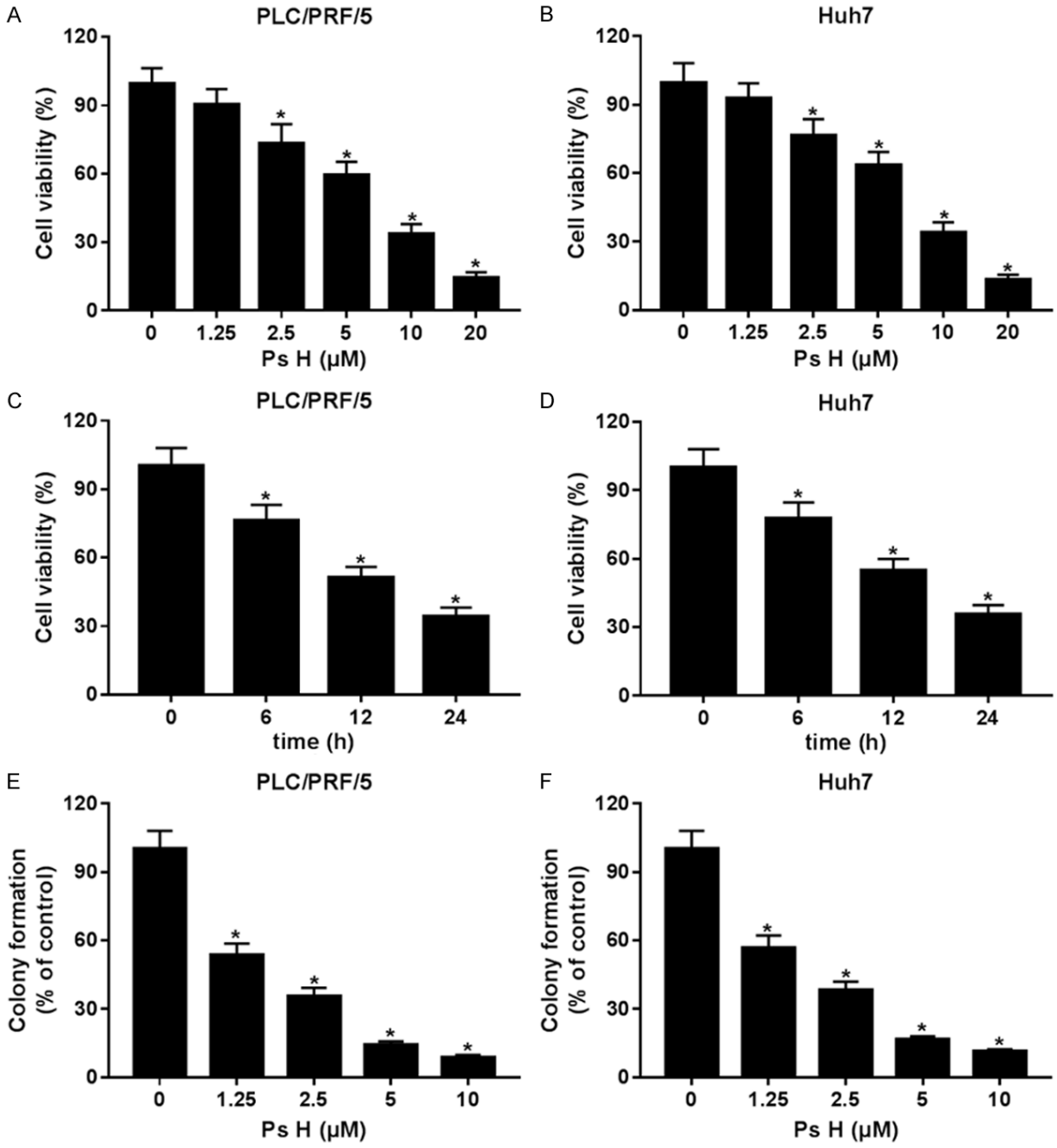

Figure 1. Roles of Paris saponin H (Ps H) on cell viability in hepatocellular carcinoma (HCC) cells. PLC/PRF/5 and Huh7 cells were exposed with Ps H. A, B. Effect of different concentrations of Ps H on cell viability. PLC/PRF/5 and Huh7 cells were treated with 1.25, 2.5, 5, 10, and 20 μM of Ps H for 24 h, and cell viability was measured with CCK-8 assay. C, D. Effect of Ps H on cell viability with time. PLC/PRF/5 and Huh7 cells were treated with 10 μM of Ps H for 6, 12 and 24 h, and cell viability was measured with CCK-8 assay. E, F. Effect of Ps H on colony formation. The number of colonies was counted after 1.25, 2.5, 5, and 10 μM of Ps H-treated PLC/PRF/5 and Huh7 cells plated and stained. All experiments were performed in triplicate and $*P < 0.05$, compared with PLC/PRF/5 (0 μM or 0 h) or Huh7 (0 μM or 0 h).

vitro. We explored whether it affected tumor growth *in vivo*. To answer the question, xenograft experiments were launched and PLC/PRF/5 cells were treateduffered with four independent processings: inoculation of cells in saline with DMSO (control group), doxorubicin (DOX, 1 mg/kg) (positive control group) and 5 mg/kg of Ps H and 10 mg/kg of Ps H (experiment groups). According to **Figure 5A** and **5C**, tumors were induced with time after inoculation, and tumor volume and weight were suppressed in Ps H groups and DOX group; the inhi-

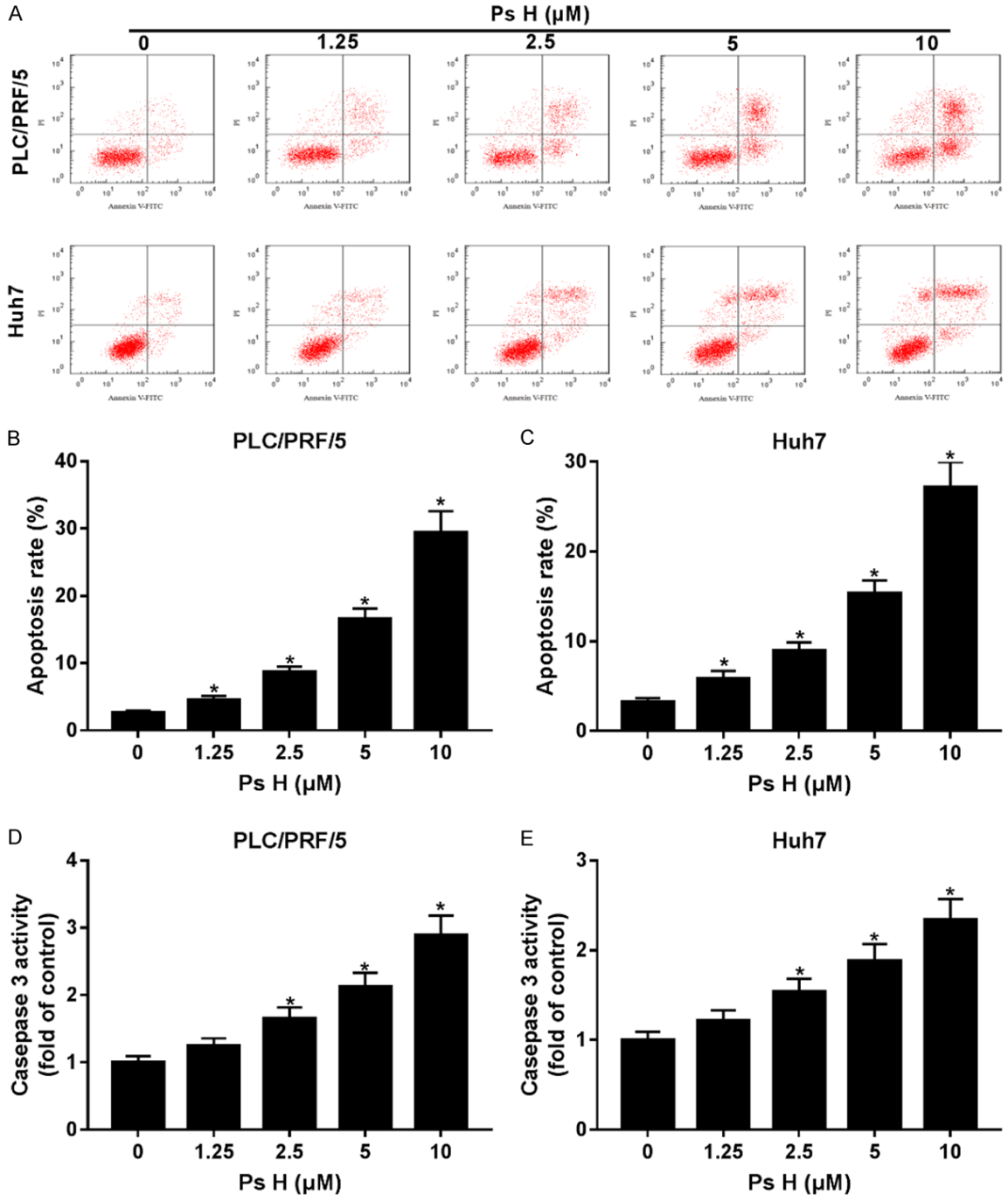

Figure 2. Roles of Ps H on cell apoptosis in HCC cells. PLC/PRF/5 and Huh7 cells were exposed with Ps H and cell apoptosis was examined. (A) Flow cytometry detected the apoptotic cells after treated with 1.25, 2.5, 5, and 10 μM of Ps H for 24 h, and (B, C) apoptosis rate was recorded. (D, E) Effect of Ps H on caspase 3 activity. Fold changes of caspase 3 activity were calculated with normalization to cells (0 μM). Data are representative of at least 3 independent experiments and $*P < 0.05$, compared with PLC/PRF/5 (0 μM) or Huh7 (0 μM).

bition of Ps H on tumor growth was correlated with dose. Meanwhile, the growth of mice exhibited no significant differences between groups (**Figure 5B**). Furthermore, expressions of β-catenin and markers of cell proliferation and apoptosis were detected by western blot in tumors (**Figure 5D**), and expressions of β-catenin and proliferating cell nuclear antigen (PCNA, cell proliferation marker) were downregulated, cleaved caspase-3 (cell apoptosis marker) ex-

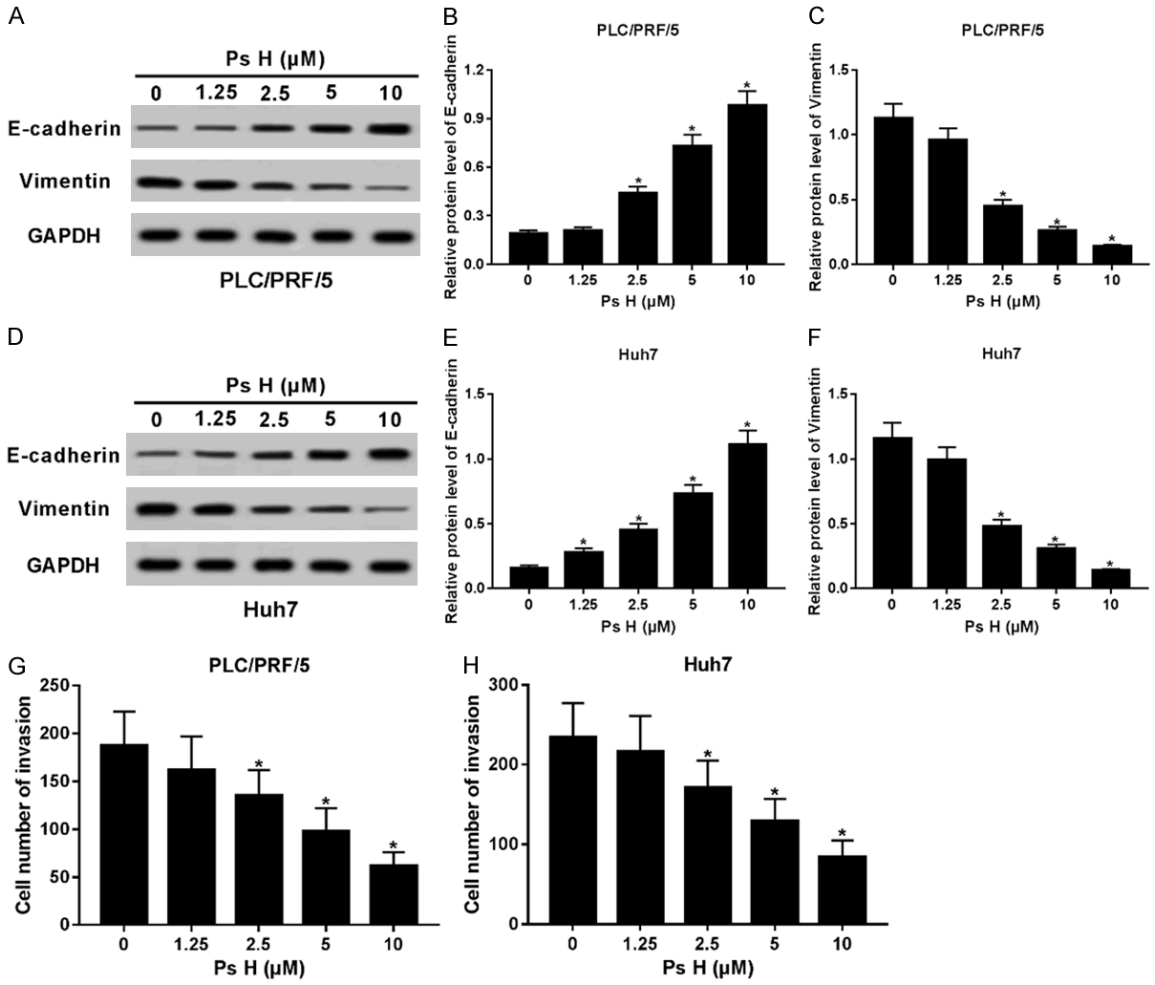

Figure 3. Roles of Ps H on epithelial-mesenchymal transition (EMT) and invasion in HCC cells. (A) Effect of Ps H on expression of EMT-related proteins in PLC/PRF/5 cells. Western blot detected expressions of E-cadherin and vimentin, and (B, C) levels of E-cadherin and vimentin relative to GAPDH were analyzed. (D) Effect of Ps H on expression of EMT-related proteins in Huh7 cells. Western blot detected expressions of E-cadherin and vimentin, and (E, F) levels of E-cadherin and vimentin relative to GAPDH were analyzed. (G, H) Effect of Ps H on cell invasion in PLC/PRF/5 and Huh7 cells. Invasive cells were counted with Transwell assay after Ps H treatment. Data are representative of 3 independent experiments and $*P < 0.05$, compared with PLC/PRF/5 (0 μM) or Huh7 (0 μM).

pression was upregulated in Ps H groups and DOX group. These findings demonstrated the anti-tumor role of Ps H *in vivo* without cytotoxicity to body growth.

Discussion

Hepatocellular carcinoma (HCC) is the most common primary hepatic malignancy, constituting around 85-90% of primary liver cancers [12]. HCC frequently develops in the setting of underlying chronic liver disease. At present, surgery, the major curative treatment for HCC, is only effective for 15% to 25% early stage of HCC patients [15] and the resistance to chemotherapeutic agents limits treatment options [16] as well. Therefore, it is imperative to search and develop new agents, such as phytochemicals from plants, for the prevention and treatment of HCC. Many anticancer drugs are obtained from natural sources. In China, there are many anticancer traditional Chinese medicine or herbal formulations that provide further leads for anticancer drug development [17], and these are receiving increasing scientific attention. In this study, the purpose was to illuminate the antitumor role of Ps H, one type of Paris saponins extracted from Chong-lou, in human HCC and the underlying mechanism was researched on Wnt/β-catenin pathway.

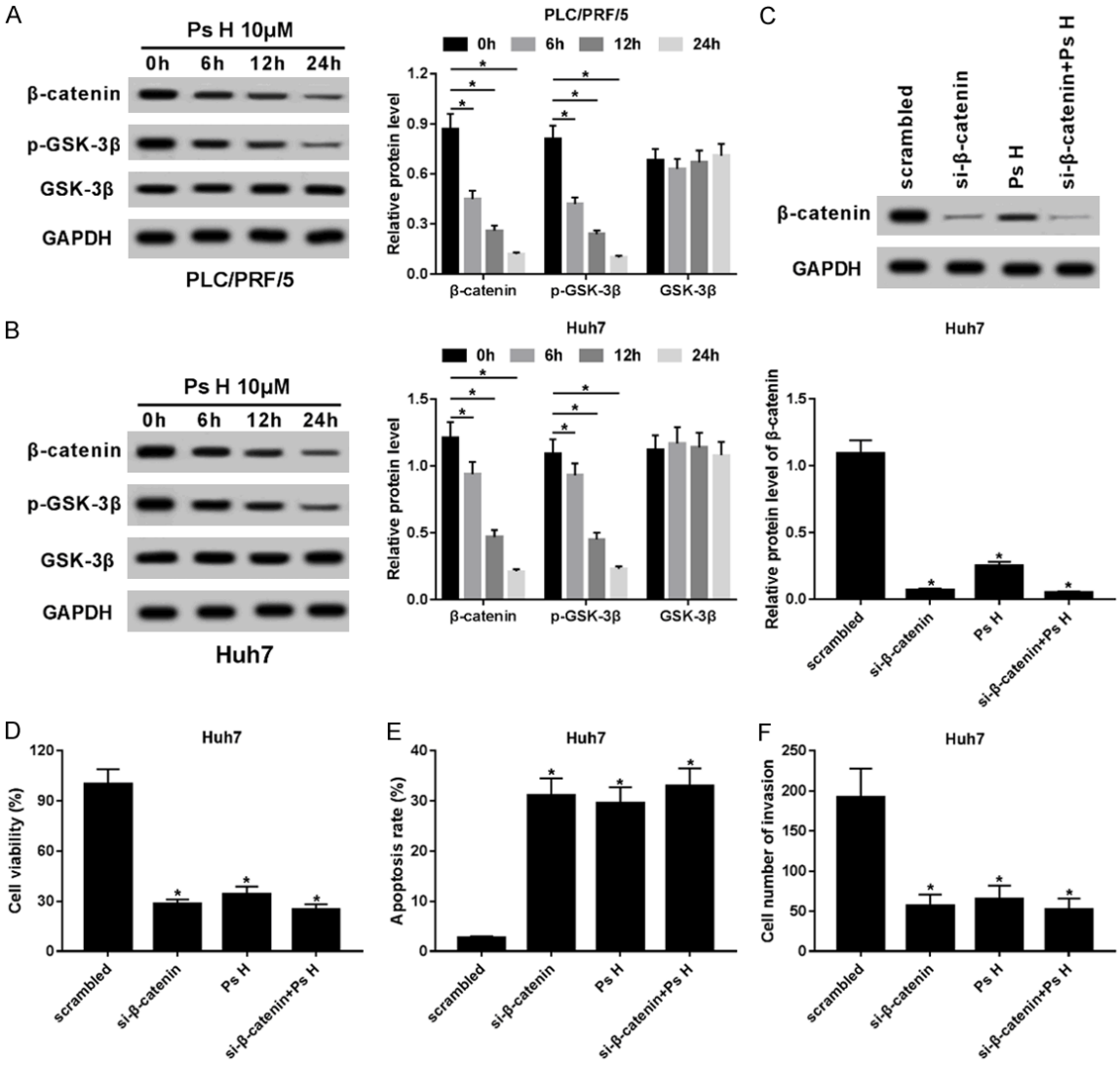

Figure 4. Effect of Ps H on Wnt/β-catenin signaling pathway and role of β-catenin in HCC cell progression. (A, B) Effects of Ps H on Wnt/β-catenin signaling pathway-relevant genes in PLC/PRF/5 and Huh7 cells. Expressions of β-catenin, p-GSK-3β and GSK-3β were monitored after Ps H exposure for 6, 12 and 24 h with western blot. (C) β-catenin was knocked down with siRNA (si-β-catenin) in PLC/PRF/5 cells. Expression of β-catenin after treated with transfection of si-β-catenin and/or incubation of Ps H. (D) cell viability was measured with CCK-8, (E) apoptosis was examined on flow cytometry, and (F) invasion was assessed with Transwell assay. All experimentswere carried out in triplicate, and $*P < 0.05$, compared with PLC/PRF/5 (0 h) or PLC/PRF/5 (scrambled).

Extracts of *Paris* have been shown to provide tumor-suppressive activity in HCC. The natural plant products are potent therapeutic as well as chemo preventive agents, including resveratrol, caffeine, quercetin, lycopene, and others, which have been found to be useful for the treatment of HCC [18]. The main antitumor active components in *Paris polyphylla* and some other *Paris* species, Rhizoma Paridis saponins (Polyphyllin) have been widely studied in HCC. Polyphyllin D (IV) was a potent apoptosis inducer through mitochondrial dysfunction in drug-resistant HepG2 cells [19]. Polyphyllin G (VII) had strong anticancer activities via suppression of proliferation, cell cycle arrest, and induction of apoptosis in modulation of drug-resistance in a wide variety of human cancer lines, especially Polyphyllin G was potentially correlated with ROS-mediated mitochondrial dysfunction and autophagic cell death through MAPK and pI3K/AKT/mTOR pathways in HepG2 cells [20, 21].

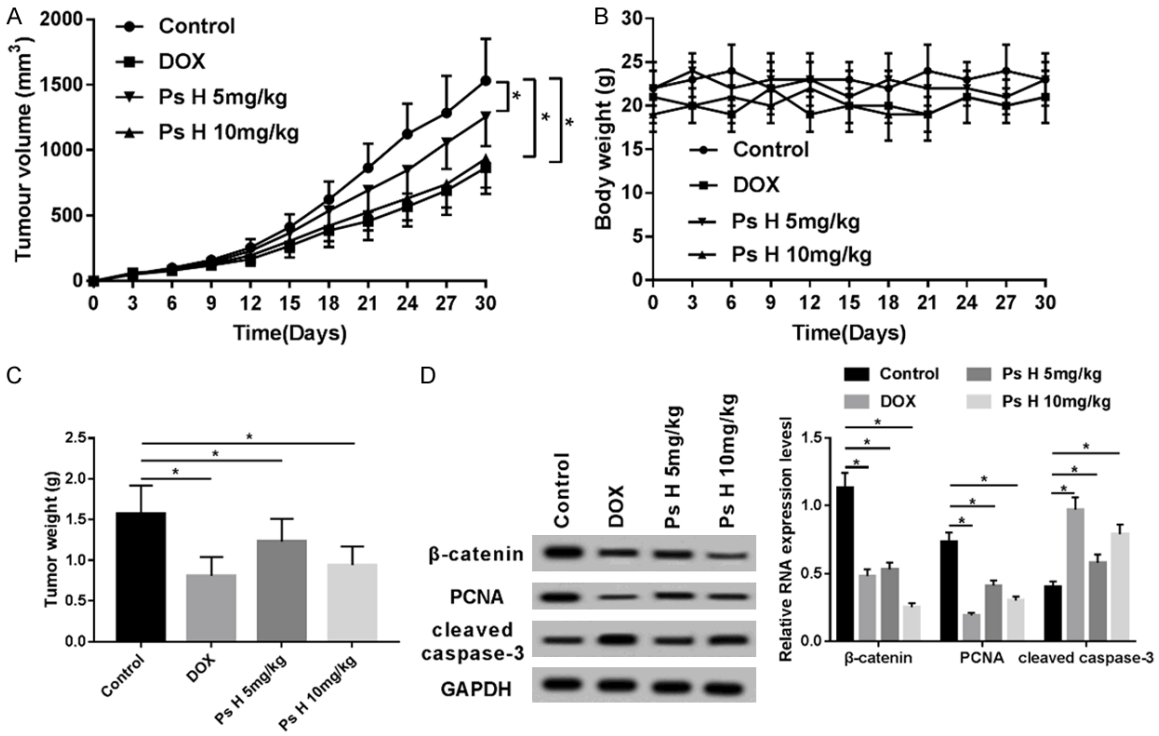

Figure 5. Effects of Ps H on xenograft growth. A. Tumor growth curve was drawn after the inoculation. B. Body weight of mice was monitored after the inoculation until 30 days. C. Tumor weight was recorded on day 30. D. Expressions of β-catenin, proliferating cell nuclear antigen (PCNA) and cleaved caspase-3 in tumors. All detections were repeated 3 times and $*P < 0.05$, compared with control (saline with DMSO). Doxorubicin (DOX, 1 mg/kg) was the positive control.

Ps H exhibits an anticancer role on cancers, including lung and hepatocellular carcinoma. Paris saponin H (Ps H) displayed some strong cytotoxicity to mouse lung cancer cells, and could inhibit lung adenocarcinoma growth and migration in LA795 cells and inhibit tumor growth and pulmonary metastasis in forms of metastases and tumor embolus [10]. Although PPC and PPY are the only two legal species of Rhizoma Paridis, other *Paris* species including *Paris fargesii* var. *brevipetala* (PFB) and *Paris forrestii* have been widely cultivated in China to meet the shortage of *Paris* resource. Chemical characteristics of saponins from PFB [11] were identified and Ps H, the predominant saponin of PFB (> 50%), was tested *in vitro* to evaluate its cytotoxic activities on HepG2, A549, RPE and L929 cells with a positive control of Cisplatin; Ps H showed a remarkable cytotoxic activity on A549 cells with an IC50 value of 1.53 μM, and on HepG2 cells with IC50 value of 5.93 μM. Very recently, Ps H from *P. forrestii* [7], a substitute of PPY, was claimed to show significant inhibitory activity against the growth of five cancer cell lines (HL-60, SMMC-7721, A549, MCF-7, and SW480); moreover, Ps H took part in the suppressive effect of total saponins on mouse hepatocellular carcinoma H22 tumor growth through intraperitoneal administration in mice. Here, the role of Ps H in HCC was investigated, and the results were that Ps H suppressed cell viability, EMT and invasion, and contributed to cell apoptosis in PLC/PRF/5 and Huh7 cells. In addition, Ps H displayed tumor cytotoxicity with IC50 value of 6.01 μM in PLC/PRF/5 cells and 6.47 μM in Huh7 cells. Moreover, the PLC/PRF/5-induced tumor growth of human hepatocellular carcinoma in nude mice was inhibited. Notably, Ps H probably shows tumor cytotoxicity without adverse effect to body growth. The weight of spleen and final body did not vary among control group and experimental group in LA795 tumor-bearing mice xenografts [10]. In this study, we monitored the body weight of PLC/PRF/5 tumor-bearing mice per 3 d from the day of inoculation till the end of the xenograft experiment. There was no significant difference between the con-

trol group, Ps H groups and the DOX group, meanwhile, the tumor volume and weight were inhibited in Ps H groups and DOX group, indicating the antitumor activity of Ps H *in vitro* and *in vivo* with no harm to other organs and the body.

Wnt/β-catenin pathway may be a promising target for future HCC therapies. Aberrant Wnt/β-catenin signaling has been shown to be common in HCC tumors and to have significant clinical impact on tumor behavior, prognosis, and response to treatment. Combretastatin A-1 phosphate (CA1P) is currently being studied as a promising treatment for HCC, and CA1P is believed to exert its antitumor activity through inactivation of AKT, which in turn inhibited the Wnt/β-catenin pathway [22]. FH535 is another small-molecule targeted therapy that is being researched in the treatment of HCC and hepatoblastoma [23-25]. This agent was shown to inhibit the proliferation by downregulating peroxisome proliferator-activated receptor (PPAR) and β-catenin/LEF/TCF pathway. To date, there are two candidate agents that specially target the Wnt/β-catenin signaling pathway in clinical phase I/II trials to study solid tumors and myeloid malignancies, such as PRI-724 and OMP-18R5 [26-29]. β-catenin is involved in the development and progression of HCC, but its role in prognostication is less clear [30]. It was uncovered that β-catenin mutation was more frequently in earlier stages (I and II) of HCC than in more advanced stages (III and IV) [30-32]. Mutation of β-catenin takes a role in prognosis of HCC. It was shown that an accumulation of mutated β-catenin [12, 33] in the nucleus implied a better 5-year survival than increased levels of wild-type β-catenin in the nucleus; cytoplasmic and membranous β-catenin expression is associated with a poorer prognosis. In the present study, β-catenin expression was downregulated *in vitro* and *in vivo* by Ps H, which exerted cytotoxicity in HCC tumors and cells. With further functional experiments, we observed that knockdown of β-catenin by ectopic expression of special siRNA could mimic Ps H-induced antitumor role in PLC/PRF/5 cells through decreasing cell viability, migration and invasion, and increasing apoptosis.

In conclusion, Ps H suppresses cell viability, migration, and invasion, and promotes apoptosis in PLC/PRF/5 and Huh7 cells. In addition, Ps H inhibits the expressions of β-catenin, p-GSK-3β and GSK-3β; moreover, β-catenin knockdown inhibits HCC progression in PLC/PRF/5 cells, suggesting the antitumor role of Ps H in HCC is mediated, at least, by β-catenin downregulation. Finally, the growth of PLC/PRF/5-induced tumors *in vivo* is slowed down by Ps H administration. These findings suggest the anticancer effect of Ps H, thereby providing a potent candidate for therapy of HCC.

Acknowledgements

This study was supported by: 1. Sichuan Science and Technology Progam (2019YFS0190, 2019YFN0096, 2018NZZJ011, 2017RZ0051, 2016JY0081). 2. The National Key R&D Program of the Ministry of Science and Technology of China (2017YFC1700705). 3. The Sichuan Science and Technology Research Project of Traditional Chinese Medicine (2018PC011, 2017PC002). 4. The Sixth Training Session for Academic Experience Inheritance of National Senior Chinese Medicine Experts, and National Talent Training Program for Inheritance of Traditional Chinese Medicine, sponsored by the State Administration of Traditional Chinese Medicine. 5. Sichuan Basic Scientific Research Project of Public Research Institutes (A-2018N-25, A-2019N-19).

Disclosure of conflict of interest

None.

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