Saponins isolated from *Schizocapsa plantaginea* inhibit human hepatocellular carcinoma cell growth *in vivo* and *in vitro* via mitogen-activated protein kinase signaling

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[ABSTRACT] The underground cane of *Schizocapsa plantaginea* (Hance) has long been used by Chinese ethnic minority as a constituent of anti-cancer formulae. Saponins are abundant secondary metabolic products located in the underground cane of this plant. The potential therapeutic effects of total saponins isolated from *Schizocapsa plantaginea* (Hance) (SSPH) on human hepatocellular carcinoma (HCC) were tested *in vitro* in human liver cancer cell lines, SMMC-7721 and Bel-7404. Apoptosis and cell cycle arrest were determined using flow cytometry, caspase activation was determined by ELISA, and PARP, cleaved PARP, mitogen-activated protein kinase (MAPK) expression and phosphorylation were measured using Western blotting analysis. *In vivo* anti-HCC effects of SSPH were verified in nude mouse xenograft model. SSPH exerted markedly inhibitory effect on HCC cell proliferation in time- and concentration-dependent manner. Moreover, SSPH significantly induced apoptosis through caspase-dependent signaling and arrested cell cycle at G2/M phase. These anti-proliferation effects of SSPH were associated with up-regulated phosphorylation of extracellular signal-regulated kinase-1/2 (Erk1/2) and c-jun-NH2-kinase-1/2 (JNK1/2) and reduced phosphorylation of p38MAPK. Furthermore, inhibitors of ERK, UO126, and JNK, SP600125 inhibited the anti-proliferation effects by SSPH, suggesting that Erk and JNK were the effector molecules in SSPH induced anti-proliferative action. During *in vivo* experiments, SSPH was found to inhibit xenograft tumor growth in nude mice, with a similar mechanism *in vitro*. Our study confirmed that SSPH exerted antagonistic effects on human liver cancer cells both *in vitro* and *in vivo*. Molecular mechanisms underlying SSPH action might be closely associated with MAPK signaling pathways. These results indicated that SSPH has potential therapeutic effects on HCC.

[KEY WORDS] Saponins; Cancer; Liver; MAPK; *Schizocapsa plantaginea*

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**Introduction**

Hepatocellular carcinoma (HCC) is one of the most common causes of solid cancer mortality. Current nonsurgical treatments, such as chemotherapy and radiotherapy, have demonstrated some effectiveness in treating patients who are diagnosed early. However, for most HCC patients, no therapy is curative [1]. In fact, developing safe and effective treatment of HCC is particularly challenging because of an array of patient-specific (medical comorbidities), tumor-specific (size, number, location, and vascular involvement), and liver-specific (parenchymal reserve) variables [2]. In previous studies, many active ingredients extracted from traditional Chinese medicine (TCM) could reduce toxicity of chemotherapy and radiotherapy and have shown synergistic effects, which relieves advanced cancer symptoms, improves quality of life and extends long-term survival [3]. TCM and natural products provide another avenue for discovery of HCC therapeutic agents. *Schizocapsa plantaginea* (Hance) is a common traditional medicine belonging to the *Taccaceae* family and incorporated into TCM anticancer formulae, indicating that this plant may possess anti-tumor properties [4-5]. Extensive investigations on the *Taccaceae* family have uncovered two types of potential active compounds, saponins and non-saponins [6]. The anticancer properties of non-saponins compounds have been
proven \[7\], but the anticancer effect of saponins compounds and underlying mechanism are poorly understood. In previous studies, total saponins harvested from Hance (SSPH) display potent cytotoxic activity in a variety of cancer cells \[6\]. Additional studies have revealed that plantagiolides are capable of inhibiting HepG2 cells and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), an important cancer target \[8\]. In our previous study, ethanol extract of *Schizocapsa plantaginea* (Hance) showed significant anti-HCC effect both in vivo and in vitro \[9\]. Collectively, these results imply the potential of SSPH as an anti-HCC agent. However, in vivo and in vitro anticancer effects of SSPH, as well as their underlying mechanisms, need further exploration.

The occurrence and development of HCC are closely associated with rapid tumor proliferation and metastasis. Several molecular pathways are involved in human HCC pathogenesis, including the MAPK signaling pathway, one of the most common anticancer targets \[10\]. More recently, many saponin compounds have been reported to induce apoptosis and cell cycle arrest in HCC cells by targeting MAPK signal pathway. For instance Chen *et al.* have shown that saponins from *Rhzoma paridis* inhibit HepG2 xenograft tumor by regulating PI3K/Akt and MAPK signal pathways \[11\]. Another study has demonstrated that Giganteaside D induces apoptosis in HCC cell line by regulating Erk1/2 and JNK \[12\]. Thus, screening of saponin compounds targeting MAPK is both effective and necessary.

In a previous study, we have concentrated the bio-active saponin fraction from *Schizocapsa plantaginea* (Hance) using bioassay-guided fractionation technology \[13\]. However, the effects of SSPH in HCC cancer remain unknown. The present study was designed to investigate the anti-HCC effects of SSPH both in vitro and in vivo and explore the underlying mechanisms of action.

### Materials and Methods

#### Preparation of total saponins from *Schizocapsa plantaginea* (Hance)

*Schizocapsa plantaginea* (Hance) tubers were collected from Guali town, Ziyuan County, Guangxi, China and identified by Prof. HUANG Yun-Feng (Guangxi Institute of Chinese Medicine & Pharmaceutical Science, Guangxi, China). A voucher specimen designated as EP No. 140729 was kept in the Pharmacology College of Guangxi Medical University.

SSPH was extracted as previously described \[13\]. Briefly, medicinal materials were dried in shade, ground, and then extracted in three-fold volume of 70% ethanol for 2 h. The solution was filtered, concentrated, and then dissolved in water. The water solution was partitioned with petroleum ether, ethyl acetate, and *n*-butanol. The *n*-butanol fraction and solute were dried in 50-fold volume of 50% ethanol, slowly flowing through 25-fold volume in a D101 Macroreticular column. The column was eluted using 150-fold volume of 50%, 60%, and 100% ethanol, respectively. The 100% ethanol elution was collected and dried, yielding dry total saponins (SSPH), which was dissolved in DMSO at a final concentration of 50 mg·mL$^{-1}$ and stored in –20 °C until use.

#### Analysis of chemical constituents in SSPH

SSPH was determined by Molish test, Liebermann-Burchard, Salkowski reaction, and other chemical constituents test reactions, respectively (Table 1).

#### Spectrophotometric analysis of SSPH

Standard curve preparation

Diosgenin (10, 12.5, 15, 17.5, 20, 22.5, and 25 μg) was dissolved in 2 mL of ethyl acetate and then mixed with 1 mL of reagent A (containing 0.5 mL of anisaldehyde and 99.5 mL of ethyl acetate) and 1 mL of reagent B (sulphuric acid : ethyl acetate = 1 : 1). After stirring, the mixtures were incubated at

### Table 1 Chemical constituents test for SSPH

<table>
<thead>
<tr>
<th>Chemical constituents</th>
<th>Reaction</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molish test</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Fehling reaction</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Liebermann-Burchard test</td>
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<tr>
<td>Salkowski test</td>
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<tr>
<td>Foam test</td>
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<td>++</td>
</tr>
<tr>
<td>Drageordoff reagent</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Nessler's reagent</td>
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<td>Bertrand reagent</td>
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<td>Lead acetate test</td>
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<tr>
<td>Magnesium and hydrochloric acid test</td>
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<tr>
<td>Magnesium acetate test</td>
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<td>Ferric chloride test</td>
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<td>Kedde reagent</td>
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<td>Baljet reagent</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Hydroxamic acid iron reaction</td>
<td>–</td>
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</tbody>
</table>

\+Positive; \-Negative
60 °C for 20 min, and then allowed to cool down for 10 min in a water-bath maintained at room temperature. The absorbance was measured at 430 nm. Each independent experiment was repeated twice.

**Determination of saponins**

SSPH (20 μg) was dissolved in methanol in the test tube, which was placed in a boiling water bath at 100 °C to remove the alcohol and, after cooling, 2 mL of ethyl acetate was added. The analysis was carried out as aforementioned standard curve preparation. Each independent experiment was repeated twice.

**Cells, antibodies, and reagents**

Human liver cancer cell lines SMMC-7721 and Bel-7404 were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were inoculated in Dulbecco’s modified Eagle’s medium (DMEM) (Solarbio, Beijing, China) containing 15% fetal bovine serum (FBS) (Gibco®, Life Technologies, Carlsbad, CA, USA) in a 37 °C incubator containing 5% CO₂ and saturated humidity. The SMMC-7721 and Bel-7404 cells at logarithmic growth phase were seeded into 6-well plates (1 × 10⁶ cells/well). After 12 h, various concentrations of SSPH (0, 2, 5, and 10 μg·mL⁻¹) were added. The cells were trypsinized 24 h after SSPH treatment, re-suspended and washed with PBS. The concentration of re-suspended cells was adjusted to 2 × 10⁶ cells/mL. 0.5 mL of the re-suspended cells were added into 2.5 mL cold absolute ethyl alcohol (75%), and placed at 4 °C for 12 h. The cells were washed with PBS twice, and DNA PREP stain were added according to manufacturer’s instructions (Beckman, CA, USA). The SMMC-7721 and Bel-7404 cells were treated with SSPH, Anisomycin (a p38 MAPK activator), UO126 (an Erk1/2 inhibitor) and SP600125 (a JNK inhibitor), respectively. After treatment, the cells were collected and washed twice with chilled PBS. Lysis buffer with 1% protein phosphatase inhibitor was added (100 μL per 1 × 10⁶ cells) and the samples were suspended, placed on ice for 30 min, and then centrifuged at 12 500 g for 10 min at 4 °C. The supernatant was collected and total cellular protein was extracted and measured using the BCA method. Next, 20 μL of loading buffer was added into 80 μL of total cellular protein, then placed in boiling water for 5 min. Samples with equal amount of total protein were loaded onto a stacking gel and then electrophoresed at 80 V for 30 min. The samples were then electrophoresed in a separation gel at 120 V for more than 90 min, until the bromophenol blue dye reached the gel bottom. Separated proteins were transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA) for 2 h at 200 mA, and then blocked with 5% milk at room temperature for 1 h. Primary and secondary antibodies were diluted in Tris-buffered saline with Tween-20 (TBST). Primary antibodies were incubated at 4 °C overnight with shaking. Secondary antibodies were incubated at room temperature for 2 h. All immunoblots were visualized by electronic chemiluminescence and then digitally scanned. The levels of target proteins were normalized by corresponding GAPDH levels.

**Cell viability assay**

Liver cancer cells SMMC-7721 and Bel-7404 at logarithmic growth phase were trypsinized by 0.25% trypsin, re-suspended in DMEM with 15% FBS, and seeded into a 96-well plate (1 × 10⁴ cells/well). The cell viability was evaluated using the MTT assay.

**Clonogenic assay**

The SMMC-7721 and Bel-7404 cells were re-suspended and washed twice with chilled DMEM. The cells were cultured in normal growth medium for 2 weeks. Then, the cells were fixed in 10% formaldehyde for 10 min and stained with Giemsa (KeyGEN, Nanjing, China). Clonogenic counts were observed under an inverted microscope.

**Apoptosis analysis**

The SMMC-7721 and Bel-7404 cells at logarithmic growth phase were seeded into 6-well plates (1 × 10⁶ cells/well). After 12 h, SSPH was added to final concentrations of 0, 2, 5, and 10 μg·mL⁻¹. The cells were trypsinized 24 h after SSPH treatment and washed with phosphate-buffered saline (PBS) thrice. The cells were re-suspended in binding buffer (100 μL/well) and Annexin V-PE (2 μL) was added to the cell suspension, which was then mixed and incubated for 15 min on ice in the dark. After incubation, binding buffer (100 μL/well) and 7-AAD (2 μL/well) were added before flow cytometry analysis. The assay was repeated thrice.

**Caspase activity assays**

The SMMC-7721 and Bel-7404 cells were treated with different concentrations of SSPH (0, 2, 5, and 10 μg·mL⁻¹) for 24 h and collected. Caspase-3, -8 and -9 expressions were measured using a Caspase Activity Assay Kit according to manufacturer’s instructions (Beyotime, Beijing, China). Absorbance was measured at 405 nm using a microplate reader.

**Cell cycle analysis**

The SMMC-7721 and Bel-7404 cells in logarithmic growth phase were seeded into 6-well plates (1 × 10⁶ cells/well). After 12 h, various concentrations of SSPH (0, 2, 5, and 10 μg·mL⁻¹) were added. The cells were trypsinized 24 h after SSPH treatment, re-suspended and washed with PBS. The concentration of re-suspended cells was adjusted to 2 × 10⁶ cells/mL. 0.5 mL of the re-suspended cells were added into 2.5 mL cold absolute ethyl alcohol (75%), and placed at 4 °C for 12 h. The cells were washed with PBS twice, and cell cycle distribution was measured by flow cytometry analysis.

**Western blot assays**

The SMMC-7721 and Bel-7404 cells were treated with SSPH, Anisomycin (a p38 MAPK activator), UO126 (an Erk1/2 inhibitor) and SP600125 (a JNK inhibitor), respectively. After treatment, the cells were collected and washed twice with chilled PBS. Lysis buffer with 1% protein phosphatase inhibitor was added (100 μL per 1 × 10⁶ cells) and the samples were suspended, placed on ice for 30 min, and then centrifuged at 12 500 g for 10 min at 4 °C. The supernatant was collected and total cellular protein was extracted and measured using the BCA method. Next, 20 μL of loading buffer was added into 80 μL of total cellular protein, then placed in boiling water for 5 min. Samples with equal amount of total protein were loaded onto a stacking gel and then electrophoresed at 80 V for 30 min. The samples were then electrophoresed in a separation gel at 120 V for more than 90 min, until the bromophenol blue dye reached the gel bottom. Separated proteins were transfered onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA) for 2 h at 200 mA, and then blocked with 5% milk at room temperature for 1 h. Primary and secondary antibodies were diluted in Tris-buffered saline with Tween-20 (TBST). Primary antibodies were incubated at 4 °C overnight with shaking. Secondary antibodies were incubated at room temperature for 2 h. All immunoblots were visualized by electronic chemiluminescence and then digitally scanned. The levels of target proteins were normalized by corresponding GAPDH levels.

**Vaccination of nude mice**

All experimental procedures involving animals were approved by the Medical Ethics Committee of the Guangxi Medical University, Nanning, China. The SMMC-7721 cells in logarithmic phase were suspended in DMEM without serum. 200 μL of cell suspension (containing 5 × 10⁶ cells) was subcutaneously injected into the right axillary of the nude mouse.
mice. After tumor volume reached 200 mm³, the nude mice were gavaged with SSPH (0, 10, 25 or 50 mg·kg⁻¹) or received intraperitoneal injection of taxol (15 mg·kg⁻¹) as positive control every day. Each dosage group included 4 male and 4 female mice. 0.5 mL of 1% DMSO was injected into each mouse and served as negative control. Tumor growth was observed every two days. Fifteen days after treatment, all the mice were sacrificed by cervical vertebral dislocation. Tumor tissues were dissected, weighed, and fixed in 10% formalin for subsequent pathological analysis.

**Immunohistochemistry analysis**

The xenograft tumors were deparaffinized and hydrated through graded alcohols. The sections were heated in citrate buffer (pH 6.0) for 1.5 min at 120 °C to retrieval the antigen. The sections were incubated in 3% hydrogen peroxide in PBS for 10 min and blocked in 10% goat serum in PBS for 10 min at room temperature. The sections were incubated with primary antibody at 4 °C overnight. After washing, the sections were incubated with biotinylated secondary antibody at 37 °C for 10 min. The sections were incubated with DAB reagent and stained with Hematoxylin. The sections were observed under a microscope and images were scanned. The sections were scored based on the percentage of positive cells (0, < 5%; 1, 6%–50%; 2, 26%–50%; and 3, > 50%) and staining intensity (0, negative; 1, weak; 2, moderate; and 3, strong).

**Statistical analysis**

The data were expressed as means ± SD or percentages. All the experimental data were analyzed with SPSS 13.0 statistical software (SPSS Inc., Chicago, IL, USA). Comparison between various groups was carried out using one-way analysis of variance. \( P < 0.05 \) was considered statistically significant.

**Results**

**The major chemical constituents in SSPH are saponins**

As shown in Table 1, the main chemical constituents of SSPH were saponins. There were no positive reactions for other kinds of chemical compounds. The spectrophotometric analysis of SSPH showed that the amount of saponins in SSPH was 85.31%.

**SSPH inhibits growth of liver cancer cells**

After human liver cancer cell lines, SMMC-7721 and Bel-7404, were treated with different concentrations of SSPH for 24, 48 and 72 h, growth inhibition occurred in a time- and concentration-dependent manner (Fig. 1A). The IC₅₀ values for SMMC-7721 cells at 24, 48 and 72 h were 3.75, 1.56, and 0.75 μg·mL⁻¹, respectively; the IC₅₀ values for Bel-7404 cells at 24, 48 and 72 h were 3.92, 1.77, and 1.10 μg·mL⁻¹, respectively.

Fig. 1  SSPH inhibits the proliferation of human HCC SMMC-7721 and Bel-7404 cells. (A), the SMMC-7721 and Bel-7404 cells were treated with different concentrations of SSPH for 24, 48 and 72 h. SSPH inhibited SMMC-7721 and Bel-7404 cells in a time- and concentration-dependent manner. Each concentration was tested in quadruplicate. The results are shown as means ± SD. (B), The effects of SSPH on cell clonogenic survival. The cells were treated with 2, 4 or 6 μg·mL⁻¹ of SSPH for 4 h and then incubated for 2 weeks. (C), the colony counts of different treatment groups. 4 and 6 μg·mL⁻¹ of SSPH significantly reduced the colony counts of the SMMC-7721 and Bel-7404 cells. Each concentration was tested in triplicate. The results are shown as means ± SD (\( * P < 0.01 \) vs control group)
SSPH inhibits colony formation of liver cancer cells

As hepatocellular carcinoma cells demonstrate high growth rates, colony formation is an important parameter reflecting the extent of tumor malignancy [15]. We designed a clonogenic assay to observe short-term effects of SSPH on SMMC-7721 and Bel-7404 cells. To avoid cell loss by long-term exposure to SSPH, we terminated the treatment at 4 h post application of SSPH, and continuously maintained the cells until colony formation. The concentrations of SSPH we used were closely relevant to its clinical dosage [10]. As shown in Fig. 1B, SSPH at 4 and 6 μg·mL⁻¹ could significantly reduce colony counts (P < 0.01), and the inhibition rate was up to 83.37% in SMMC-7721 cells and 89.02% in Bel-7404 cells when 6 μg·mL⁻¹ of SSPH was applied.

SSPH induces apoptosis in liver cancer cells

We employed a PE/7-AAD double stain flow cytometry method to determine the apoptosis of the cells, after treatment with SSPH at various concentrations. Our data showed that SSPH could induce apoptosis in both SMMC-7721 and Bel-7404 cells in a concentration-dependent manner (P < 0.01; Fig. 2A). SSPH at 2 μg·mL⁻¹ could trigger early apoptotic event in SMMC-7721 cells but not Bel-7404 cells and higher concentrations resulted in necrosis in SMMC-7721 cells and late apoptosis in Bel-7404 cells. Therefore, SSPH exerted its anti-tumor functions by induction of apoptosis in Bel-7404 cells while trigger of necrosis and apoptosis in SMMC-7721 cells (Fig. 2B).

Fig. 2  SSPH induces apoptosis in SMMC-7721 and Bel-7404 cells. (A), Flow cytometry analysis of cell apoptosis. The SMMC-7721 and Bel-7404 cells were treated with 0, 2, 5, and 10 μg·mL⁻¹ of SSPH for 24 h, and each independent experiment was repeated 3 times. SSPH increased early apoptosis at 2 μg·mL⁻¹, and late apoptosis and necrosis at 10 μg·mL⁻¹ in SMMC-7721 cells. SSPH induced early and late apoptosis in Bel-7404 cells in a concentration-dependent manner. The results are shown as means ± SD (*P < 0.01 vs control group; #P < 0.01 vs 5 and 10 μg·mL⁻¹ dosage; ^P < 0.01 vs 2 μg·mL⁻¹ dosage). (B), Representative results. Early apoptotic cells were defined as PE-positive, 7-AAD-negative cells; Necrosis cells were defined as 7-AAD-positive, PE-negative cells; Late apoptotic cells were defined as 7-AAD- and PE-positive cells

SSPH induces cell cycle arrest in liver cancer cells

To reveal the influence of SSPH on cell cycle, flow cytometry assays were employed after treating cells with different concentrations of SSPH for 24 h. We found that the
percentage of SMMC-7721 and Bel-7404 cells in G2/M phase gradually increased along with the increase of SSPH concentration. Additionally, the percentage of SMMC-7721 cells in S phase was slightly increased. SSPH also caused a Sub-G1 phase increase at 5 and 10 μg·mL\(^{-1}\) (Fig. 3A). Thus, we inferred that most cells were arrested at G2/M phase (Fig. 3). **SSPH activates caspase cascade and causes PARP cleavage in liver cancer cells**

Since caspase cascade plays central roles in apoptosis, we next investigated whether SSPH could alter the expression of caspases. Our data showed that SSPH treatment increased the expression of caspase-3, -8 and -9 in both liver cancer cells. Activation of these caspases occurred in a concentration-dependent manner, indicating that SSPH-induced apoptosis may be associated with caspase signaling (Fig. 4A). Cleaved PARP is a marker of apoptosis. SSPH caused PARP cleaved at 5 and 10 μg·mL\(^{-1}\), verifying that SSPH induced apoptosis in SMMC-7721 and Bel-7404 cells (Fig. 4B).

**SSPH regulates MAPK pathway in vitro**

To analyze molecular mechanisms of SSPH-induced inhibition, Western blot assays were performed to evaluate the total level and phosphorylation level of key proteins in MAPK signaling pathway (Fig. 4). Compared with the control group, phosphorylation level of Erk1/2, JNK1/2 in both cell lines were significantly increased (\(P < 0.01\)) by SSPH treatment while the phosphorylation level of p38 were significantly reduced (\(P < 0.01\); Fig. 4C). The total level of MAPK expression was not influenced.

**SSPH inhibits SMMC-7721 and Bel-7404 cells proliferation through MAPK signaling pathway**

Based on our preliminary data, it seemed that MAPK signaling pathway interfered in SSPH-mediated anti-proliferation in SMMC-7721 and Bel-7404 cells, we further employed UO126, SP600125, and anisomycin to manipulate MAPK pathway. As shown in Fig. 5, UO126 and SP600125 abolished SSPH-induced phosphorylation of Erk1/2 and JNK. Anisomycin reversed the decrease of p-P38 induced by SSPH. Pre-condition of liver cancer cells with UO126 and SP600125 could
Fig. 4  SSPH regulates the protein expression and phosphorylation in SMMC-7721 and Bel-7404 cells. (A), SSPH induces activation of the caspase family in SMMC-7721 and Bel-7404 cells. The SMMC-7721 and Bel-7404 cells were treated with 2, 5 or 10 μg·mL\(^{-1}\) of SSPH for 24 h, demonstrating significantly increased caspase activity. Each independent experiment was repeated three times. The results are shown as means ± SD (\(P < 0.01\) vs control group). (B), SSPH induces PARP cleavage in SMMC-7721 and Bel-7404 cells. The SMMC-7721 and Bel-7404 cells were treated with 2, 5 or 10 μg·mL\(^{-1}\) of SSPH for 24 h. Cleaved PARP was observed after 5 or 10 μg·mL\(^{-1}\) SSPH treatment, indicating that SSPH induced apoptosis in SMMC-7721 and Bel-7404 cells. (C), the histogram shows the protein phosphorylation levels. The phosphorylation level of Erk1/2 and JNK were significantly increased (\(P < 0.01\)), and the phosphorylation level of p38 was significantly decreased (\(P < 0.01\)). Each independent experiment was repeated three times. The results are shown as means ± SD (\(P < 0.01\) vs control group; \(P < 0.01\) vs 3 μg·mL\(^{-1}\) group). (D), SSPH regulates MAPK signaling pathway in SMMC-7721 and Bel-7404 cells. SMMC-7721 and Bel-7404 cells were treated with 0, 3, 6 and 9 μg·mL\(^{-1}\) of SSPH for 6 h. Western blot analysis was used to determine the expression and phosphorylation level of Erk1/2, JNK1/2 and p38MAPK. GAPDH was used as a loading control. SSPH increased the phosphorylation of Erk1/2 and JNK1/2, but decreased the phosphorylation level of p38MAPK. SSPH showed no influence in Erk1/2, JNK1/2 or p38MAPK expression.
Fig. 5  The regulatory effects of SSPH combined with Anisomycin, UO126, or SP600125 on MAPK signal pathway. (A), the histograms show the protein phosphorylation levels. The UO126 and SP600125 treatments abolished the SSPH-induced phosphorylation in Erk1/2 and JNK. Treatment with Anisomycin abolished the SSPH-induced p-p38 decreased. Each independent experiment was repeated three times. The results are shown as means ± SD (* P < 0.01 vs control group). (B), Western blot analysis detected proteins expression. The SMMC-7721 and Bel-7404 cells were treated with UO126 (10 μmol·L⁻¹), SP600125 (20 μmol·L⁻¹) and Anisomycin (0.2 μmol·L⁻¹) for 1 h prior to exposure to additional SSPH (6 μg·mL⁻¹) for a further 6 h. GAPDH was used as a loading control. (C), UO126 and SP600125 blocked SSPH’s anti-proliferation effect. SMMC-7721 and Bel-7404 cells were treated with UO126 (10 μmol·L⁻¹), SP600125 (20 μmol·L⁻¹), or Anisomycin (0.2 μmol·L⁻¹) for 1 h prior to exposure to additional SSPH (6 μg·mL⁻¹) for a further 24 h. Each treatment had quadruplicate wells. The results are shown as mean ± SD. (D), UO126 and SP600125 blocked SSPH’s cleaved PARP induction effect. The SMMC-7721 and Bel-7404 cells were treated with UO126 (10 μmol·L⁻¹), SP600125 (20 μmol·L⁻¹) and Anisomycin (0.2 μmol·L⁻¹) for 1 h prior to exposure to additional SSPH (6 μg·mL⁻¹) for a further 24 h. Anisomycin showed no influence on SSPH induced PARP cleavage.
restore the growth inhibition by SSPH (Fig. 5C). In addition, UO126 and SP600125 could block the SSPH induced PARP cleaved (Fig. 5D). Our results indicated that Erk1/2 and JNK played important roles in SSPH-mediated liver cancer cell growth inhibition.

SSPH inhibits xenograft tumor growth in nude mice

After treatment with varying doses of SSPH, we found both tumor volume and weight were significantly decreased in a dose-dependent manner, compared with the vehicle control. Positive control group demonstrated a significant inhibition of tumor volume and weight. Further, tumor volume in the high dosage group was significant smaller than low dosage ($P < 0.01$). The inhibition levels of tumor weight and volume between positive groups and SSPH treated group either at high or middle dosage did not show significant difference ($P > 0.1$; Figs. 6B and 6C). Thus, we inferred that SSPH had similar potency to Taxol at high and middle dosages.

SSPH regulates MAPK signaling pathway in nude mice

Taxol caused a significantly activation of p38MAPK, JNK and Erk1/2 in xenograft tumors. As shown in Fig. 7, positive cells and staining intensity were significantly increased.

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Fig. 6  SSPH inhibits tumor growth in a nude mouse xenograft model. (A), Photo of xenograft tumors in different groups. (B), Nude mice were gavaged with SSPH at different dosages (10, 25 or 50 mg·kg$^{-1}$) or administered with intraperitoneal injection of Taxol (15 mg·kg$^{-1}$) every day for 15 days, the volume of tumors was then measured every two days. The high and middle dosages of SSPH and positive group significantly inhibited the tumor volume; SSPH showed dosage-dependent inhibitory effects. The results are shown as means ($^*P < 0.01$ vs control group; $^#P < 0.01$ vs low dosage group). (C), Weight of tumors at end of administration. High and middle dosages of SSPH and positive group showed significant inhibition of tumor weight at end of administration. The results are shown as means ± SD, $n = 8$($P < 0.01$ for high dosage of SSPH and positive group, $P < 0.05$ for middle dosage of SSPH; $^*P < 0.01$ vs control group; $^#P < 0.05$ vs control group). (D), Body weight of mice during the administration. There were no significant differences in body weight among different groups ($n = 8$)
SSPH showed similar activation effect as Taxol in JNK and Erk1/2 signal pathway; SSPH at high dosage and Taxol significantly activated JNK and Erk1/2 \((P < 0.01)\), but a p38MAPK inactivation was observed after SSPH treatment. These results indicated that SSPH had similar mechanism both \textit{in vivo} and \textit{in vitro}.

**Discussion**

\textit{Schizocapsa plantaginea} (Hance) belongs to the \textit{Taccaceae} family and is widely applied in traditional Chinese medicine (TCM). TCM prescriptions use this plant as one of the constituents of anti-cancer formulae. However, the bioactivity of saponins found in \textit{Schizocapsa plantaginea} (Hance) is rarely mentioned. Our present study examined potential anti-HCC effects of SSPH both \textit{in vitro} and \textit{in vivo}.

The bioactivity saponins fractions from \textit{Schizocapsa plantaginea} (Hance) (SSPH) were separated by D101 macroporous resin \cite{16}. SSPH significantly inhibited the growth of hepatocarcinoma cell lines SMMC-7721 and Bel-7404 in a time- and dose-dependent manner. Short-term treatment with SSPH produced long-term growth inhibition. Our results indicated SSPH could efficiently suppress HCC cell proliferation.

Overgrowth and metastasis represent the two major characteristics of HCC, multiple kinds of saponins inhibited HCC cells proliferation by inducing apoptosis. Studies in Paris saponins showed that pennogenyl saponins I and II caused caspase-8-dependent apoptosis in Hela cell \cite{17}; another...
saponin Dioscin caused HCC cells Bel-7402, SMMC-7721 and HepG2 apoptosis by activating caspase-3, 8 and 9 [18]. We for the first time reported the effects of SSPH on HCC cell apoptosis and cell cycle distribution. Our data showed the number of cells undergoing late apoptosis kept increasing along with dosage boosting, indicating the capability of SSPH in induction of apoptosis. The mitochondrial-dependent apoptosis pathway involves the sequential activation of caspase-9 and -3 [19]. In the present study, caspase-9 and -3 expression levels increased in SMMC-7721 cells after exposure to SSPH, reflecting that mitochondrial apoptotic pathway was activated in SSPH-induced apoptosis, and cleaved PARP induced by SSPH also verified that SSPH induced apoptosis in SMMC-7721 and Bel-7404 cells. Furthermore, SSPH arrested cell cycle at G2/M phase and slightly increased the cell number at G1 phase, and an increasing sub-G1 phase. Collectively, these results suggested SSPH significantly inhibited HCC cells in vitro and triggered apoptosis.

The P38 and JNK MAPK pathways are often activated by many factors, like radiation, cytotoxic drugs, and UV. In most HCC cells, MAPK is often overexpressed or phosphorylated, indicating its pivotal roles in HCC proliferation and apoptosis [20-21]. However, applying MAPK-targeted drugs requires actual tumor type analysis due to crosstalk between descending pathways of p38MAPK, JNK, and Erk1/2 [10]. To further analyze molecular mechanisms underlying the anti-HCC effects of SSPH, we performed Western blot to investigate the alteration of MAPK signaling pathway, which is widely proven to mediate cell proliferation and apoptosis.

SSPH-induced JNK phosphorylation played important roles in HCC inhibition. JNKs belong to the MAP kinases superfamily and participate in the regulation of cell proliferation, differentiation, and apoptosis. JNK1 and JNK2 are essential signaling molecules for normal cell physiology, and deregulation of JNK signaling pathway occurs frequently in human cancers. Studies have shown different kinds of saponins exert anticancer effect by activating JNK. A ginseng saponin, compound K, mediates generation of ROS, leading to apoptosis through activation of p38 MAPK and JNK [22]. Ziyuglycoside II, a triterpenoid saponin, also induces cell cycle arrest and apoptosis through activation of ROS/JNK pathway in breast carcinoma cells [21]. In our study, the phosphorylation level of JNK was increased and most cells underwent early apoptosis after SSPH treatment. Suppression of JNK with specific inhibitors could attenuate hepatocytes apoptosis [24]. We applied the JNK inhibitor SP600125 to mitigate SSPH-induced JNK phosphorylation, and found the growth inhibition effect and cleaved PARP induction were compromised by JNK inhibitor, implicating that activation of JNK1/2 signaling pathway by SSPH could promote apoptosis of liver carcinoma.

p38MAPK, an important regulator of cell homeostasis, is deregulated during HCC tumor growth; its activation triggers apoptosis in HCC cells [25]. However, long-term activation of p38MAPK by hepatitis B virus (HBV) X-protein causes carcinogenesis in liver rather than induces apoptosis, indicating different functions due to different types of stimuli as well as their strength [26]. In our present study, SSPH could block the phosphorylation of p38MAPK and anisomycin could reverse SSPH-mediated inhibitory effects, but p38MAPK activator anisomycin did not block the inhibitory effect of SSPH on cell proliferation. SSPH was not a pure compound, it is necessary to separate components of SSPH and investigate the function of individual ingredient on p38MAPK activity in the future.

Previous study has shown that ERK activation facilitates cell proliferation, autophagy, and apoptosis. Lots of natural active products induce apoptosis through activation of ERK [27]. Previous study has shown that Astragalus saponins promote apoptosis through long-term activation of Erk1/2 [28]. In the present study, we provided evidence supporting that activation of ERK was important for the induction of SSPH-induced apoptosis in human liver cancer cells. Manipulation of ERK activity by SSPH promoted the apoptosis and proliferation inhibition; however, downregulation of ERK phosphorylation level by its inhibitor U0126 led to suppress of SSPH-induced apoptosis and proliferation inhibition. Phosphorylation of ERK contributed to cell apoptosis upon treatment with SSPH. Thus, SSPH exerted its anti-HCC effect through Erk1/2 signaling pathway.

Taxol is an anticancer drug demonstrating anti-HCC effects in the clinic [29]. Studies have shown that taxol causes JNK, p38MAPK and Erk activation in different cancer cell lines [30-31]. MAPK activated by taxol is also observed in different kinds of xenograft tumors [32-33]. To display the anti-HCC and MAPK regulation effects of SSPH, we utilized taxol as positive control in our nude mouse xenograft model. We established a nude mouse SMMC-7721 xenograft model to verify antitumor effects of SSPH. Taxol significantly inhibited tumor growth and activated JNK, Erk1/2, and p38MAPK signaling in nude mouse xenograft tumors. SSPH possessed the similar potency in anti-tumor growth to taxol, activated Erk1/2 and JNK1/2 and inhibited p38MAPK phosphorylation in vivo. Our study proved that SSPH inhibited xenograft growth in vivo, and immunohistochemistry stain showed SSPH may have similar mechanism in vivo as in vitro, confirming the potential of SSPH as an anti-HCC drug.

Conclusion

Our present study demonstrated that SSPH exerted anti-tumor effects both in vitro and in vivo. The anti-HCC effects of SSPH in vivo and in vitro were linked to manipulation of p38MAPK, ERK, and JNK signaling pathways. Our data indicated that SSPH might be used to target MAPK as an anti-HCC drug.

References


