# A novel cell cycle blocker extracted from *Stellera chamaejasme* L. inhibits the proliferation of hepatocarcinoma cells

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**Abstract.** Currently, liver cancer is the sixth most prevalent cancer and the third most common cause of cancer-related death. However, effective chemotherapeutic drugs with low drug resistance and few side-effects for the clinical treatment of liver cancer are lacking. Therefore, the search for novel drugs to compensate for the defects of existing drugs is urgently needed. Herein, we successfully screened an extract named from *Stellera chamaejasme* L. (SCL), a historically confimed antitumor plant, through a novel extraction platform. In the present study, we firstly screened the anticancer effect of ESC by the sulforhodamine B (SRB) cell proliferation assay in a wide range of malignant cell lines, including A549, NCI-H157, NCI-H460, SK-HEP-1 and HepG2. With the highest inhibitory rate in hepatocarcinoma cells, we further identified the tumor-suppressive efficacy and the safety of ESC in an H22 hepatocarcinoma xenograft model *in vivo*. In a mechanistic study, flow cytometry and western blot analysis were performed to evaluate the effects of ESC on the induction of cell apoptosis, intervention of cell cycle distribution and its influence on key G2/M-phase regulators. The results showed that ESC significantly inhibited the cell growth of liver cancer cell lines. Accordingly, the tumor inhibition rate was also increased following ESC administration with little systemic toxicity in H22-transplanted mice. Mechanistically, ESC caused obvious G2/M-phase arrest in both the SK-HEP-1 and HepG2 cell lines without cell apoptosis. Furthermore, cyclin B1 was downregulated, while the phosphorylation level of CDK1 was increased in response to ESC treatment. All these data confirmed that ESC possesses potent anti-proliferative efficacy for hepatocarcinoma through the induction of cyclin-mediated cell cycle arrest. Thus, ESC is a promising candidate for hepatocarcinoma treatment in the future.

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*Abbreviations:* CDKs, cyclin-dependent kinases; SCL, *Stellera chamaejasme* L.; ESC, extract of *Stellera chamaejasme* L.

*Key words:* cell cycle arrest, hepatocarcinoma, cyclin B1, CDK1 *Stellera chamaejasme* L.

## Introduction

As one of the major global public health issues, cancer greatly threatens human survival and has become the leading cause of mortality of humankind in this century (1). Clinically, during the rapid development of anticancer strategies, chemotherapy has consistently played an important role in cancer treatment. To date, classical botanical agents, as represented by paclitaxel or vincristine, are still first-line drugs and are widely applied as the primary choice against multiple malignancies (2). Benefitting from these agents, treatment effectiveness and the survival rate of cancer patients have greatly improved in recent years. However, in spite of the satisfactory tumor-suppressive activities, with their extensive application worldwide, growing evidence reveals that side-effects and drug resistance are still the leading obstacles largely limiting the clinical use of classic tumor-toxic agents (3).

In recent years, liver cancer has become one of the most prevalent types of cancer and is the third most common cause of cancer-related mortality (4,5). Notably, its incidence and mortality rank second among Chinese cancer patients (6) and liver cancer patients in China account for more than half of all cases worldwide (7).

In current clinical practice, surgical resection and liver transplantation are the most recommended treatment strategies against hepatocarcinoma (8). However, the statistics show that only a small portion of patients are suitable for these therapeutics (9), and even for those qualified patients, the recurrence rate is over 50% (10). This situation indicates that chemotherapy is one of the few remaining options for the vast majority of patients (11). Unfortunately, effective chemotherapeutic strategies for liver cancer patients are still not well established and are under extensive investigation. Therefore, the search for novel potential anticancer compounds with high specificity and sensitivity for liver cancer patients is urgently needed. During such research, natural products provide a precious reservoir with high-level chemical diversity. Thus, the

efficacy-based high-throughput screening of natural products serves as an efficient approach to drug discovery (12).

*Stellera chamaejasme* L. (SCL), a wide-spread perennial plant in Northwest China (13), was firstly recorded in Chinese medical ancient classic Sheng Nong's Herbal Classic. Historically, SCL has been applied to a variety of diseases including edema, tuberculosis, carbuncle, hemorrhoids, scrofula, scabies, and tumors over centuries (14,15). In modern times, characterized by high therapeutic efficacy and low cost, SCL shows great benefit in anticancer drug research and development and such has attracted increasing attention.

Recent pharmacologic studies have revealed that extracts from SCL, eluted with water, petroleum ether, ethyl acetate, acetone, ethanol, methanol and other solvents, possess clear antitumor effects in a wide-spectrum of malignancies (16,17). They have been confirmed to be strong inhibitors of tumor progression mainly due to unselected cytotoxic effects. Additionally, some of the extracts show higher efficiency in terms of cell death induction compared with commonly used chemotherapeutic agents. Although this promising evidence has greatly revealed its feasibility for clinical application, its material basis and the detailed pharmacological mechanism are still obscure. Particularly, limited to the isolation procedures and the establishment of disease models, current studies for SCL are merely restricted to crude extracts with little disease specificity. The targeted efficacy identification and optimization are still lacking. More importantly, most of the known extracts from SCL unexpectedly exhibit multi-organ damaging effects, mainly represented by hematological and immunological toxicities, which adds another obstacle for its clinical application.

Under such circumstances, the systematic efficacy screening, verification and the detailed pharmacological analysis for SCL are needed and will be beneficial for the comprehensive understanding of SCL during clinical application.

Based on the above analysis, our study was designed to screen and identify a novel extract from SCL with explicit material basis, high activity and low systemic toxicity. In addition, we also aimed to clarify its detailed molecular mechanism in hepatocarcinoma. Here, we report that, by screening dozens of *Stellera chamaejasme* extracts with a different polarity and optimizing the extraction process, a new fraction from SCL, named ESC, was identified with potent activities against the proliferation of multiple types of cancer cell lines, particularly SK-HEP-1 and HepG2 liver cancer cell lines. Inspired by this, we further confirmed the growth inhibitory capacity of ESC as well as its low systemic toxicity on hepatocarcinoma *in vivo*, and finally confirmed the cyclin-dependent mechanism in the regulation of cell cycle distribution. Taken together, our study experimentally identified a novel cell cycle blocker for hepatocarcinoma and provides more convincing evidence of *Stellera chamaejasme* L. as a valuable resource in the research and development of anticancer agents.

## Materials and methods

*Cell culture and reagents*. The cancer cell lines A549, NCI-H157, NCI-H460, HepG2 and SK-HEP-1 were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in RPMI-1640 or Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 1% Pen/Strep. The H22 cell line was kindly donated by Dr Lanfang Li from the Institute of Chinese Materia Medica. RPMI-1640, DMEM and fetal bovine serum were purchased from Invitrogen (Carlsbad, CA, USA). ESC was extracted and prepared by the Dalian Institute of Chemical Physics, Chinese Academy of Sciences. An HPD-100 macroporous absorbent column was produced by Cangzhou Bon Absorber Technology Co., Ltd. Sulforhodamine B (SRB) was purchased from Sigma (USA). A cell cycle and apoptosis detection kit was purchased from CWBio (Beijing, China). Primary antibodies against cyclin B1 and total-CDK1 were purchased from Boster (Wuhan, China); the phosphor-CDK1 (Tyr15) antibody was obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA); β-actin antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

*SCL extraction and ESC preparation*. *Stellera chamaejasme* L. (2 kg) was decocted thrice with ethanol at 70˚C (15 lx5 h, 7.5 lx3 h, 7.5 lx3 h). The liquids were merged and concentrated to extracts (230 g) on a rotary vacuum evaporator at 60˚C. The further purification of the extracts (222 g) was performed by means of HPD-100 macroporous absorbent column chromatography eluted with different concentrations of alcohol (40-100%). Furthermore, the fractions were concentrated on a rotary vacuum evaporator at 60˚C, dried to constant weight in vacuo and crushed into powder (ESC) before the experiments. The yield of the ESC was 32.4% (72 g).

*Cell proliferation assay*. The cell proliferation intensity was analyzed using the SRB assay. Cells were seeded onto 96-well plates (4,000 cells/well) and treated with ESC at different concentrations for 24, 48, 72 h. At the indicated time-points, the cells were fixed with 50% trichloroacetic acid (TCA) for 1 h at 4˚C. Then the wells were washed with deionized water for 5 times. After drying, 100 $\mu$l SRB was added to each well and reacted for 10 min, the unbound SRB was washed out with 1% acetic acid and then dried completely. After being dissolved in 10 mmol/l unbuffered Tris-base, the $OD_{490}$ values were detected and calculated to assess the cell proliferation level in response to drug treatment.

*H22 xenograft model*. H22 cells were passaged in the peritoneal cavity of ICR mouse. After 10 days, the mouse was sacrificed by cervical dislocation, the ascites was collected and diluted with cold sterile saline. The cell suspension was planted subcutaneously at the axillary fossa, $2x10^6$ cells/mouse. On the next day, the mice were randomly divided into 4 groups (10 mice/group), and then received an intragastric administration of ESC (135.85, 271.7, 543.4 mg/kg) and the control group was gastric transfused with an equivalent volume of distilled water for 10 days. The mice were weighed every day, and the volume of the tumors was measured every 2 days. On the 11th day of drug administration, the blood was collected and the mice were sacrificed. The tumors, spleens and thymus were dissected and weighed to calculate the tumor volume, spleen index and thymus index according to the following equations: Tumor volume = (length x width$^2$)/2 (18). The spleen (thymus) index (mg/g) = spleen (thymus) weight/body weight after tumor removal.

The number of white blood cells was detected with an automatic blood cell analyzer (Siemens Ltd., Japan).

*Histological analysis*. All of the tumor tissue was paraffin-fixed, and H&E staining was performed according to a standard protocol. The results were scored by a pathologist in a blinded manner. Tumors were classified using the WHO classification (19). Two standard morphological features of malignancy: cellular heteromorphism and apoptosis were assessed, each symbolized with -, +, ++ and +++.

*Apoptosis analysis*. HepG2 and SK-HEP-1 cells were plated onto a 6-well plate and exposed to ESC at concentrations of 25, 50 and 100 $\mu$g/ml for 24 h. Then the apoptotic rate was detected using the Annexin V-FITC/PI apoptosis detection kit. The protocol was strictly designed according to the manufacturer's instructions. Briefly, the cells were harvested and washed with PBS for 3 times and then re-suspended with 150 $\mu$l binding buffer containing 10 $\mu$l Annexin V-FITC and 5 $\mu$l PI. After incubation for 20 min in the dark, the apoptotic rates were analyzed by flow cytometry (BD FACSCalibur).

*Cell cycle analysis*. HepG2and SK-HEP-1 cells were plated into a 6-well plate and treated with 25, 50 and 100 $\mu$g/ml ESC. After 24 h, the cells were washed with PBS and trypsinized. The cell suspension of each group was centrifuged for 5 min, at 1,000 x g, and then the cells were fixed with 70% cold ethanol overnight. The fixative solution was discarded, and the cells were washed with PBS twice. Cells were re-suspended with 500 $\mu$l PBS containing 50 $\mu$g/ml PI and 50 $\mu$g/ml RNase A, incubated at 37˚C for 30 min, and then the samples were analyzed with a flow cytometer. The detailed method was previously described (20).

*Western blot analysis*. SK-HEP-1 and HepG2 cells were seeded onto 6-well plates ($1x10^5$ cells/well) and treated with different concentrations of ESC (25, 50, 100 $\mu$g/ml). The cells were next harvested after 24 h and lysed with the appropriate volume of lysis buffer (including 20 mM Tris-HCl, pH, 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF and 1% Triton X-100). Tumor tissues were collected from three randomly chosen mice in each group, and the tumor lysate samples were prepared followed the steps as previously described (21). The protein concentration for each lysate was determined using a BCA protein quantification kit (Pierce, USA). Then the denatured protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the gels were electrically blotted onto NC membranes (Pall, USA). The membranes were blocked with 5% albumin from bovine serum (BSA) and then incubated with the primary antibodies at 4˚C for 8 h. After that, the membranes were incubated with the relevant secondary antibody conjugated with HRP. The target bands were visualized using chemiluminescence detection reagents (Thermo Fisher Scientific Inc., USA). β-actin was detected as the internal control for each experiment.

*Statistical analysis*. All the quantitative data shown in this manuscript are presented as arithmetic means ± standard errors. Statistical analysis was performed with SPSS 17.0 and one-way analysis of variance (one-way ANOVA) was used to analyze the quantitation. $P<0.05$ indicates statistical significance ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$).

## Results

*Screening of the tumor-suppressive efficacy of ESC in different tumor cell lines*. Based on the historical clinical record of SCL, lung and liver cancer have been proven to be the most sensitive disease models. Based on this, we firstly examined the inhibitory effect of ESC on cell proliferation in lung cancer (A549, NCI-H157, NCI-H460) and liver cancer cell lines (HepG2, SK-HEP-1), respectively. During the efficacy screening, cells were treated with different concentrations of ESC for 24, 48 and 72 h. At each time-point, the tumor proliferative intensity was quantified by the SRB incorporative assay, and the inhibitory rates were calculated in the different groups. As expected, the results showed that ESC exerted clear but different levels of suppressive influence on all 5 types of cell lines in a time- and dose-dependent manner (Fig. 1A-E).

Additionally, the inhibitory sensitivity toward different cancers was further analyzed through calculation of the $IC_{50}$ value. The minimum $IC_{50}$ was 49.00 and 9.50 $\mu$g/ml in the SK-HEP-1 and HepG2 cells, respectively, which clearly suggested that ESC exhibited more powerful anti-proliferation activity in the liver cancer cell lines and hepatocarcinoma was determined to be the targeted cancer model for further study.

*Safety assessment of ESC application in vivo*. As mentioned previously, the multi-organ-involved toxic effect of SCL was the widely existed and severely restrictive factor for SCL application. To exclude this *in vivo*, we conducted a safety test on the liver cancer xenograft mice, which was confirmed to be the most sensitive model for ESC.

According to the results, 135.85, 271.7 and 543.4 mg/kg (equivalent to 1/10, 1/5, 1/2.5 of the $LD_{50}$ value, respectively) were chosen as a low, medium and high dose for ESC oral application. The tumor-bearing mice were randomly grouped, regularly treated and weighed. The most susceptible tissues, including peripheral blood, thymus gland and spleen, were collected after 10 days of drug administration and prepared for further detailed analysis.

The results showed that intragastric administration of a low and medium dose of ESC had little impact on body weight (Fig. 2A), and the spleen and thymus indices (Fig. 2B and C). Moreover, whole blood analysis revealed that the quantity of leukocytes was also maintained at a similar level when compared with the negative control mice (Fig. 2D). It is notable that, although the leukocyte count in the high dose group decreased to $5.05\pm1.66x10^3/mm^3$, its value was maintained within the physiological level, which is $4.0\text{-}12.0x10^3/mm^3$. The spleen and thymus indices were not obviously affected by ESC treatment. Collectively, our test clearly indicated that, different from the known extracts from SCL, ESC exhibited minimal hematotoxicity and had little influence on the immune system following oral application, which fully guaranteed the application safety and met the requirement for further study.

*Identification of the tumor inhibitory effect of ESC in vivo*. Based on the above results, we further aimed to ascertain

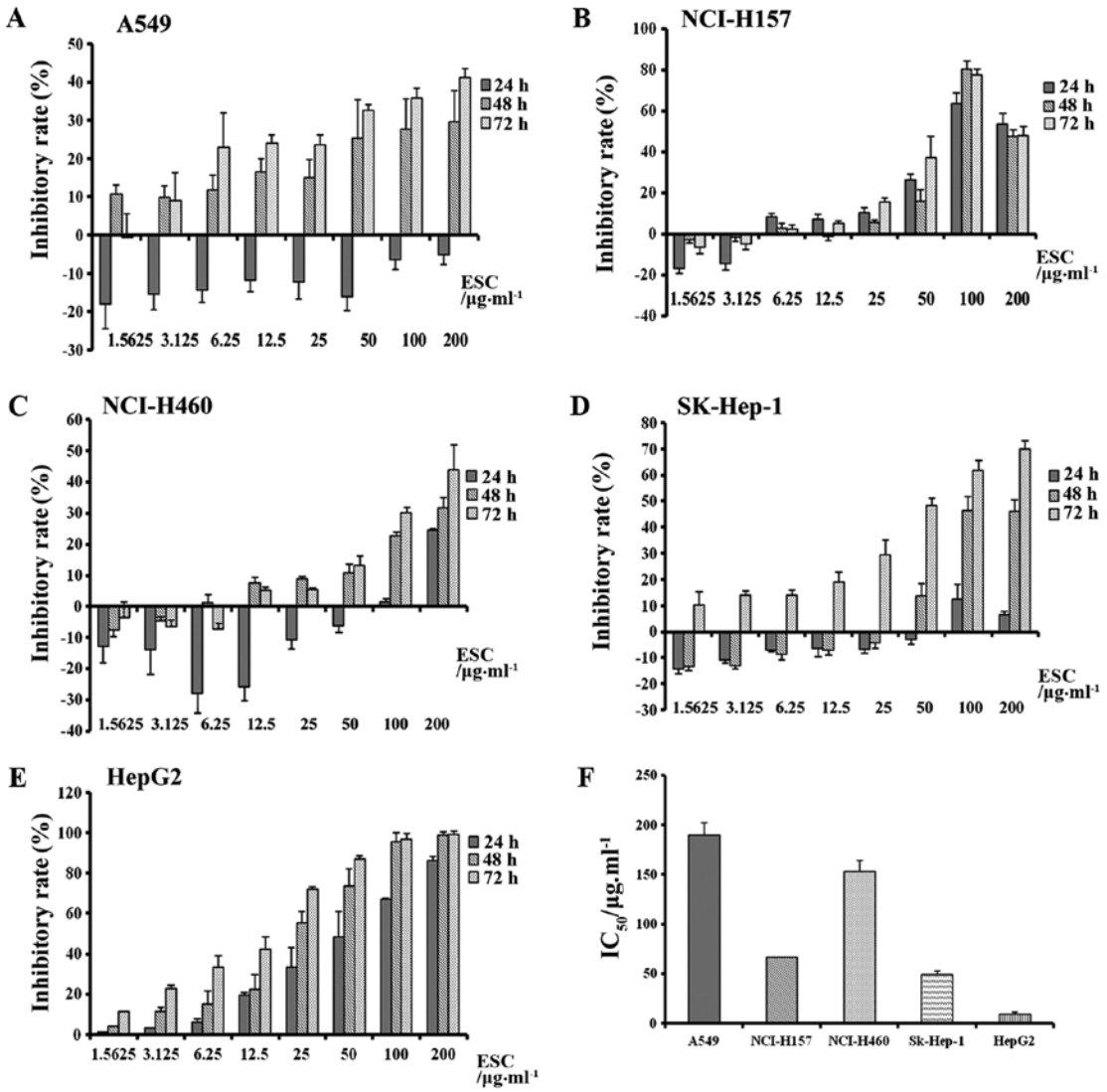


Figure 1. Anti-proliferative effect of ESC on different cancer cell lines. ESC inhibited cell growth in a dose- and time-dependent manner. In (A) A549, (B) NCI-H157 and (C) NCI-H460 cells, ESC exhibited a moderate anti-proliferative effect, whereas in (D) SK-HEP-1 and (E) HepG2 cell lines, ESC markedly inhibited cell growth in an explicit dose- and time-dependent manner. (F) $IC_{50}$ value for each cell line after 72 h of ESC treatment. The $IC_{50}$ values for the liver cancer cell lines, SK-HEP-1 and HepG2, were obviously lower than these values in the lung cancer cell lines. Results are represented and quantified as mean ± standard error of triplicate findings.

whether the anti-proliferative effects of ESC *in vitro* functionally contribute to the suppression of tumor burden *in vivo*.

To accomplish this, an H22 hepatocarcinoma model was established and tumor morphological observation, volume calculation and pathological analysis were performed. Firstly, through general observation, tumors in the negative control group were formed by the red and crisp tissues and characterized by their incomplete pseudomembrane and bleeding tendency. Importantly, at the margin of the tumors, part of the cancer cells invaded into the surrounding tissue, which is the morphological marker for an aggressive growth pattern and high malignancy.

In contrast, the drug-exposed tumors were characterized by a rounded shape with a low perfusion level and firm texture (Fig. 3A). In line with this observation, the average tumor weight and volume in the ESC-treated groups were obviously and dose dependently decreased in response to ESC treatment (Fig. 3B and C). Moreover, the tumor growth curve clearly showed that ESC exerted a tumor growth suppressive effect since the 4th day after modeling in a dose-dependent manner (Fig. 3D), which further statistically confirms that ESC can efficiently and negatively regulate primary hepatocarcinoma growth.

Accordingly, the pathological analysis further supported our results. As noted from the microscopic observation and pathological scoring, in the negative control mice, 80% of tumors showed high heteromorphism (Table I). In addition, cell death was detected at a low level (Table II). By H&E staining, the surrounding muscles and connective tissue were extensively and widely infiltrated by tumor cells. In contrast from the above results, the cell heteromorphism was gradually alleviated along with the elevated level of cell death following ESC treatment (Fig. 3E and Tables I and II). Taken together, our results clearly revealed the potent activity of ESC on the regulation of malignant growth behavior, which finally resulted in the inhibition of proliferation of hepatocarcinoma *in vivo*.

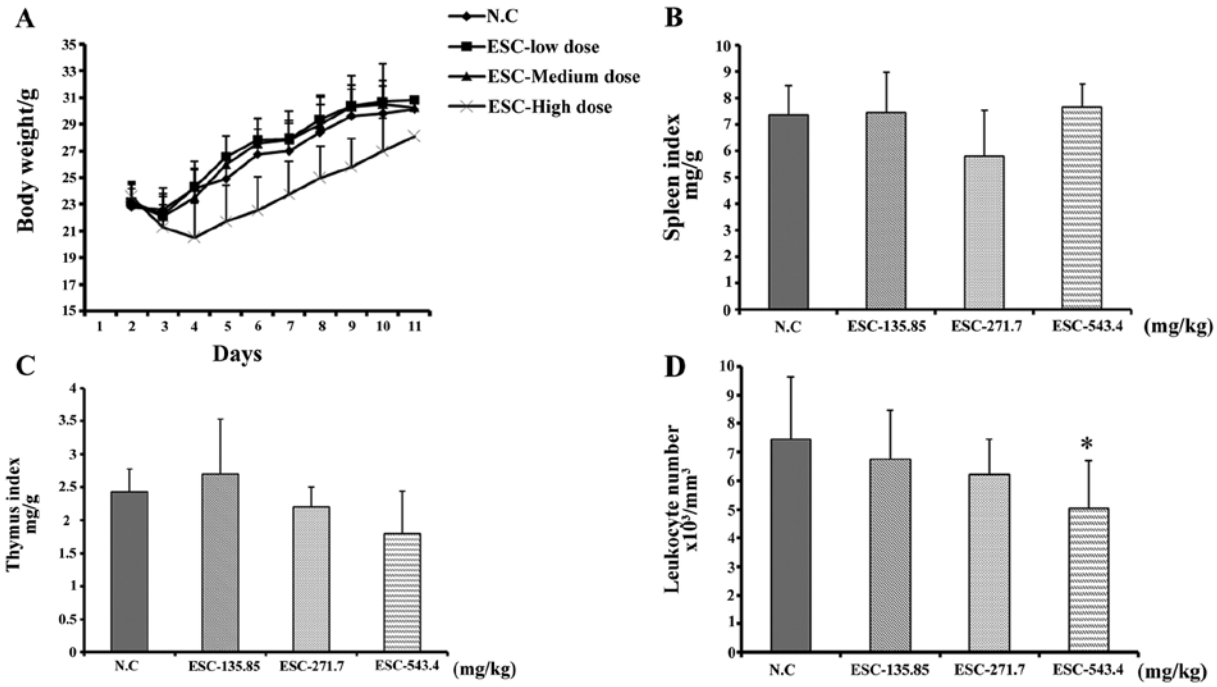


Figure 2. Evaluation of the systemic toxicity of ESC *in vivo*. (A) The body weight of H22 tumor-bearing mice. Following administration of ESC, the spleen index (B) and thymus index (C) were recorded and calculated according to the formula provided in the 'Material and methods' section. The blood was collected from each group to analyze the quantity of leukocytes (D). The results showed that, although high-dose ESC had a slight impact on body weight and the amount of leukocytes ($^{*}P<0.05$), the above indices were within a physiological range. Low-dose and medium-dose ESC did not affect all of the observed indices. Data are represented as means ± standard error, p-value was determined by one-way ANOVA analysis.

Table I. Tumor cell heteromophism in each group.

| Group | No. of animals | Heteromorphism - | + | ++ | +++ | P-value |
|---|---|---|---|---|---|---|
| N.C | 10 | 0 | 0 | 2 | 8 | |
| ESC - low dose | 9 | 0 | 0 | 6 | 3 | >0.05 |
| ESC - medium dose | 10 | 0 | 0 | 7 | 3 | <0.05 |
| ESC - high dose | 8 | 0 | 0 | 7 | 1 | <0.01 |

Table II. The apoptosis of tumor cells in each group.

| Group | No. of animals | Apoptosis - | + | ++ | P-value |
|---|---|---|---|---|---|
| N.C | 10 | 9 | 1 | 0 | |
| ESC - low dose | 9 | 6 | 3 | 0 | >0.05 |
| ESC - medium dose | 10 | 4 | 4 | 0 | <0.10 |
| ESC - high dose | 8 | 3 | 5 | 0 | <0.05 |

*ESC induces G2/M arrest in hepatocarcinoma cells*. Indicated by the above evidence, we next investigated the underlying mechanism of ESC in the regulation of tumor cell growth and proliferation.

In the present study, we firstly noted that, with the same initial cell density, the cultural confluency was significantly and time-dependently lower in the ESC groups than that in the negative control group (Fig. 4A). Initially, we speculated this phenomenon was closely associated with apoptosis induction. However, the results of Annexin V-FITC/PI staining explicitly showed that the percentage of apoptotic cells was maintained at the approximate level in each group in both cell lines (Fig. 4B and C), which excluded an apoptosis-inducing effect of ESC. Therefore, we naturally hypothesized that ESC treatment may lead to cell cycle arrest.

In order to clarify this, flow cytometry assay was carried out to detect changes in the cell cycle distribution of the SK-HEP-1 and HepG2 cells.

As expected, in the HepG2 and SK-HEP-1 negative control groups, the percentages of cells in the G2/M phase were 7.86 and 13.95%, respectively. In contrast, ESC treatment induced a 3- to 6-fold increase in G2/M-phase cells; the percentages at most reached 25.42% in the HepG2 and 75.90% in the SK-HEP-1 cells (Fig. 4D and E). In addition, these data further revealed the typical dose-dependent characteristic of ESC in the regulation of cell cycle distribution in hepatocarcinoma cells. This result clearly proved our hypothesis and identified that ESC functions as a potent cell cycle regulator which results in arrest of proliferation and tumor growth inhibition *in vivo*.

*Regulatory effects of ESC on cell cycle-associated molecules*. Based on the above results, we further analyzed the influence of ESC on cell cycle regulation at the molecular level. It is widely accepted that mitosis is strictly controlled by a molecular complex at different checkpoints consisting of phase-specific cyclins and cyclin-dependent kinases (CDKs).

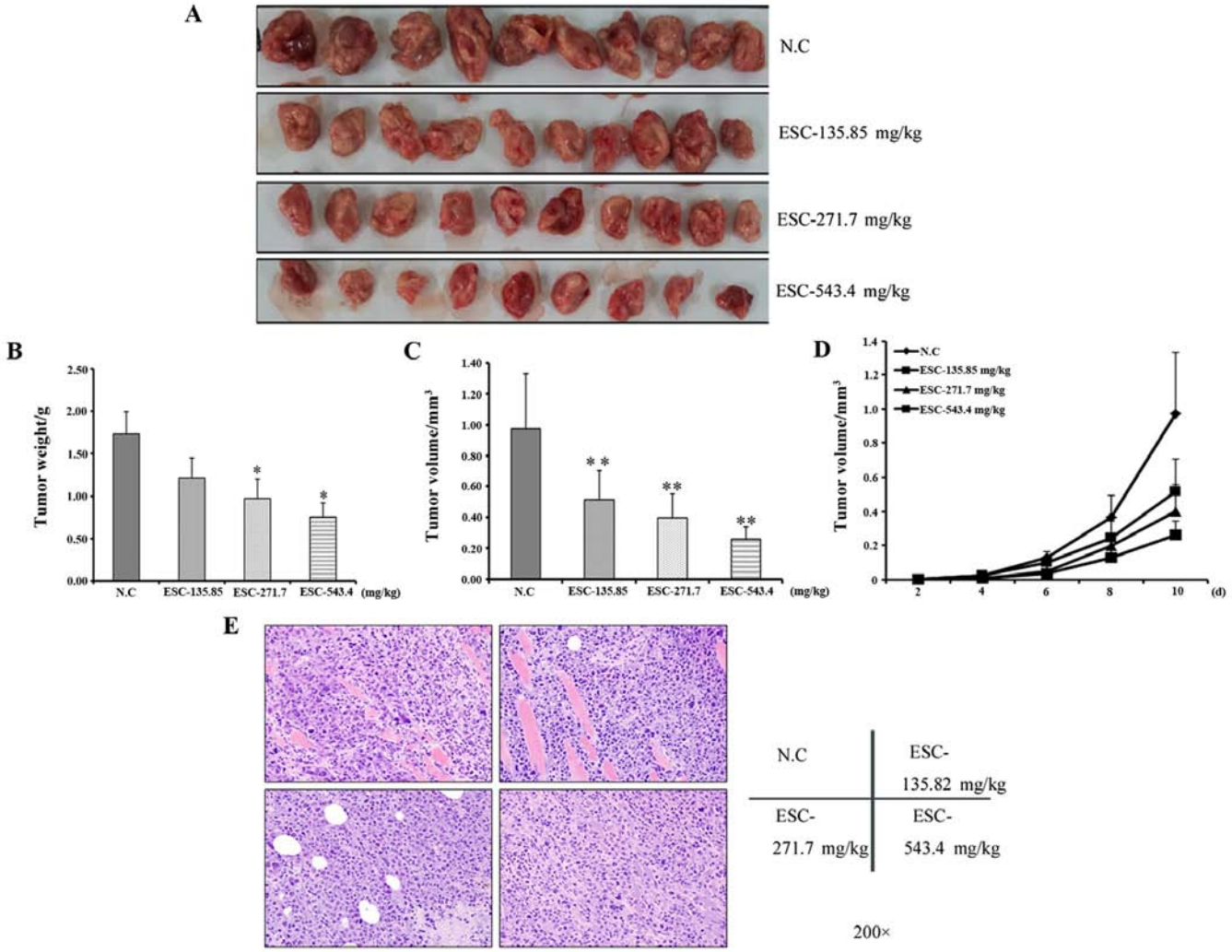


Figure 3. The H22 tumor inhibitory effect of ESC *in vivo*. (A) The image of H22 tumor samples resected from mice in each group. The volume of the tumors was decreased in a dose-dependent manner. (B) Tumor weight analysis. The tumor weight was markedly decreased in each drug-treated group. (C) The tumor volume index in each group. The results were calculated according to the formula described in the 'Material and methods' section. The tumor volume was decreased along with an increase in the ESC dose. (D) The tumor growth curve of each group in the H22 xenograft model. The diagrams represent the increase in tumor volume. The line chart illustrates that the tumor mass was clearly detected on the 4th day in each group, and tumors in the ESC-treated groups grew at a lower rate compared with the control group. (E) The histological analysis of tumor samples collected from ESC-treated or non-treated mice. Based on H&E staining, the morphological features of malignancy were observed. The detailed histological scores can be found in Tables I and II (samples were collected from 8-10 mice in the different groups, x200 magnification). Compared with the control group, $^{*}$P<0.05 was defined to have statistical significance. Data are represented as means ± standard error, p-value was determined by one-way ANOVA analysis. $^{*}$P<0.05, $^{**}$P<0.01, $^{***}$P<0.001.

Sequential activation of such a complex is necessary for motivating the cell cycle physiologically. In contrast, any prolonged or excessive signals will lead to uncontrolled mitosis and pathological cell cycle progression, which functionally causes the malignant proliferation in transformed cells. Led by this theory, we detected the expression levels of CDK1 and cyclin B1, the essential regulatory components specifically for the G2/M phase.

By immune blot analysis, the results clearly revealed that ESC treatment at 25, 50, 100 $\mu$g/ml dose-dependently decreased the expression level of cyclin B1. Accordingly, the phosphorylation level of CDK1 at the site of Tyr15 was significantly upregulated in both the SK-HEP-1 and HepG2 cells (Fig. 5A and B).

To further determine whether ESC exhibits the same function *in vivo*, samples from the xenograft model described in Fig. 3 were used, and the key cell cycle regulators were detected. Consistent with the results *in vitro*, ESC dose-dependently induced a decrease in cyclin B1 and upregulated the phosphorylation of CDK1$^{Tyr15}$ in the mixed tumor lysate (Fig. 5C) or in single tumor samples (Fig. 5D). Thus, we concluded that ESC played an important role in cell cycle arrest by suppressing the monitor protein in the G2/M phase.

## Discussion

During this century, with advancements in technology and scientific research, improvements have been achieved in the area of cancer therapy, and various types of effective drugs have been discovered. However, to date, the incidence and mortality rate of liver cancer remain at a high level. Even though locoregional therapies such as hepatic artery ligation

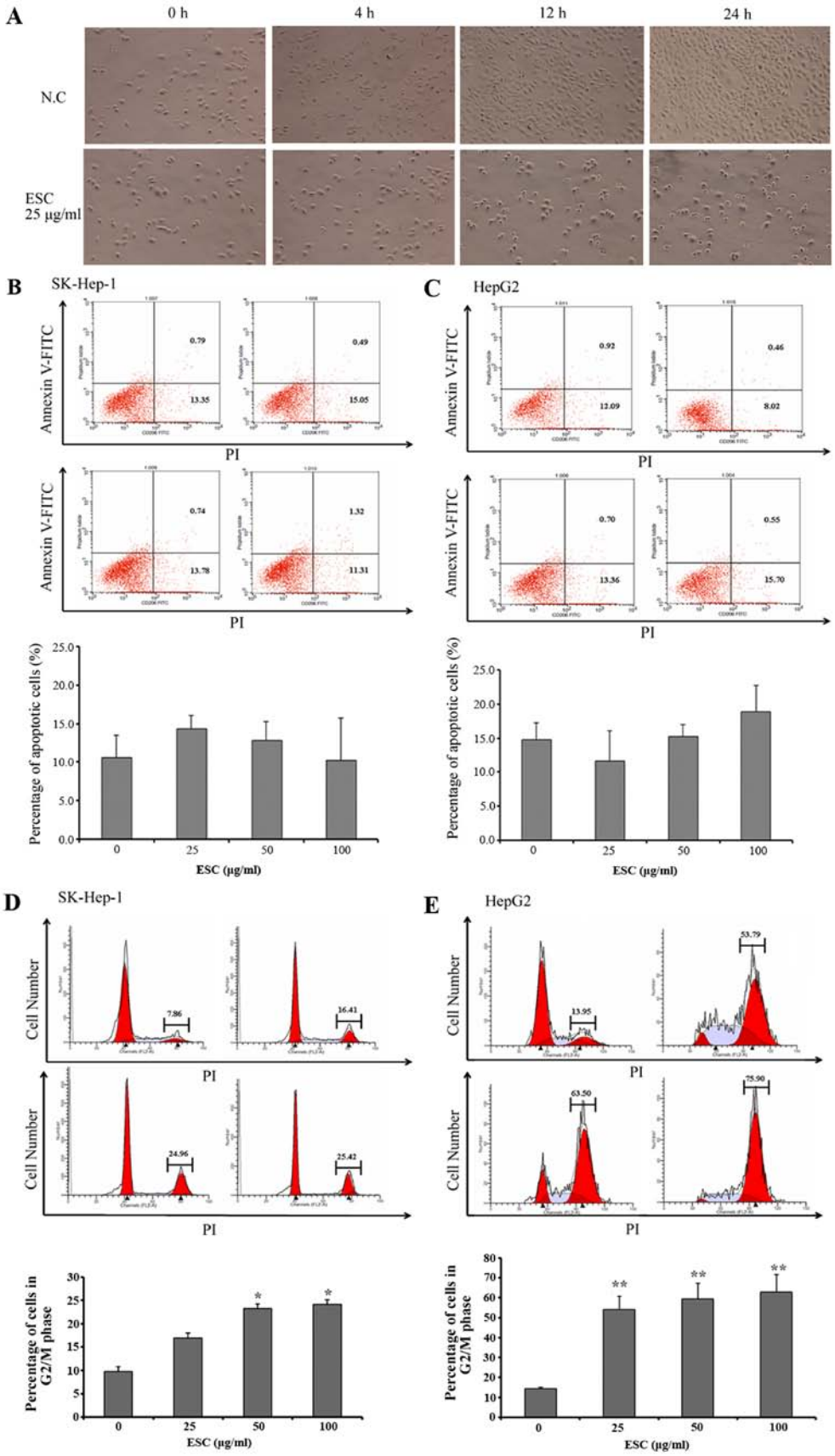


Figure 4. ESC arrests the cell cycle in the G2/M phase. (A) The morphological changes in ESC-treated SK-HEP-1 cells. After treating the cells with 25 $\mu$g/ml ESC for 4, 12 and 24 h, the light-microscopic images (x100) were collected. Results showed the dual influence of ESC on cell morphology and growth intensity. (B and C) Cell apoptosis detection in the SK-HEP-1 (B) and HepG2 (C) cells. Annexin V-FITC/PI staining demonstrated that ESC at the indicated doses had little impact on the induction of apoptosis in both cell lines after a 24-h treatment. (D and E) Cell cycle analysis by PI staining in the SK-HEP-1 (D) and HepG2 (E) cells. The results clearly showed that treatment with ESC effectively increased the ratio of cells in the G2/M phase in a dose-dependent manner. All the quantitative data were collected from at least three repeated experiments. Compared with the control group, $^{*}$P<0.05 was defined to have statistical significance. Data are represented as means ± standard error (B and C), p-value was determined by one-way ANOVA analysis. $^{*}$P<0.05, $^{**}$P<0.01, $^{***}$P<0.001.

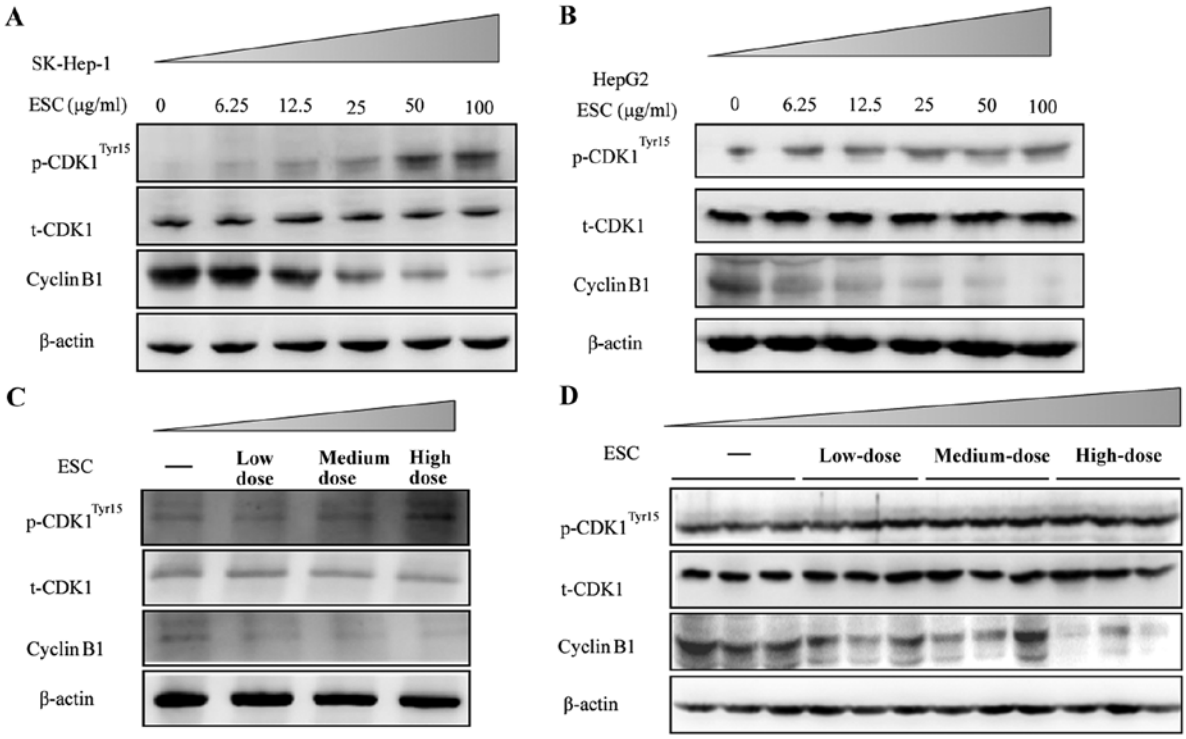


Figure 5. Effect of ESC on key proteins in the G2/M phase of the cell cycle. (A and B) Detection of the expression of cyclin B1, CDK1 and the phosphorylation of CDK1$^{Tyr15}$ in the SK-HEP-1 (A) and HepG2 (B) cells. ESC downregulated cyclin B1 expression in a dose-dependent manner. With basically the same expression level of t-CDK, the phosphorylation level of CDK1$^{Tyr15}$ was upregulated significantly in both cell lines. To further prove this result, we detected the same molecules in mixed tumor lysate collected from three randomly chosen mice (C) and in individual tumor samples (D). As shown in the diagram, the expression of the detected molecules *in vivo* exhibited similar trends as those *in vitro*. The low, medium and high dose of ESC corresponds to 135.85, 271.7 and 543.4 mg/kg, respectively. ESC induced G2/M arrest by regulating the cyclin B1-CDK1 complex. All the results were obtained from at least three independent repeats.

or embolization have been developed in the past decades, they have failed to significantly prolong the overall survival of liver cancer patients (22-24). Despite decades of efforts made by researchers worldwide, the investigation of anti-liver cancer drugs is stagnant (25-28). Conventionally, the commonly used drugs for liver cancer chemotherapy are 5-fluorouracil, mitomycin C, cisplatin, doxorubicin and their derivatives. In recent years, etoposide (VP-16) and paclitaxel have also been used in the clinic, but the efficacy has not obviously improved (29,30).

Unfortunately, chemoresistance accompanies the extensive and long-term use of chemotherapeutic drugs in clinical liver cancer treatment (29). Moreover, the commonly used chemical agents usually have unexpected side-effects, such as hair loss, nausea, vomiting, and diarrhea (31). Additionally, after resection or transarterial chemoembolization, the impaired physiological function of the liver and gallbladder leads to further severe side-effects and systemic toxicity (32). Therefore, the lack of effective chemotherapy for liver cancer contributes to the death of patients as early as one year after diagnosis (33). It is imperative to search for novel and effective anti-hepatocarcinoma drugs. Thus, natural extracts and their derivatives are valuable sources for drug discovery.

SCL is a wide-spread and historical-recorded plant, which has been used to cure cancer for over a thousand years. In previous studies of SCL, the extraction processes were immature and the efficiency was unstable. In addition, the unselective toxicity was another inevitable obstacle in SCL application. In our study, we established a novel extraction platform and made a reasonable optimization on SCL extraction. Through high throughput screening, we found a new extract, ESC, which exerted a specific inhibitory effect against liver cancer both *in vitro* and *in vivo*. Importantly, the antitumor activity showed little toxicity for immune and hematological systems *in vivo*. Thus, ESC has the potential to become an ideal candidate for clinical liver cancer treatment.

Based on a previous study, sustaining proliferative signaling, evading growth factors and enabling replicative immortality are widely regarded as the most important biological capabilities required in tumor progression (34). Whereas, the oncogenic cell cycle regulation is the foundation of all these three hallmarks. Therefore, therapeutics targeting the cell cycle are reliable strategies for cancer treatment. Inspired by this, in addition to identification of efficacy, we also carried out a preliminary but indicative exploration on the mechanism of ESC against liver cancer. In this study, we found that ESC induced cell cycle arrest at the G2/M phase in both HepG2 and SK-HEP-1 liver cancer cell lines. It is widely accepted that the cell cycle is governed by a series of checkpoint molecular complexes consisting of cyclins and CDKs. The sequential paired combination and separation between specific cyclin and CDK is the foundation of a normal cell cycle. Controlled by cyclin B-CDK1 complex activation, the G2/M checkpoint prevents DNA-damaged cells from entering mitosis and allowing DNA repair (35). During this process, the cyclin B-CDK1 complex activation is dependent on expression of cyclin B1 and dephosphorylation of CDK1$^{Tyr15}$. By analyzing the expression level of cyclin B1 and the phosphorylation level of CDK1$^{Tyr15}$, we found that the activity of the cyclin B1-CDK1

complex was downregulated in a dose-dependent manner by ESC.

In conclusion, ESC exhibited a potent effect against liver cancer under a relatively safe condition via arresting tumor cells in the G2/M phase. Thus, ESC is a promising chemotherapeutic candidate to treat liver cancer. This may provide patients, who are not qualified for surgery, with another option and offer a novel comprehensive therapeutic strategy combining liver surgical resection and hepatic artery ligation.

Encouraged by the results mentioned previously, further experiments will be conducted to clarify the detailed and precise mechanism in ESC-induced cell cycle arrest. The purified chemical compound isolated from ESC would certainly be another aim of future study.

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## References

1. Wu F, Lin GZ and Zhang JX: An overview of cancer incidence and trend in China. China Cancer: 81-85, 2012.
2. Rabindran SK, Ross DD, Doyle LA, Yang W and Greenberger LM: Fumitremorgin C reverses multidrug resistance in cells transfected with the breast cancer resistance protein. Cancer Res 60: 47-50, 2000.
3. Florea AM and Büsselberg D: Cisplatin as an anti-tumor drug: Cellular mechanisms of activity, drug resistance and induced side effects. Cancers (Basel) 3: 1351-1371, 2011.
4. Parkin DM, Bray F, Ferlay J and Pisani P: Estimating the world cancer burden: Globocan 2000. Int J Cancer 94: 153-156, 2001.
5. Forner A, Llovet JM and Bruix J: Hepatocellular carcinoma. Lancet 379: 1245-1255, 2012.
6. Li J and Luo R: Advances of biochemotherapy in hepatocellular carcinoma. J Mol Diagnostics Therapy 1: 60, 2009.
7. Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D: Global cancer statistics. CA Cancer J Clin 61: 69-90, 2011.
8. Hasegawa K, Kokudo N and Makuuchi M: Surgical management of hepatocellular carcinoma. Liver resection and liver transplantation. Saudi Med J 28: 1171-1179, 2007.
9. Poon RT, Fan ST, Tsang FH and Wong J: Locoregional therapies for hepatocellular carcinoma: A critical review from the surgeon's perspective. Ann Surg 235: 466-486, 2002.
10. Thomas MB and Zhu AX: Hepatocellular carcinoma: The need for progress. J Clin Oncol 23: 2892-2899, 2005.
11. Dizon DS and Kemeny NE: Intrahepatic arterial infusion of chemotherapy: Clinical results. Semin Oncol 29: 126-135, 2002.
12. Harvey AL: Natural products in drug discovery. Drug Discov Today 13: 894-901, 2008.
13. Shi GL, Liu SQ, Cao H, Zhao LL, Li J and Li SY: Acaricidal activities of extracts of *Stellera chamaejasme* against *Tetranychus viennensis* (Acari: Tetranychidae). J Econ Entomol 97: 1912-1916, 2004.
14. Yang BY: Inhibitory effects of *Stellera chamaejasme* on the growth of a transplantable tumor in mice. Zhong Yao Tong Bao 11: 58-59, 1986 (In Chinese).
15. Yoshida M, Feng W, Saijo N and Ikekawa T: Antitumor activity of daphnane-type diterpene gnidimacrin isolated from *Stellera chamaejasme* L. Int J Cancer 66: 268-273, 1996.
16. Yang G, Liao Z, Xu Z, Zhang H and Chen D: Antimitotic and antifungal C-3/C-3"-biflavanones from *Stellera chamaejasme*. Chem Pharm Bull (Tokyo) 53: 776-779, 2005.
17. Yang G and Chen D: Biflavanones, flavonoids, and coumarins from the roots of *Stellera chamaejasme* and their antiviral effect on hepatitis B virus. Chem Biodivers 5: 1419-1424, 2008.
18. Naito S, von Eschenbach AC, Giavazzi R and Fidler IJ: Growth and metastasis of tumor cells isolated from a human renal cell carcinoma implanted into different organs of nude mice. Cancer Res 46: 4109-4115, 1986.
19. Wittekind C: The new WHO classification of liver tumors - what is really new?. Verh Dtsch Ges Pathol 85: 212-218, 2001 (In German).
20. Pozarowski P and Darzynkiewicz Z: Analysis of cell cycle by flow cytometry. Methods Mol Biol 281: 301-311, 2004.
21. Novotny-Diermayr V, Sangthongpitag K, Hu CY, *et al*: SB939, a novel potent and orally active histone deacetylase inhibitor with high tumor exposure and efficacy in mouse models of colorectal cancer. Mol Cancer Ther 9: 642-652, 2010.
22. Yim HJ, Suh SJ and Um SH: Current management of hepatocellular carcinoma: An Eastern perspective. World J Gastroenterol 21: 3826-3842, 2015.
23. Okuda K, Ohtsuki T, Obata H, Tomimatsu M, Okazaki N, Hasegawa H, Nakajima Y and Ohnishi K: Natural history of hepatocellular carcinoma and prognosis in relation to treatment. Study of 850 patients. Cancer 56: 918-928, 1985.
24. Nagasue N, Yukaya H, Hamada T, Hirose S, Kanashima R and Inokuchi K: The natural history of hepatocellular carcinoma. A study of 100 untreated cases. Cancer 54: 1461-1465, 1984.
25. Park SH, Lee Y, Han SH, Kwon SY, Kwon OS, Kim SS, Kim JH, Park YH, Lee JN, Bang SM, *et al*: Systemic chemotherapy with doxorubicin, cisplatin and capecitabine for metastatic hepatocellular carcinoma. BMC Cancer 6: 3, 2006.
26. Boucher E, Corbinais S, Brissot P, Boudjema K and Raoul JL: Treatment of hepatocellular carcinoma (HCC) with systemic chemotherapy combining epirubicin, cisplatinum and infusional 5-fluorouracil (ECF regimen). Cancer Chemother Pharmacol 50: 305-308, 2002.
27. Pohl J, Zuna I, Stremmel W and Rudi J: Systemic chemotherapy with epirubicin for treatment of advanced or multifocal hepatocellular carcinoma. Chemotherapy 47: 359-365, 2001.
28. Feun LG, O'Brien C, Molina E, Rodriguez M, Jeffers L, Schiff ER, Marini A, Savaraj N and Ardalan B: Recombinant leukocyte interferon, doxorubicin, and 5FUDR in patients with hepatocellular carcinoma-A phase II trial. J Cancer Res Clin Oncol 129: 17-20, 2003.
29. Alexandre J, Tigaud JM, Gross-Goupil M, Gornet JM, Romain D, Azoulay D, Misset JL and Goldwasser F: Combination of topotecan and oxaliplatin in inoperable hepatocellular cancer patients. Am J Clin Oncol 25: 198-203, 2002.
30. Hsu JL, Chiang PC and Guh JH: Tunicamycin induces resistance to camptothecin and etoposide in human hepatocellular carcinoma cells: Role of cell-cycle arrest and GRP78. Naunyn Schmiedebergs Arch Pharmacol 380: 373-382, 2009.
31. Ueda H, Fukuchi H and Tanaka C: Toxicity and efficacy of hepatic arterial infusion chemotherapy for advanced hepatocellular carcinoma (Review). Oncol Lett 3: 259-263, 2012.
32. Basile A, Carrafiello G, Ierardi AM, Tsetis D and Brountzos E: Quality-improvement guidelines for hepatic transarterial chemoembolization. Cardiovasc Intervent Radiol 35: 765-774, 2012.
33. Gerunda GE, Neri D, Merenda R, Barbazza F, Zangrandi F, Meduri F, Bisello M, Valmasoni M, Gangemi A and Faccioli AM: Role of transarterial chemoembolization before liver resection for hepatocarcinoma. Liver Transpl 6: 619-626, 2000.
34. Hanahan D and Weinberg RA: Hallmarks of cancer: The next generation. Cell 144: 646-674, 2011.
35. Wang Y, Ji P, Liu J, Broaddus RR, Xue F and Zhang W: Centrosome-associated regulators of the G(2)/M checkpoint as targets for cancer therapy. Mol Cancer 8: 8, 2009.