**Name of Journal:** *World Journal of Gastrointestinal Oncology*

**Manuscript NO:** 71434

**Manuscript Type:** ORIGINAL ARTICLE

***Basic Study***

**Xihuang pills induce apoptosis in hepatocellular carcinoma by suppressing phosphoinositide 3-kinase/protein kinase-B/mechanistic target of rapamycin pathway**

Teng YJ *et al*. Xihuang pills induce apoptosis in HCC

Yong-Jie Teng, Zhe Deng, Zhao-Guang Ouyang, Qing Zhou, Si Mei, Xing-Xing Fan, Yong-Rong Wu, Hong-Ping Long, Le-Yao Fang, Dong-Liang Yin, Bo-Yu Zhang, Yin-Mei Guo, Wen-Hao Zhu, Zhen Huang, Piao Zheng, Di-Min Ning, Xue-Fei Tian

**Yong-Jie Teng, Qing Zhou, Hong-Ping Long, Le-Yao Fang,** The First Hospital of Hunan University of Chinese Medicine, Hunan University of Chinese Medicine, Changsha 410208, Hunan Province, China

**Zhe Deng, Yong-Rong Wu, Yin-Mei Guo, Wen-Hao Zhu, Zhen Huang, Piao Zheng, Di-Min Ning, Xue-Fei Tian,** College of Integrated Chinese and Western Medicine, Hunan Key Laboratory of Translational Research in Formulas and Zheng of Traditional Chinese Medicine, Hunan University of Chinese Medicine, Changsha 410208, Hunan Province, China

**Zhao-Guang Ouyang,** Department of Preventive Dentistry, Guangzhou Key Laboratory of Basic and Applied Research of Oral Regenerative Medicine, Guangzhou Medical University, Guangzhou 510132, Guangdong Province, China

**Si Mei,** Department of Physiology, Hunan University of Chinese Medicine, Changsha 410208, Hunan Province, China

**Xing-Xing Fan,** State Key Laboratory of Quality Research in Chinese Medicine, Macau Institute For Applied Research in Medicine and Health, Macau University of Science and Technology, Macau, China

**Dong-Liang Yin,** School of Pharmacy, Hunan University of Chinese Medicine, Changsha 410208, Hunan Province, China

**Bo-Yu Zhang,** College of Acupuncture and Massage, Hunan University of Chinese Medicine, Changsha 410208, Hunan Province, China

**Author contributions:** Teng YJ, Deng Z, and Wu YR completed the experiment and wrote the paper; Teng YJ, Deng Z, and Ouyang ZG reviewed the manuscript; Tian XF and Zhou Q provided research ideas; Long HP and Fang LY performed HPLC analysis; Zhang BY has processed the figures of the article; Fan XX, Yin DL, Guo YM, Zheng P, Huang Z and Ning DM conducted statistical analysis; all authors have read and approved the manuscript to ensure its accuracy and completeness.

**Supported by** National Natural Science Foundation of China, No. U20A20408 and No. 82074450; Natural Science Foundation of Hunan Province, No. 2020JJ4066; Hunan Province "Domestic First-class Cultivation Discipline" Integrated Traditional Chinese and Western Medicine Open Fund Project, No. 2020ZXYJH34 and No. 2020ZXYJH35; Hunan Graduate Scientific Research Innovation Project, No. QL20210173 and No. CX20210730; Hunan Province Science and Technology Innovation Talents Plan College Students Science and Technology Innovation and Entrepreneurship Project, No. 2020RC1004; Guangzhou Health Science and Technology Project, No. 20221A011102; and Hunan Traditional Chinese Medicine Scientific Research Project, No. 202101.

**Corresponding author: Xue-Fei Tian, PhD, Professor,** College of Integrated Chinese and Western Medicine, Hunan Key Laboratory of Translational Research in Formulas and Zheng of Traditional Chinese Medicine, Hunan University of Chinese Medicine, No. 300 Xueshi Road, Yuelu District, Changsha 410208, Hunan Province, China. 003640@hnucm.edu.cn

**Received:** September 7, 2021

**Revised:** December 30, 2021

**Accepted: March 14, 2022**

**Published online:**

**Abstract**

BACKGROUND

The phosphoinositide 3-kinase/protein kinase-B/mechanistic target of rapamycin (PI3K/Akt/mTOR) signalling pathway is crucial for cell survival, differentiation, apoptosis and metabolism. Xihuang pills (XHP) are a traditional Chinese preparation with antitumour properties. They inhibit the growth of breast cancer, glioma, and other tumours by regulating the PI3K/Akt/mTOR signalling pathway. However, the effects and mechanisms of action of XHP in hepatocellular carcinoma (HCC) remain unclear. Regulation of the PI3K/Akt/mTOR signalling pathway effectively inhibits the progression of HCC. However, no study has focused on the XHP-associated PI3K/Akt/mTOR signalling pathway. Therefore, we hypothesized that XHP might play a role in inhibiting HCC through the PI3K/Akt/mTOR signalling pathway.

AIM

To confirm the effect of XHP on HCC and the possible mechanisms involved.

METHODS

The chemical constituents and active components of XHP were analysed using ultra-performance liquid chromatography-quadrupole time of flight mass spectrometry (UPLC-Q-TOF-MS). Cell-based experiments and *in vivo* xenograft tumour experiments were utilized to evaluate the effect of XHP on HCC tumorigenesis. First, SMMC-7721 cells were incubated with different concentrations of XHP (0, 0.3125, 0.625, 1.25, and 2.5 mg/mL) for 12 h, 24 h and 48 h. Cell viability was assessed using the CCK-8 assay, followed by an assessment of cell migration using a wound healing assay. Second, the effect of XHP on the apoptosis of SMMC-7721 cells was evaluated. SMMC-7721 cells were stained with fluorescein isothiocyanate and annexin V/propidium iodide. The number of apoptotic cells and cell cycle distribution were measured using flow cytometry. The cleaved protein and mRNA expression levels of caspase-3 and caspase-9 were detected using Western blotting and quantitative reverse-transcription polymerase chain reaction (RT-qPCR), respectively. Third, Western blotting and RT–qPCR were performed to confirm the effects of XHP on the protein and mRNA expression of components of the PI3K/Akt/mTOR signalling pathway. Finally, the effects of XHP on the tumorigenesis of subcutaneous hepatocellular tumours in nude mice were assessed.

RESULTS

The following 12 compounds were identified in XHP using high-resolution mass spectrometry: Valine, 4-gingerol, myrrhone, ricinoleic acid, glycocholic acid, curzerenone, 11-keto-β-boswellic acid, oleic acid, [germacrone](https://www.chemsrc.com/en/cas/6902-91-6_895786.html), 3-acetyl-9,11-dehydro-β-boswellic acid, 5β-androstane-3,17-dione, and 3-acetyl-11-keto-β-boswellic acid. The cell viability assay results showed that treatment with 0.625 mg/mL XHP extract decreased HCC cell viability after 12 h, and the effects were dose- and time-dependent. The results of the cell scratch assay showed that the migration of HCC cells was significantly inhibited in a time-dependent manner by the administration of XHP extract (0.625 mg/mL). Moreover, XHP significantly inhibited cell migration and resulted in cell cycle arrest and apoptosis. Furthermore, XHP downregulated the PI3K/Akt/mTOR signalling pathway, which activated apoptosis executioner proteins (*e.g.*, caspase-9 and caspase-3). The inhibitory effects of XHP on HCC cell growth were determined *in vivo* by analysing the tumour xenograft volumes and weights.

CONCLUSION

XHP inhibited HCC cell growth and migration by stimulating apoptosis *via* the downregulation of the PI3K/Akt/mTOR signalling pathway, followed by the activation of caspase-9 and caspase-3. Our findings clarified that the antitumour effects of XHP on HCC cells are mediated by the PI3K/Akt/mTOR signalling pathway, revealing that XHP may be a potential complementary therapy for HCC.

**Key Words:** Hepatocellular carcinoma; Xihuang pills; Apoptosis; Antitumour; Phosphoinositide 3-kinase/protein kinase-B/mechanistic target of rapamycin pathway

Teng YJ, Deng Z, Ouyang ZG, Zhou Q, Mei S, Fan XX, Wu YR, Long HP, Fang LY, Yin DL, Zhang BY, Guo YM, Zhu WH, Huang Z, Zheng P, Ning DM, Tian XF. Xihuang pills induce apoptosis in hepatocellular carcinoma by suppressing phosphoinositide 3-kinase/protein kinase-B/mechanistic target of rapamycin pathway. *World J Gastrointest Oncol* 2022; In press

**Core Tip:** The study revealed that Xihuang pills (XHP) increases caspase-9 and caspase-3 activities by inhibiting the phosphoinositide 3-kinase/protein kinase-B/mechanistic target of rapamycin signalling pathway and induces apoptosis and cell cycle arrest. Consequently, our study indicated that XHP inhibits the growth, migration, and proliferation of hepatocellular carcinoma (HCC) cells. Our study provides a better understanding of the antitumour effects of XHP and reveals the underlying mechanism. The findings of this study suggest that XHP might serve as a supplementary medicine in HCC treatment.

**INTRODUCTION**

Hepatocellular carcinoma (HCC) is a primary liver cancer with a poor prognosis, and limited treatments are available for patients with advanced HCC[1]. The absence of specific clinical signs and symptoms makes the early diagnosis of HCC even more difficult[2]. Although diagnostic and therapeutic methods have improved in recent years, the efficacy of HCC treatments is only 30%–40%[3]. In addition, the toxicity and side effects associated with conventional treatments remain a clinical challenge that demands a prompt solution. Therefore, the development of effective antitumour drugs with reduced toxicity is needed. The carcinogenesis of HCC is regulated by several signalling pathways, and the phosphoinositide 3-kinase/protein kinase-B/mechanistic target of rapamycin (PI3K/Akt/mTOR) pathway is one of the most important pathways[4].

The PI3K/Akt/mTOR signalling pathway is crucial for cell survival, differentiation, apoptosis and metabolism[5-7]. PI3K is a member of the lipid kinase family[8], and its activation initiates the expression of the second messenger phosphatidylinositol 3,4,5-triphosphate (PIP3). PIP3 induces the activation of multiple protein kinases, such as Akt. Activated Akt then facilitates cell differentiation, proliferation, metabolism, apoptosis and angiogenesis *via* the upregulation of several downstream effectors, including mTOR, B-cell lymphoma 2 (Bcl-2) family proteins, glycogen synthase kinase 3, S6 protein kinase, and caspase-9[3]. Activated caspase-9 induces the function of caspase-3 zymogen and induces apoptosis through proteolysis[9]. As a highly conserved mechanism of programmed cell death, apoptosis maintains tissue homeostasis. A reduction in apoptosis can induce the occurrence of tumours and promote their development[10]. PI3K/Akt/mTOR signalling, an apoptosis-related pathway[11], is often abnormally activated in HCC[4]. Therefore, apoptosis may be induced by suppressing the PI3K/Akt/mTOR pathway, which inhibits the proliferation of HCC cells.

Xihuang pills (XHP), a traditional Chinese antitumour prescription, are composed of four Chinese herbs, namely, *Bos taurus domesticus Gmelin*, *Boswellia carteri Birdwood*, *Moschus berezovskii Flerov* and *Commiphora myrrha (Nees) Engl*[12]. XHP exerts antitumour effects, reduces side effects, and improves the quality of life and survival rate of patients receiving tumour therapy[12]. Clinical studies have shown that XHP combined with chemotherapy effectively enhances the tumour response in patients with breast cancer, and reduces the toxicity and side effects of chemotherapy[13]. Several studies have confirmed that the antitumour activity of XHP depends on the PI3K-Akt-mTOR signalling pathway. Li *et al*[14] reported that XHP promotes apoptosis of Treg cells through the PI3K/Akt/AP-1 signalling pathway, improves the immunosuppressive state of the tumour microenvironment, and inhibits tumour growth. Fu *et al*[15] reported that XHP enhances the antitumour effect of temozolomide on glioblastoma-transplanted tumours through the Akt/mTOR pathway. According to Shao *et al*[16], XHP regulates the apoptosis of U-8MG glioblastoma cells through the ROS-mediated Akt/mTOR/FOXO1 pathway.

Collectively, the antitumour effect of XHP is associated with suppression of the PI3K/Akt/mTOR signalling pathway. Regulation of the PI3K/Akt/mTOR signalling pathway effectively inhibits the progression of HCC[4]. A growing number of studies have documented the antitumour effects of XHP on breast cancer, lung cancer, colon cancer, glioma, *etc.* However, few studies have been performed on HCC. No research has focused on the XHP-associated PI3K/Akt/mTOR signalling pathway. Therefore, we hypothesized that XHP might play a role in inhibiting HCC through the PI3K/Akt/mTOR signalling pathway. Our results confirm that XHP induces apoptosis and inhibits proliferation, both *in vivo* and *in vitro*. Moreover, this inhibitory effect depends on the PI3K/Akt/mTOR signalling pathway. Thus, this research revealed a potential antitumour effect of XHP on HCC cells.

**MATERIALS AND METHODS**

***Chemicals***

XHP was purchased from Tong Ren Tang Technologies Co., Ltd. (Beijing, China, Lot number: 17043278). All study parameters fulfilled the requirements of standard quality. For extraction, XHP was soaked in 6 mL of double distilled water for 24 h, followed by ultrasonic dissolution. The precipitates were collected *via* centrifugation at 3000 rpm for 5 min and resuspended in 6 mL of dimethyl sulfoxide (DMSO; Sigma–Aldrich, Shanghai, China) using an ultrasonic dissolver. Another centrifugation step was performed, and the precipitates were collected. An XHP extract was then prepared using the obtained precipitates and stored at 4 °C for future use. According to the pharmacological dosage regulations of XHP, the daily dose of XHP for adults is 6 g/d, and the body weight of each mice is about 20 g. The dose of XHP was determined by dose extrapolation based on dose-body surface area normalization, this dosage was converted to 78 mg/kg[17].

***The study of XHP using high resolution mass spectrometry***

The compounds present in XHP were analysed using high-resolution mass spectrometry (MS). XHP (3 g) was ground into a powder and extracted with 10 mL of 100% methanol, followed by ultrasonic extraction for 45 min. The supernatant was collected by centrifugation at 8000 rpm for 5 min and then filtered with a 0.22 μm microporous filter membrane. The filtrate was collected and measured using a UPLC-Q-TOF-MS (1290 UPLC-6540, Agilent Technologies Inc., United States) system. An Agilent ZORBAX Eclipse Plus C18 column (100 mm × 3.0 mm, 1.8 μm) was utilized for chromatographic separation. The mobile phase consisted of acetonitrile (A) and 0.1% formic acid, which were prepared as solutions with different gradients: 0–10 min containing 5%–15% A; 10–15 min containing 15%–20% A; 15–25 min containing 25%–45% A; and 25–40 min containing 45%–80% A. The flow rate of the mobile phase was 0.4 mL/min, and the injection volume was 1 μL/sample. Parameters for the MS system were fixed according to the manufacturer’s recommendation. In detail, the ionization mode, electrospray ionization and accurate mass data correction were performed using electrospray ionization-L Low Concentration Tuning Mix (G1969-85000). Positive and negative ion switching and the MRE scan model were used, and the mass range was set from 100 m/z to 1700 m/z. The electrospray capillary voltage was 4.0 kV. The sheath gas temperature and drying gas temperature were set to 350 °C. The drying gas (nitrogen) flow rate was 6.8 L/min. After the first mass analysis in full scan mode, a secondary MS scan was conducted in data-dependent mode, and collision-induced dissociation (CID) was used to fragment the first three strong peaks. Subsequently, the secondary scan data were obtained from 50 to 1000 m/z with fragmentation voltages of 10, 20, and 30 kV.

***Cell culture and treatment***

The SMMC-7721 HCC cell line was purchased from the Beinac Biotechnology Research Institute (Beijing, China) and was not contaminated, as determined by short tandem repeat (STR) identification. SMMC-7721 cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco, New York, United States) supplemented with 10% heat-inactivated foetal bovine serum (Gibco, New York, United States) and 1% (v/v) penicillin–streptomycin (Gibco, New York, United States). Cells were cultured in a humidified atmosphere containing 5% CO2 at 37 °C. When cells reached 80% confluence, they were treated with different concentrations of the XHP extract (0, 0.3125, 0.625, 1.25, and 2.5 mg/mL) for various durations (0 h, 6 h, 12 h, 24 h, and 48 h).

***Cell viability assay***

Cell Counting Kit-8 (CCK-8, Gibco, New York, United States) was used to measure cell viability. Briefly, 100 μL of suspended SMMC-7721 cells (1 × 105 cells/mL) in logarithmic growth phase were inoculated in 96-well plates. Subsequently, cells were treated with different concentrations of XHP extract (0, 0.625, 1.25, and 2.5 mg/mL) for different durations. Cells treated with the same volume of 0.1% DMSO and cultured for the same time were considered the control group. After an incubation for 0 h, 6 h, 12 h, 24 h, or 48 h, the absorbance was measured using a microplate reader (Awareness Stat Fax ®2600, Qingdao, China) at 450 nm. Cell viability was determined by comparing treated cells to controls. All assays were repeated at least three times.

***Wound healing assay***

SMMC-7721 cells were inoculated into 12-well plates after digestion. After spreading cells over the bottom of the plate, a pipette tip (1 mL) was used to create scratch wounds in the cell layer, ensuring that all wounds had a consistent width. The cell culture media were then aspirated, and cell debris created by the scratch were removed by rinsing the plate three times with phosphate-buffered saline (PBS). Then, serum-free culture medium was added to the plate, and an inverted fluorescence microscope (Olympus, type IX71) was used for imaging. The culture plate was placed in a cell incubator (Thermo) for 48 h to allow cell migration. The plate was removed from the incubator every 12 h for imaging. The assay results were analysed according to the collected imaging data.

***Apoptosis analysis***

Apoptosis was detected using the Annexin V-APC Apoptosis Detection Kit (KeyGEN, Nanjing, Jiangsu, China) according to the manufacturer’s instructions. Briefly, cells were digested with 0.25% trypsin (Beyotime Biotechnology Co., Ltd., Shanghai, China) after washes with PBS, followed by centrifugation at 3000 rpm for 5 min. The collected cells were washed with PBS twice and suspended in 500 μL of binding buffer. Then, 5 μL of fluorescein isothiocyanate-labelled annexin V-APC were added and evenly mixed with 5 μL of propidium iodide (PI) at room temperature for 15 min in the dark. Subsequently, a flow cytometer (Beckman Coulter, Brea, CA, United States) was used to quantify the apoptotic cells.

***Analysis of cell cycle arrest***

The DNA content and cell cycle distribution were measured using a FACSCalibur flow cytometer (Beckman Coulter, Brea, CA, United States). SMMC-7721 cells were inoculated in 6-well plates at a density of 5 × 105 cells/well. After a 24 h incubation, cells were treated with or without XHP extract for 48 h. Subsequently, the cells were fixed with 75% ethanol at 4 °C overnight, followed by washes with PBS. The cells were incubated at room temperature for 15–30 min in the dark and then analysed using flow cytometry.

***Western blotting***

Mouse tumour tissue specimens were obtained and lysed in RIPA lysis buffer (20 mmol/L HEPES, 1 mmol/L EDTA, 1% Triton X-100, 2 mmol/L EGTA, 150 mmol/L NaCl, 20 mmol/L phosphoglycerol, and 10% protease glycerol). The suspension was then homogenized and subsequently centrifuged at 12000 rpm for 15 min at 4 °C. A BCA detection kit (Beyotime, Shanghai, China) was used to determine the protein concentration. Polyacrylamide gel electrophoresis was used for protein separation Then, the proteins were transferred to a polyvinylidene fluoride membrane with a transfer device in cold buffer. A freshly prepared 5% skim milk solution was utilized for blocking at room temperature for 2 h. Afterwards, specific primary antibodies were incubated with the membrane at 4 °C overnight. The membrane was then washed with Tris-buffered saline supplemented with 0.1% Tween-20 (TBST) and incubated with peroxidase-conjugated secondary antibodies. Visualization was performed using the Super Sight West Pico Blotting kit (Pierce, Massachusetts, United States). The following primary antibodies were used: Cleaved caspase-3 (1:4000), cleaved caspase-9 (1:2000), Akt (1:2000), mTOR (1:2000), P-PI3K (1:1000), P-AKT (1:2000), and P-mTOR (1:2000), which were obtained from Abcam plc. (Cambridge, England). Antibodies against PI3K (1:5000) and β-actin (1:5000) were obtained from Proteintech Group, Inc. (Chicago, United States). Trypsin and PBS were purchased from HyClone Company (Logan, Utah, United States), and DMSO was obtained from Sigma (Germany)

***Quantitative reverse-transcription polymerase chain reaction***

Total RNA was extracted from SMMC7721 cells using TRIzol reagent (Takara) according to the manufacturer’s instructions. Reverse transcription was then performed using reverse transcriptase to produce cDNA templates. The quantitative reverse-transcription polymerase chain reaction (RT–qPCR) conditions were set up as follows: 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. The following primers were used: F-TGGCAACAGAATTTGAGTCCT and R-ACCATCTTCTCACTTGGCAT for caspase 3, F-AAGCCAACCCTAGAAAACCTTACCC and R-AGCACCGACATCACCAAATCCTC for caspase-9, F-TGCGTCTACTAAAATGCATGG and R-AACTGAAGGTTAATGGGTCA for PI3K, F-AGCCCTGGACTACCTGCACTCG, R-CTGTGATCTTAATGTGCCCGTCCT for AKT, and F-CCAAAGGCAACAAGCGATCCCGAA and R-CTCCAAGTTCCACACCGTCCA for mTOR.

***Animal and tumour xenograft experiments***

All laboratory animals were carefully monitored, and the animal experiments were reviewed and approved by the Ethical Review Committee of Experimental Animal Welfare at Central South University. Additionally, experiments were performed according to the European Community guidelines for laboratory animal use and care. All animals were housed under specific pathogen-free conditions. Subcutaneous xenograft tumours were established by subcutaneously injecting SMMC7721 cells (1 × 107 cells/mouse) (Shanghai SLAC Laboratory Animal Co., Ltd., Shanghai, China) into 5-week-old male BALB/c nude mice. Mouse weights and tumour volumes were measured every other day using the formula for an ellipsoid (length × width2 × 0.5). When the tumour volume reached approximately 100 mm3, mice were randomly classified into two groups (*n* = 5 mice per group). Mice in the control group received oral administration of distilled water daily, whereas mice in the XHP group received XHP extract (78 mg/kg body weight/day) by oral gavage. After 2 wk, mice were sacrificed to investigate the effect of XHP on subcutaneous xenograft tumours.

***Statistical analysis***

All statistical calculations were performed using GraphPad Prism 7 software (GraphPad Software Company, United States). Data are presented as the means ± SD. One-way analysis of variance (ANOVA) followed by the least significant difference test were conducted to analyse the differences between two groups. A *P* < 0.05 was considered statistically significant.

**RESULTS**

***Components of XHP***

The main components of XHP responsible for its inhibitory effects on HCC were analysed using high-resolution MS. Ion flow diagrams were extracted, and the molecular formulas of the compounds were compared with the information in the literature and database for identification (Figure 1). The following 12 compounds were identified: Valine, 4-gingerol, myrrhone, ricinoleic acid, glycocholic acid, curzerenone, 11-keto-β-boswellic acid, oleic acid, [germacrone](https://www.chemsrc.com/en/cas/6902-91-6_895786.html), 3-acetyl-9,11-dehydro-β-boswellic acid, 5β-androstane-3,17-dione, and 3-acetyl-11-keto-β-boswellic acid (Table 1).

***The inhibitory effects of XHP extract on HCC cell viability and migration***

The effects of XHP extract on HCC cell viability were investigated. Cells were separated into five groups and treated with different concentrations of the XHP extract according to their human equivalent doses (0, 0.3125, 0.625, 1.25, and 2.5 mg/mL), and cell viability was determined using the CCK-8 assay at 12, 24, and 48 h posttreatment. The lowest concentration at which the XHP extract exerted an antitumour effect was 0.625 mg/mL (Figure 2A) at 12 h post-treatment (Figure 2B). The XHP extract inhibited SMMC-7721 cell viability in a dose- and time-dependent manner (Figure 2A and B). Treatment with the effective XHP extract dose, *i.e.*, 0.625 mg/mL, significantly inhibited the migration of SMMC-7721 cells in a time-dependent manner (Figure 2C), as determined using a cell scratch assay.

***XHP extract promotes HCC cell apoptosis in a dose-dependent manner***

Apoptosis plays an important role in anti-tumour therapy. Therefore, annexin V and PI staining were performed to confirm the apoptosis-inducing effect of the XHP extract on SMMC-7721 cells. As shown in Figure 3A, the proportion of apoptotic cells among XHP-treated SMMC-7721 cells was significantly increased, ranging from 11.87% to 82.42%. Moreover, flow cytometry showed that the proportion of SMMC-7721 cells in G2/M phase was substantially increased, from 18.83% to 42.66% (Figure 3B), indicating cell cycle arrest in SMMC-7721 cells after XHP extract treatment. Subsequently, the expression levels of apoptosis-related proteins caspase-3 and caspase-9 were determined. Similar to the results of the flow cytometry analysis, *in vivo* experiments revealed that the protein expression levels of cleaved caspase-3 and cleaved caspase-9 were significantly increased in the subcutaneous xenograft tumours from nude mice. The mRNA expression levels of caspase-3 and caspase-9 also showed similar trends. (Figure 3C). These results were confirmed by *in vitro* experiments. After treatment with different concentrations of the XHP extract, mRNA expression, as well as protein levels of caspase-3 and caspase-9, were increased in SMMC-7721 cells in a dose-dependent manner (Figure 3D). These results were consistent with the data obtained from the cell viability and migration assays. Collectively, our results indicated that XHP extract promotes HCC cell apoptosis in a dose-dependent manner.

***The inhibitory effects of XHP extract on the expression of components of the PI3K/Akt/mTOR signalling pathway in HCC cells***

The PI3K/Akt/mTOR signalling pathway plays significant roles in regulating the cell cycle, apoptosis, and proliferation of HCC. The protein and mRNA expression levels of PI3K, Akt and mTOR were detected to clarify the mechanism involving the PI3K/Akt/mTOR signalling pathway in XHP-induced apoptosis and migration. *In vivo* experiments showed that the ratios of phosphorylated PI3K, Akt, and mTOR to the total protein were noticeably reduced after treatment with the proper concentration of XHP (*i.e.*, 78 mg/kg) (Figure 4A) in the subcutaneous xenograft HCC mouse model. RT–qPCR analysis further confirmed the inhibitory effects of XHP on PI3K, Akt, and mTOR mRNA expression levels (Figure 4B). Moreover, *in vitro* cell experiments showed that XHP extract inhibited the phosphorylation and mRNA expression levels of components of the PI3K/Akt/mTOR signalling pathway in a dose-dependent manner (Figure 4C and D). The data described above indicate that XHP extract inhibits the PI3K/Akt/mTOR signalling pathway in a dose-dependent manner.

***Inhibitory effects of XHP on the growth of subcutaneous xenograft tumours in HCC mice***

A xenograft tumour model was established using male BALB/c nude mice to confirm the antitumour effect of XHP *in vivo*. As shown in Figure 5A, the subcutaneous xenograft tumours were significantly reduced after XHP treatment compared to the control group. This result was further confirmed by calculating the volume (Figure 5B) and weight (Figure 5C). However, the overall body weight of the nude mice did not decrease. A significant difference in body weight was not observed between the two groups (Figure 5D). The *in vivo* experiment revealed the protective effect of XHP on preventing the progression of HCC tumorigenesis in the established mouse model.

**DISCUSSION**

HCC is an aggressive tumour characterized by a high degree of proliferation and invasion. Considering the limitations of current treatment methods, more effective treatments with reduced toxicity must be established. Notably, traditional Chinese medicine and its prepared compounds may serve as supplementary treatments for HCC[16]. XHP is an effective antitumour drug included in the traditional Chinese medicine system. Studies have shown that XHP inhibits the proliferation and metastasis of tumour cells[12]. However, the specific effects and mechanisms of XHP in HCC progression have not been discussed. In this study, we showed that XHP exerts an inhibitory effect on HCC cells by *in vivo* and *in vitro* experiments. Moreover, we identified that XHP reduces the viability and migration of tumour cells *via* the PI3K/Akt/mTOR signalling pathway. This pathway is involved in tumour cell apoptosis of HCC. Thus, the results of our study confirmed the antitumour properties of XHP in HCC, as well as the potential mechanism.

Abnormal activation of the PI3K/Akt/mTOR signalling pathway is a crucial factor promoting HCC development. It induces HCC cell proliferation and cell cycle arrest by inhibiting apoptosis[18-20]. The combination of growth factors and receptors in tumour cells activates PI3K, increasing cell viability, proliferation, and migration[21,22]. PI3K increases the viability of tumour cells in HCC by regulating apoptosis[23-25]. Moreover, Akt serves as a downstream target and mediates the antiapoptotic effects of PI3K, with Akt regulating the sequential steps of apoptotic signalling[26]. Akt activates many downstream proteins, including mTOR, Bcl-2-associated agonist of cell death (Bad), and GSK3[27,28]. In terms of cell cycle regulation, Akt expression suppresses the activity of GSK3β, reduces the expression of cyclin D1 and promotes the expression of Rb, thereby promoting cell cycle progression[29]. Caspase-3 is the primary executioner of apoptosis. It specifically lyses poly (ADP-ribose) polymerase and other substrates, leading to DNA fragmentation and eventual apoptosis[30]. Thus, this mechanism plays an important role in the inhibition of cancer cell invasion and metastasis[31]. The caspase-3 zymogen is regulated by caspase-9[19]. Activation of PI3K may inhibit caspase 3 activity and DNA fragmentation in several cell types. Therefore, PI3K is essential for maintaining cell viability. Furthermore, Bcl-2 has an important role in caspase-3-mediated apoptosis[32]. mTOR, a downstream target of Akt, phosphorylates Bad (an apoptotic molecule) and induces the expression of antiapoptotic Bcl-2 family proteins. Moreover, it inhibits the release of cytochrome c from the mitochondria, thereby inhibiting the activation of caspase-9 and caspase-3 and increasing cell viability[19,23,33]. Thus, the PI3K/Akt/mTOR signalling pathway regulates apoptosis by modulating caspase-9 and caspase-3 activity.

In this study, the high resolution MS was used to analyze the compound composition of XHP. Twelve compounds in XHP were identified, which were valine, 4-gingerol, myrrhone, ricinoleic acid, glycocholic acid, curzerenone, 11-keto-β-boswellic acid, oleic acid, [germacrone](https://www.chemsrc.com/en/cas/6902-91-6_895786.html), 3-acetyl-9,11-dehydro-β-boswellic acid, 5β-androstane-3,17-dione, and 3-acetyl-11-keto-β-boswellic acid. Among them, Curcuzederone, 11-keto-α-Boswellic Acid, Oleic Acid and 3-acetyl-11-keto-beta-Boswellic Acid have been reported to have good anticancer activity. They inhibit tumor growth by promoting apoptosis of cancer cells[34-38]. We speculate that the anti-tumor effect of XHP may be through these active ingredients, and we will further clarify the role of the active ingredients in future studies.

Activation of apoptosis is one of the crucial strategies for anti-tumour therapy[10,39-41]. The results of this study revealed that XHP effectively suppressed the growth of HCC cells at a concentration of 0.625 mg/mL in a dose- and time-dependent manner. The scratch assay revealed that XHP treatment also inhibited HCC cell migration in a time-dependent manner. Apoptosis experiments further confirmed that XHP treatment arrested the cell cycle in G2/M phase, facilitating the apoptosis of HCC cells. Based on these results, XHP exerts an inhibitory effect on the growth and migration of tumour cells by inducing apoptosis.

We further investigated the effects of XHP on the PI3K/Akt/mTOR pathway. XHP treatment inhibited the PI3K/Akt/mTOR pathway both *in vivo* and *in vitro* in a dose-dependent manner. Moreover, the activities of cleaved caspase-9 and cleaved caspase-3 were increased in response to XHP treatment. Therefore, we hypothesized that XHP may cause cell cycle arrest *via* the PI3K/Akt/mTOR signalling pathway and regulates the expression of the apoptosis executioner proteins cleaved caspase-9 and cleaved caspase-3, thereby promoting apoptosis in HCC and inhibiting the growth and migration of tumour cells. In addition, an *in vivo* experiment in nude mice further confirmed the inhibitory effects of XHP on HCC cells, as evidenced by decreases in tumour volume and weight. However, the effect of XHP on the mitochondrial apoptotic pathway, such as autophagy, was not detected in this study. The exact step that XHP modulated in the PI3K/Akt/mTOR pathway is unclear. Therefore, further research is needed to obtain a comprehensive interpretation of the XHP-regulated antitumour effect.

**CONCLUSION**

In conclusion, XHP increases cleaved caspase-9 and cleaved caspase-3 activities by inhibiting the PI3K/Akt/mTOR signalling pathway and induces apoptosis and cell cycle arrest. Consequently, XHP inhibits the growth, migration, and proliferation of HCC cells. Our study provides a better understanding of the antitumour effects of XHP and reveals the underlying mechanism. The findings of this study suggest that XHP may serve as a supplementary medicine in HCC treatment.

**ARTICLE HIGHLIGHTS**

***Research background***

Xihuang pills (XHP) are a traditional Chinese preparation with antitumour properties. They inhibit the growth of breast cancer, glioma, and other tumours by regulating the phosphoinositide 3-kinase/protein kinase-B/mechanistic target of rapamycin (PI3K/Akt/mTOR) signalling pathway. However, the effects and mechanisms of action of XHP in hepatocellular carcinoma (HCC) remain unclear. Regulation of the PI3K/Akt/mTOR signalling pathway effectively inhibits the progression of HCC.

***Research motivation***

We hypothesized that XHP might play a role in inhibiting HCC through the PI3K/Akt/mTOR signalling pathway.

***Research objectives***

To confirm the effect of XHP on HCC and the possible mechanisms involved.

***Research methods***

The chemical constituents and active components of XHP were analysed using ultra-performance liquid chromatography-quadrupole time of flight mass spectrometry (MS) (UPLC-Q-TOF-MS). First, cell-based experiments and *in vivo* xenograft tumour experiments were utilized to evaluate the effect of XHP on HCC tumorigenesis. Cell viability was assessed using the CCK-8 assay, followed by an assessment of cell migration using a wound healing assay. Second, the effect of XHP on the apoptosis of SMMC-7721 cells was evaluated. Third, Western blotting and reverse-transcription polymerase chain reaction were performed to confirm the effects of XHP on the protein and mRNA expression of components of the PI3K/Akt/mTOR signalling pathway. Finally, the effects of XHP on the tumorigenesis of subcutaneous hepatocellular tumours in nude mice were assessed.

***Research results***

The 12 compounds were identified in XHP by high-resolution MS. The cell viability assay results showed that treatment with 0.625 mg/mL XHP extract decreased HCC cell viability after 12 h. Moreover, XHP significantly inhibited cell migration and resulted in cell cycle arrest and apoptosis. Furthermore, XHP downregulated the PI3K/Akt/mTOR signalling pathway, which activated apoptosis executioner proteins (*e.g.*, caspase-9 and caspase-3). The inhibitory effects of XHP on HCC cell growth were determined *in vivo* by analysing the tumour xenograft volumes and weights.

***Research conclusions***

XHP inhibited HCC cell growth and migration by stimulating apoptosis *via* the downregulation of the PI3K/Akt/mTOR signalling pathway, followed by the activation of caspase-9 and caspase-3. Our findings clarified that the antitumour effects of XHP on HCC cells are mediated by the PI3K/Akt/mTOR signalling pathway.

***Research perspectives***

Our findings revealed that XHP may be a potential complementary therapy for HCC.

**REFERENCES**

1 **Dasgupta P**, Henshaw C, Youlden DR, Clark PJ, Aitken JF, Baade PD. Global Trends in Incidence Rates of Primary Adult Liver Cancers: A Systematic Review and Meta-Analysis. *Front Oncol* 2020; **10**: 171 [PMID: 32185125 DOI: 10.3389/fonc.2020.00171]

2 **Ko KL**, Mak LY, Cheung KS, Yuen MF. Hepatocellular carcinoma: recent advances and emerging medical therapies. *F1000Res* 2020; **9** [PMID: 32595940 DOI: 10.12688/f1000research.24543.1]

3 **Rahmani F**, Ziaeemehr A, Shahidsales S, Gharib M, Khazaei M, Ferns GA, Ryzhikov M, Avan A, Hassanian SM. Role of regulatory miRNAs of the PI3K/AKT/mTOR signaling in the pathogenesis of hepatocellular carcinoma. *J Cell Physiol* 2020; **235**: 4146-4152 [PMID: 31663122 DOI: 10.1002/jcp.29333]

4 **Zhou Q**, Lui VW, Yeo W. Targeting the PI3K/Akt/mTOR pathway in hepatocellular carcinoma. *Future Oncol* 2011; **7**: 1149-1167 [PMID: 21992728 DOI: 10.2217/fon.11.95]

5 **Brotelle T**, Bay JO. [PI3K-AKT-mTOR pathway: Description, therapeutic development, resistance, predictive/prognostic biomarkers and therapeutic applications for cancer]. *Bull Cancer* 2016; **103**: 18-29 [PMID: 26582734 DOI: 10.1016/j.bulcan.2015.09.011]

6 **Fresno Vara JA**, Casado E, de Castro J, Cejas P, Belda-Iniesta C, González-Barón M. PI3K/Akt signalling pathway and cancer. *Cancer Treat Rev* 2004; **30**: 193-204 [PMID: 15023437 DOI: 10.1016/j.ctrv.2003.07.007]

7 **Polivka J Jr**, Janku F. Molecular targets for cancer therapy in the PI3K/AKT/mTOR pathway. *Pharmacol Ther* 2014; **142**: 164-175 [PMID: 24333502 DOI: 10.1016/j.pharmthera.2013.12.004]

8 **Janku F**. Phosphoinositide 3-kinase (PI3K) pathway inhibitors in solid tumors: From laboratory to patients. *Cancer Treat Rev* 2017; **59**: 93-101 [PMID: 28779636 DOI: 10.1016/j.ctrv.2017.07.005]

9 **Zhang Y**, Xie C, Li A, Liu X, Xing Y, Shen J, Huo Z, Zhou S, Liu X, Xie Y, Cao W, Ma Y, Xu R, Cai S, Tang X, Ma D. PKI-587 enhances chemosensitivity of oxaliplatin in hepatocellular carcinoma through suppressing DNA damage repair pathway (NHEJ and HR) and PI3K/AKT/mTOR pathway. *Am J Transl Res* 2019; **11**: 5134-5149 [PMID: 31497229]

10 **Pistritto G**, Trisciuoglio D, Ceci C, Garufi A, D'Orazi G. Apoptosis as anticancer mechanism: function and dysfunction of its modulators and targeted therapeutic strategies. *Aging (Albany NY)* 2016; **8**: 603-619 [PMID: 27019364 DOI: 10.18632/aging.100934]

11 **Hong SW**, Jung KH, Lee HS, Choi MJ, Son MK, Zheng HM, Hong SS. SB365 inhibits angiogenesis and induces apoptosis of hepatocellular carcinoma through modulation of PI3K/Akt/mTOR signaling pathway. *Cancer Sci* 2012; **103**: 1929-1937 [PMID: 22909393 DOI: 10.1111/j.1349-7006.2012.02409.x]

12 **Guo Q**, Xu X, He S, Yuan Y, Chen S, Hua B. Xi huang pills enhance the tumor treatment efficacy when combined with chemotherapy: A meta-analysis and systematic review. *J Cancer Res Ther* 2018; **14**: S1012-S1018 [PMID: 30539838 DOI: 10.4103/0973-1482.192795]

13 **Mao D**, Feng L, Huang S, Zhang S, Peng W, Zhang S. Meta-Analysis of Xihuang Pill Efficacy When Combined with Chemotherapy for Treatment of Breast Cancer. *Evid Based Complement Alternat Med* 2019; **2019**: 3502460 [PMID: 30992708 DOI: 10.1155/2019/3502460]

14 **Li XY**, Su L, Jiang YM, Gao WB, Xu CW, Zeng CQ, Song J, Xu Y, Weng WC, Liang WB. The Antitumor Effect of Xihuang Pill on Treg Cells Decreased in Tumor Microenvironment of 4T1 Breast Tumor-Bearing Mice by PI3K/AKT~AP-1 Signaling Pathway. *Evid Based Complement Alternat Med* 2018; **2018**: 6714829 [PMID: 29849718 DOI: 10.1155/2018/6714829]

15 **Fu J**, Zhu SH, Xu HB, Xu YQ, Wang X, Wang J, Kong PS. Xihuang pill potentiates the anti-tumor effects of temozolomide in glioblastoma xenografts through the Akt/mTOR-dependent pathway. *J Ethnopharmacol* 2020; **261**: 113071 [PMID: 32603676 DOI: 10.1016/j.jep.2020.113071]

16 **Shao M**, He Z, Yin Z, Ma P, Xiao Q, Song Y, Huang Z, Ma Y, Qiu Y, Zhao A, Zhou T, Wang Q. Xihuang Pill Induces Apoptosis of Human Glioblastoma U-87 MG Cells *via* Targeting ROS-Mediated Akt/mTOR/FOXO1 Pathway. *Evid Based Complement Alternat Med* 2018; **2018**: 6049498 [PMID: 30046342 DOI: 10.1155/2018/6049498]

17 **Nair A**, Morsy MA, Jacob S. Dose translation between laboratory animals and human in preclinical and clinical phases of drug development. *Drug Dev Res* 2018; **79**: 373-382 [PMID: 30343496 DOI: 10.1002/ddr.21461]

18 **Golob-Schwarzl N**, Krassnig S, Toeglhofer AM, Park YN, Gogg-Kamerer M, Vierlinger K, Schröder F, Rhee H, Schicho R, Fickert P, Haybaeck J. New liver cancer biomarkers: PI3K/AKT/mTOR pathway members and eukaryotic translation initiation factors. *Eur J Cancer* 2017; **83**: 56-70 [PMID: 28715695 DOI: 10.1016/j.ejca.2017.06.003]

19 **Wu T**, Dong X, Yu D, Shen Z, Yu J, Yan S. Natural product pectolinarigenin inhibits proliferation, induces apoptosis, and causes G2/M phase arrest of HCC *via* PI3K/AKT/mTOR/ERK signaling pathway. *Onco Targets Ther* 2018; **11**: 8633-8642 [PMID: 30584322 DOI: 10.2147/OTT.S186186]

20 **Wu Y**, Zhang Y, Qin X, Geng H, Zuo D, Zhao Q. PI3K/AKT/mTOR pathway-related long non-coding RNAs: roles and mechanisms in hepatocellular carcinoma. *Pharmacol Res* 2020; **160**: 105195 [PMID: 32916254 DOI: 10.1016/j.phrs.2020.105195]

21 **Goncalves MD**, Hopkins BD, Cantley LC. Phosphatidylinositol 3-Kinase, Growth Disorders, and Cancer. *N Engl J Med* 2018; **379**: 2052-2062 [PMID: 30462943 DOI: 10.1056/NEJMra1704560]

22 **Jiang S**, Wang Q, Feng M, Li J, Guan Z, An D, Dong M, Peng Y, Kuerban K, Ye L. C2-ceramide enhances sorafenib-induced caspase-dependent apoptosis *via* PI3K/AKT/mTOR and Erk signaling pathways in HCC cells. *Appl Microbiol Biotechnol* 2017; **101**: 1535-1546 [PMID: 27807662 DOI: 10.1007/s00253-016-7930-9]

23 **Khemlina G**, Ikeda S, Kurzrock R. The biology of Hepatocellular carcinoma: implications for genomic and immune therapies. *Mol Cancer* 2017; **16**: 149 [PMID: 28854942 DOI: 10.1186/s12943-017-0712-x]

24 **Song L**, Luo Y, Li S, Hong M, Wang Q, Chi X, Yang C. ISL Induces Apoptosis and Autophagy in Hepatocellular Carcinoma *via* Downregulation of PI3K/AKT/mTOR Pathway *in vivo* and in vitro. *Drug Des Devel Ther* 2020; **14**: 4363-4376 [PMID: 33116421 DOI: 10.2147/DDDT.S270124]

25 **Xue S**, Zhou Y, Zhang J, Xiang Z, Liu Y, Miao T, Liu G, Liu B, Liu X, Shen L, Zhang Z, Li M, Miao Q. Anemoside B4 exerts anti-cancer effect by inducing apoptosis and autophagy through inhibiton of PI3K/Akt/mTOR pathway in hepatocellular carcinoma. *Am J Transl Res* 2019; **11**: 2580-2589 [PMID: 31105864]

26 **Lee YI**, Kang-Park S, Do SI, Lee YI. The hepatitis B virus-X protein activates a phosphatidylinositol 3-kinase-dependent survival signaling cascade. *J Biol Chem* 2001; **276**: 16969-16977 [PMID: 11278872 DOI: 10.1074/jbc.M011263200]

27 **Aoki M**, Fujishita T. Oncogenic Roles of the PI3K/AKT/mTOR Axis. *Curr Top Microbiol Immunol* 2017; **407**: 153-189 [PMID: 28550454 DOI: 10.1007/82\_2017\_6]

28 **Coutte L**, Dreyer C, Sablin MP, Faivre S, Raymond E. [PI3K-AKT-mTOR pathway and cancer]. *Bull Cancer* 2012; **99**: 173-180 [PMID: 21742593 DOI: 10.1684/bdc.2011.1384]

29 **Li A**, Zhang R, Zhang Y, Liu X, Wang R, Liu J, Liu X, Xie Y, Cao W, Xu R, Ma Y, Cai W, Wu B, Cai S, Tang X. BEZ235 increases sorafenib inhibition of hepatocellular carcinoma cells by suppressing the PI3K/AKT/mTOR pathway. *Am J Transl Res* 2019; **11**: 5573-5585 [PMID: 31632530]

30 **Crowley LC**, Waterhouse NJ. Detecting Cleaved Caspase-3 in Apoptotic Cells by Flow Cytometry. *Cold Spring Harb Protoc* 2016; **2016** [PMID: 27803251 DOI: 10.1101/pdb.prot087312]

31 **Zhou M**, Liu X, Li Z, Huang Q, Li F, Li CY. Caspase-3 regulates the migration, invasion and metastasis of colon cancer cells. *Int J Cancer* 2018; **143**: 921-930 [PMID: 29524226 DOI: 10.1002/ijc.31374]

32 **Tsujimoto Y**. Role of Bcl-2 family proteins in apoptosis: apoptosomes or mitochondria? *Genes Cells* 1998; **3**: 697-707 [PMID: 9990505 DOI: 10.1046/j.1365-2443.1998.00223.x]

33 **Li TT**, Zhu D, Mou T, Guo Z, Pu JL, Chen QS, Wei XF, Wu ZJ. IL-37 induces autophagy in hepatocellular carcinoma cells by inhibiting the PI3K/AKT/mTOR pathway. *Mol Immunol* 2017; **87**: 132-140 [PMID: 28433890 DOI: 10.1016/j.molimm.2017.04.010]

34 **Al-Amin M**, Eltayeb NM, Khairuddean M, Salhimi SM. Bioactive chemical constituents from *Curcuma caesia* Roxb. rhizomes and inhibitory effect of curcuzederone on the migration of triple-negative breast cancer cell line MDA-MB-231. *Nat Prod Res* 2021; **35**: 3166-3170 [PMID: 31726856 DOI: 10.1080/14786419.2019.1690489]

35 **Schmiech M**, Ulrich J, Lang SJ, Büchele B, Paetz C, St-Gelais A, Syrovets T, Simmet T. 11-Keto-α-Boswellic Acid, a Novel Triterpenoid from *Boswellia* spp. with Chemotaxonomic Potential and Antitumor Activity against Triple-Negative Breast Cancer Cells. *Molecules* 2021; **26** [PMID: 33445710 DOI: 10.3390/molecules26020366]

36 **Jung S**, Lee S, Lee H, Yoon J, Lee EK. Oleic acid-embedded nanoliposome as a selective tumoricidal agent. *Colloids Surf B Biointerfaces* 2016; **146**: 585-589 [PMID: 27424089 DOI: 10.1016/j.colsurfb.2016.06.058]

37 **Mizushina Y**, Takeuchi T, Sugawara F, Yoshida H. Anti-cancer targeting telomerase inhibitors: β-rubromycin and oleic acid. *Mini Rev Med Chem* 2012; **12**: 1135-1143 [PMID: 22876944 DOI: 10.2174/138955712802762220]

38 **Riaz A**, Rasul A, Kanwal N, Hussain G, Shah MA, Sarfraz I, Ishfaq R, Batool R, Rukhsar F, Adem Ş. Germacrone: A Potent Secondary Metabolite with Therapeutic Potential in Metabolic Diseases, Cancer and Viral Infections. *Curr Drug Metab* 2020; **21**: 1079-1090 [PMID: 32723267 DOI: 10.2174/1389200221999200728144801]

39 **Goldar S**, Khaniani MS, Derakhshan SM, Baradaran B. Molecular mechanisms of apoptosis and roles in cancer development and treatment. *Asian Pac J Cancer Prev* 2015; **16**: 2129-2144 [PMID: 25824729 DOI: 10.7314/apjcp.2015.16.6.2129]

40 **Lin Y**, Chen Y, Wang S, Ma J, Peng Y, Yuan X, Lv B, Chen W, Wei Y. Plumbagin induces autophagy and apoptosis of SMMC-7721 cells *in vitro* and in vivo. *J Cell Biochem* 2019; **120**: 9820-9830 [PMID: 30536473 DOI: 10.1002/jcb.28262]

41 **Wang K**. Autophagy and apoptosis in liver injury. *Cell Cycle* 2015; **14**: 1631-1642 [PMID: 25927598 DOI: 10.1080/15384101.2015.1038685]

**Footnotes**

**Institutional animal care and use committee statement:** This study was reviewed and approved by the Ethics Review Committee of Experimental Animal Welfare at the Central South University in Changsha, China.

**Conflict-of-interest statement:** The authors declare that they have no competing interests.

**Data sharing statement:** Dataset available from the corresponding author at 003640@hnucm.edu.cn. Participants gave informed consent for data sharing.

**ARRIVE guidelines statement:** The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

**Open-Access:** This article is an open-access article that was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution NonCommercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: https://creativecommons.org/Licenses/by-nc/4.0/

**Provenance and peer review:** Unsolicited article; Externally peer reviewed.

**Peer-review model:** Single blind

**Peer-review started:** September 7, 2021

**First decision:** December 4, 2021

**Article in press:**

**Specialty type:** Oncology

**Country/Territory of origin:** China

**Peer-review report’s scientific quality classification**

Grade A (Excellent): 0

Grade B (Very good): B, B

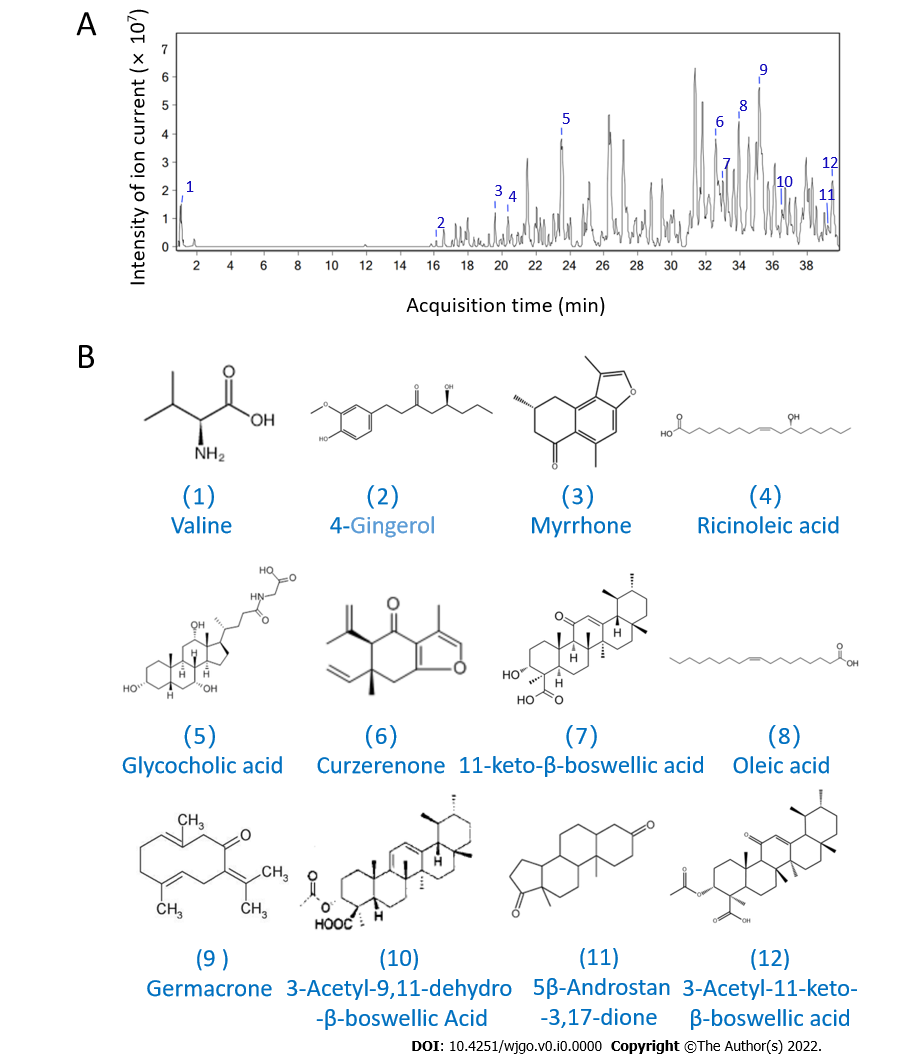
Grade C (Good): 0

Grade D (Fair): 0

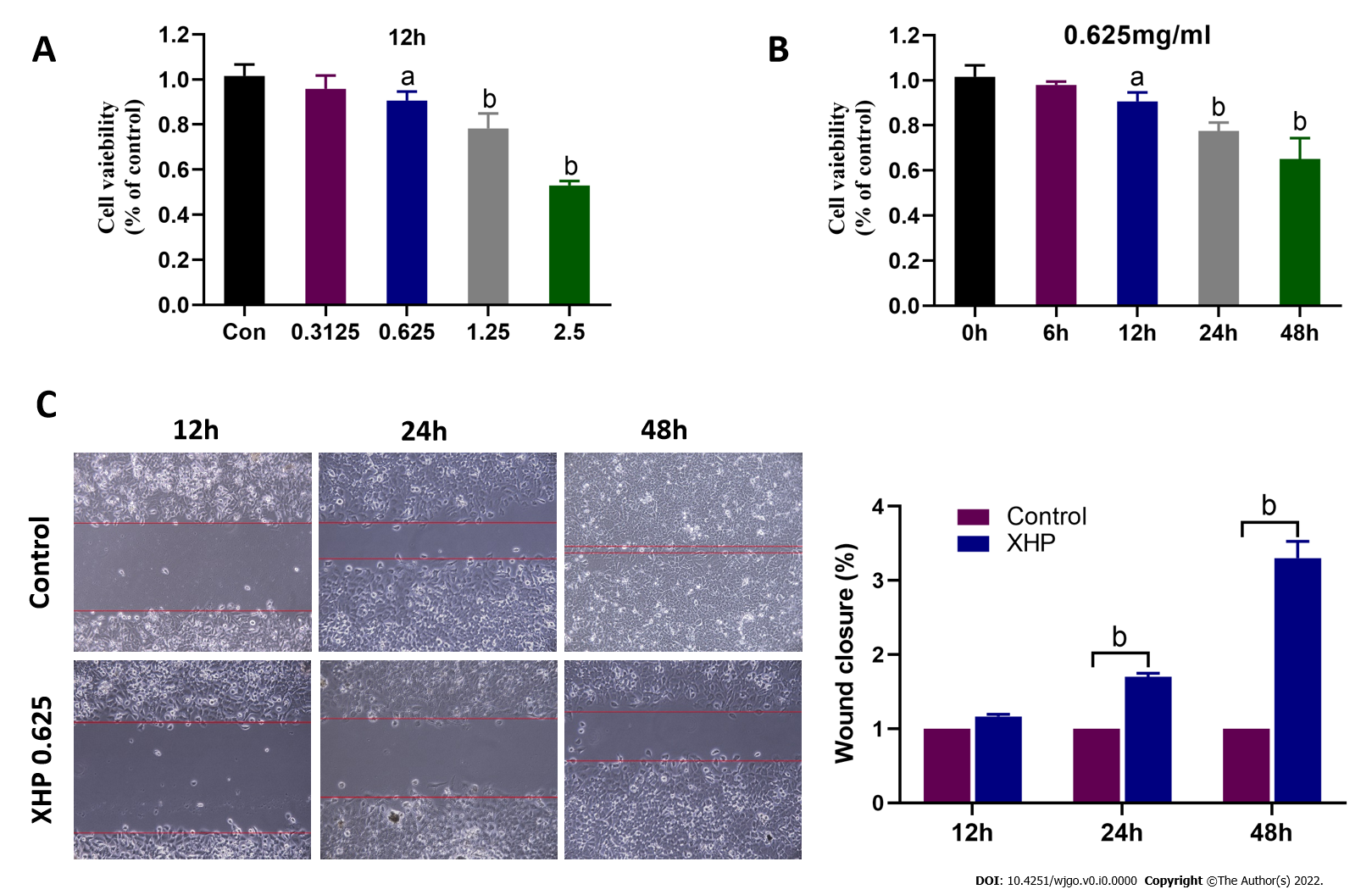
Grade E (Poor): 0

**P-Reviewer:** Hassaan NA, Egypt; Prasetyo EP, Indonesia **S-Editor:** Fan JR **L-Editor:** A **P-Editor:** Fan JR

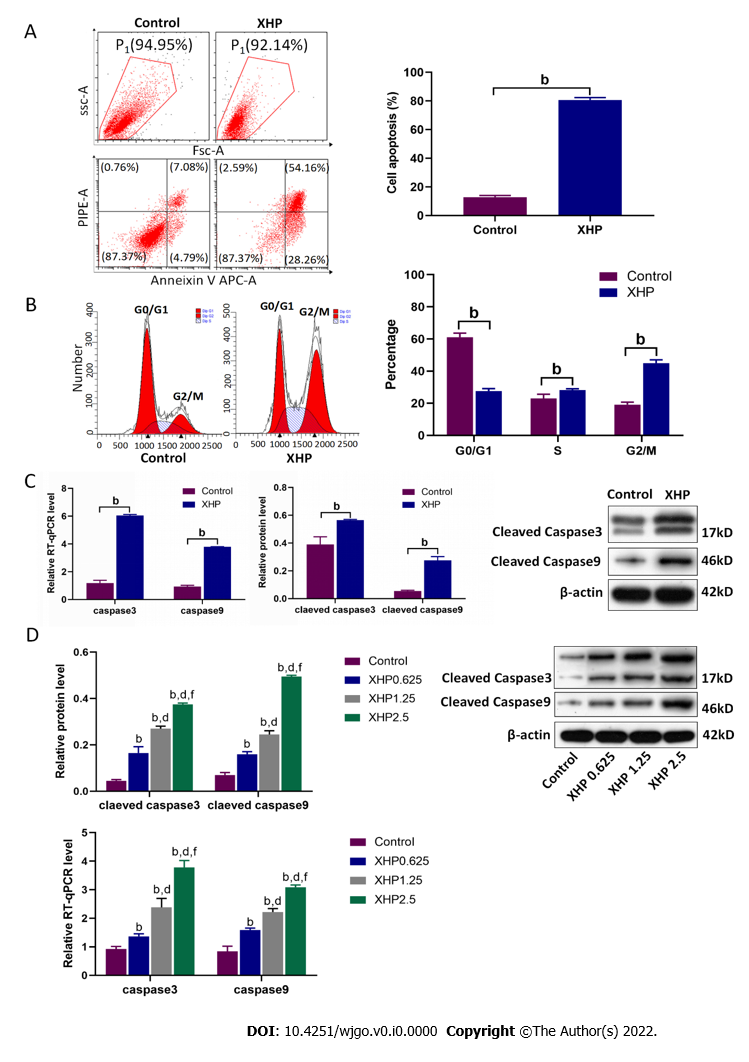
**Figure Legends**



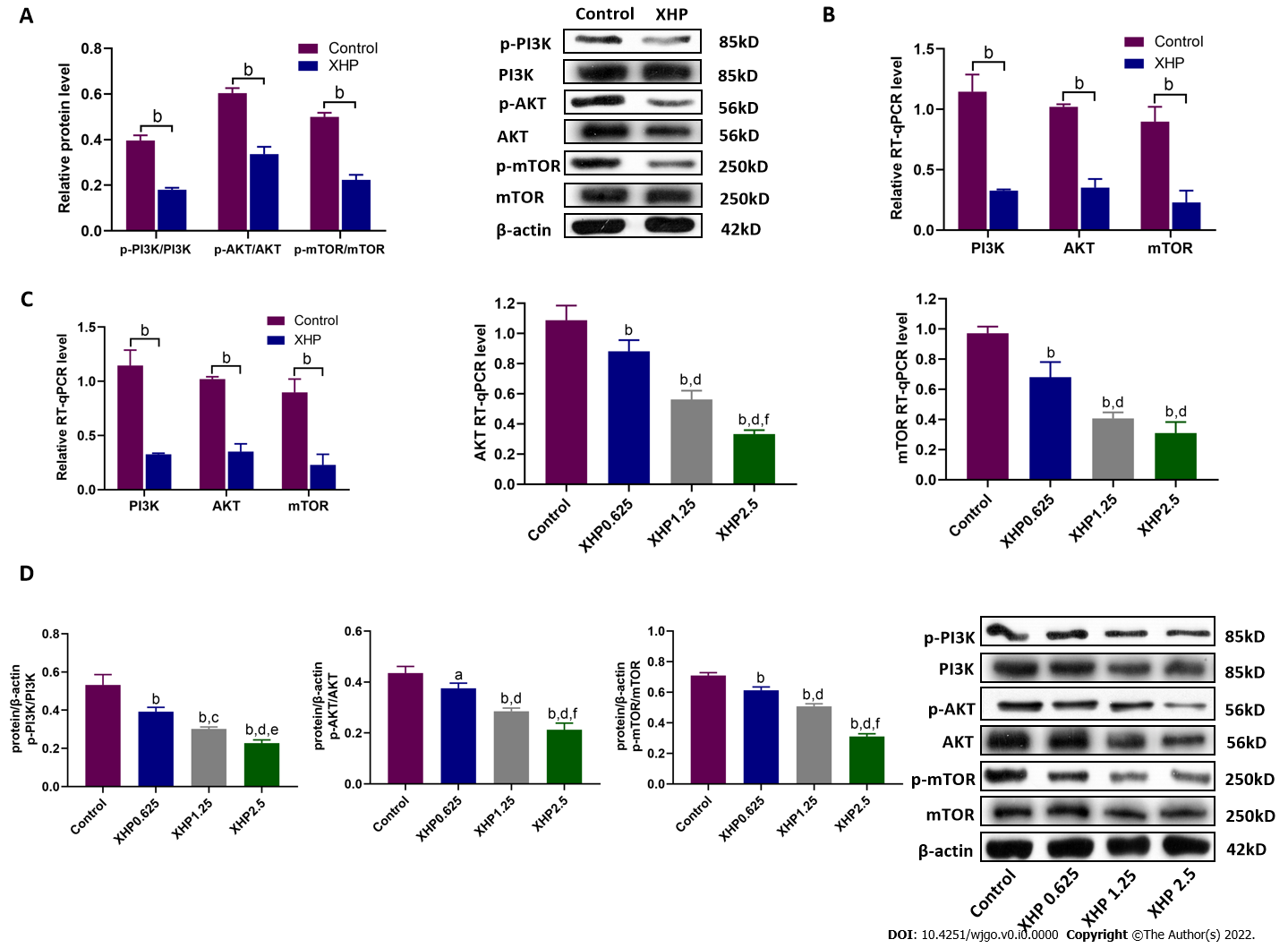
**Figure 1** **Components of Xihuang Pills.** The fingerprint of the Xihuang pill was determined using high-resolution mass spectrometry. A: The 12 compounds were labelled according to chromatographic retention times, and their molecular structures were analysed; B: The chemical formulae of 12 compounds. The chemical structures of 12 compounds and chemical formulae obtained from chemsrc (https://m.chemsrc.com/mip/), according to the compound numbering scheme in Table 1.



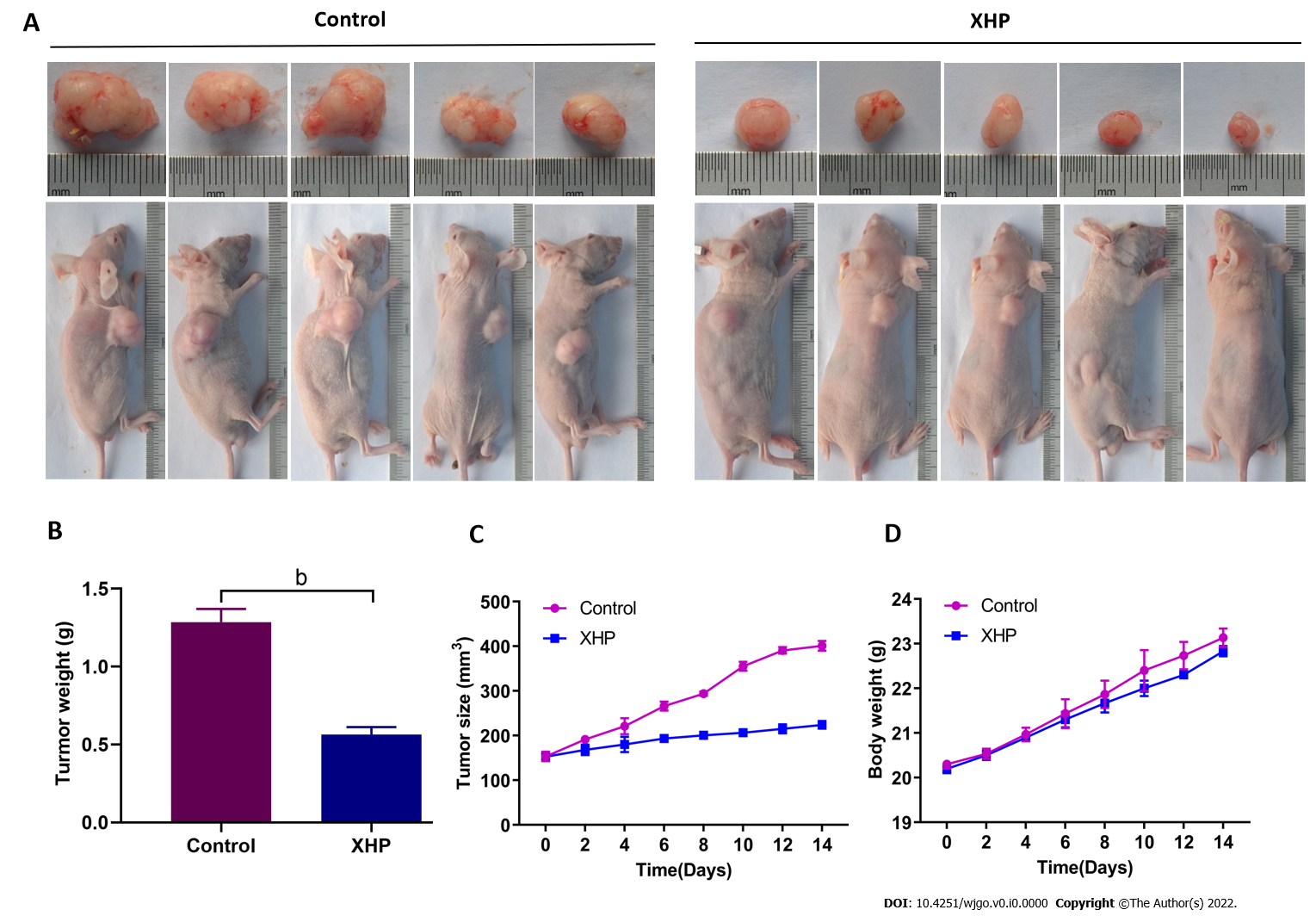
**Figure 2** **Xihuang pills extract inhibits the growth and migration of SMMC7721 cells.** Cells were treated with different concentrations of the Xihuang pills (XHP) extract (0, 0.3125, 0.625, 1.25, and 2.5 mg/mL) for 12 h, 24 h, and 48 h. Cell viability was measured using Cell Counting Kit-8. A: A representative graph showing the effects of treatment with various concentrations of the XHP extract for 12 h; B: A representative graph showing that treatment with 0.625 mg/mL XHP extract induces a decrease in cell viability after 12 h; C: The migration of SMMC-7721 cells was measured using a cell scratch assay with or without the administration of 0.625 mg/mL XHP for 48 h. Data are presented as the means ± SD. a*P* < 0.05 and b*P* < 0.01compared with the control group.



**Figure 3** **SMMC-7721 cell apoptosis is induced by Xihuang pills extract.** SMMC-7721 cells were stained with annexin V/propidium iodide and fluorescein isothiocyanate after treatment with or without 0.625 mg/mL Xihuang pills (XHP) extract for 12 h. A and B: Flow cytometry was used to detect the number of apoptotic cells (A) and the cell cycle distribution (B); C: Cleaved caspase-3 and cleaved caspase-9 protein expression levels were detected by Western blotting, and caspase-3 and caspase-9 mRNA expression levels were detected by reverse-transcription polymerase chain reaction (RT–qPCR); D: After treating SMMC-7721 cells with different concentrations of the XHP extract, cleaved caspase-3 and cleaved caspase-9 protein expression levels were determined using Western blotting, whereas caspase-3 and caspase-9 mRNA expression levels were determined using RT–qPCR. The experiment was repeated three times, and the data are presented as the means ± SD. a*P* < 0.05 and b*P* < 0.01compared with the control group; c*P* < 0.05 and d*P* < 0.01compared with the XHP 0.625 group; e*P* < 0.05 and f*P* < 0.01compared with the XHP 1.25 group.



**Figure 4** **Inhibitory effects of Xihuang pills extract on the expression of components of the phosphoinositide 3-kinase/protein kinase-B/mechanistic target of rapamycin signalling pathway both *in vivo* and *in vitro*.** A: The protein expression levels and ratios of phosphoinositide 3-kinase/protein kinase-B/mechanistic target of rapamycin (PI3K/Akt/mTOR) and p-PI3K/p-Akt/p-mTOR in SMMC-7721 cells were detected using Western blotting; B: PI3K/Akt/mTOR mRNA expression levels in SMMC-7721 cells, as determined using reverse-transcription polymerase chain reaction (RT-qPCR); C: PI3K/Akt/mTOR mRNA expression levels in SMMC-7721 cells after treatment with different concentrations of Xihuang pills (XHP), as measured using RT–qPCR; D: Levels of the PI3K/Akt/mTOR and p-PI3K/p-Akt/p-mTOR proteins in SMMC-7721 cells treated with different concentrations of the XHP extract were detected using Western blotting. Relative protein and mRNA expression levels are shown in representative histograms. The experiment was repeated three times, and the data are presented as the means ± SD. a*P* < 0.05 and b*P* < 0.01compared with the control group; c*P* < 0.05 and d*P* < 0.01compared with the XHP 0.625 group; e*P* < 0.05 and f*P* < 0.01compared with the XHP 1.25 group.



**Figure 5 Xihuang pills treatment inhibits tumour growth *in vivo*.** SMMC7721 cells were injected subcutaneously into the lower right side of 5-week-old BALB/c male nude mice. After a model of subcutaneous xenograft tumours was successfully established in nude mice, animals were randomly divided into two groups. Each group was then treated with either 0.2 mL of distilled water (control) or 78 mg/kg of body weight Xihuang pills. A: Representative images of subcutaneous xenograft tumours at the end of treatment; B: Subcutaneous tumour weights measured at the end of the treatment; C: Average subcutaneous tumour volume measured every 2 d; D: Average mouse body weight measured every 2 d. The experiment was repeated three times, and the data are presented as the means ± SD. The results were analysed using one-way analysis of variance, followed by the least significant difference test. a*P* < 0.05 and b*P* < 0.01compared with the control group. XHP: Xihuang pills.

**Table 1 Analysis information of 12 compounds in Xihuang pills**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| [**Number**](javascript:;) | **RT (min)** | **Mass** | **Molecular formula** | **Name** |
| 1 | 1.088 | 117.0778 | C5H11NO2 | Valine |
| 2 | 16.110 | 267.1588 | C15H22O4 | 4-Gingerol |
| 3 | 19.579 | 228.1131 | C15H16O2 | Myrrhone |
| 4 | 20.357 | 321.2403 | C18H34O3 | Ricinoleic acid |
| 5 | 23.486 | 465.3060 | C26H43NO6 | Glycocholic acid |
| 6 | 32.635 | 231.1366 | C15H18O2 | Curzerenone |
| 7 | 33.057 | 470.3377 | C30H46O4 | 11-keto-β-boswellic acid |
| 8 | 33.977 | 282.4610 | C18H34O2 | Oleic acid |
| 9 | 35.078 | 218.1674 | C15H22O | [Germacrone](https://www.chemsrc.com/en/cas/6902-91-6_895786.html) |
| 10 | 36.543 | 496.3517 | C32H48O4 | 3-Acetyl-9,11-dehydro-β-boswellic Acid |
| 11 | 38.816 | 288.2084 | C19H28O2 | 5β-androstane-3,17-dione |
| 12 | 39.500 | 512.3485 | C32H48O5 | 3-Acetyl-11-keto-β-boswellic acid |