

MULBERROSIDE A EXTRACT ENHANCES THE ANTITUMOR EFFICACY OF PACLITAXEL BY INHIBITING THE HIF-1 α /SHH SIGNALING PATHWAY IN LEWIS LUNG CARCINOMA-BEARING MICE

A. Q. Liu, B. Yan and R. C. Jiang*

Department of Cancer Precision and Conversion, Tianjin Cancer Hospital Airport Hospital, 300308, Tianjin, China.

*Corresponding author's Email: jiangrich88688@126.com

ABSTRACT

The present work set out to determine whether Mulberroside A (MA) extract could boost the antitumor efficacy of paclitaxel (PTX) in Lewis lung carcinoma (LLC)-bearing mice through modulation of the HIF-1 α /SHH signaling pathway. LLC-bearing C57BL/6 mice were randomized into control, model, PTX, MA, PTX+MA, PTX+MA+HIF-1 α inhibitor (YC-1) and PTX+MA+SHH inhibitor (Cyclopamine) groups. Tumor growth, apoptosis (TUNEL), microvascular density (MVD and CD31 staining), and histopathology (H&E) were assessed. Expression of HIF-1 α /SHH pathway components and downstream targets (VEGF, Bcl-2 and caspase-3) was measured by qPCR, and Western blot. PTX+MA synergistically inhibited tumor growth and reduced MVD versus PTX alone ($P \leq 0.05$), while a higher level of apoptotic cells was observed ($P \leq 0.05$). This was accompanied by downregulation of HIF-1 α , SHH, SMO, GLI1, VEGF, and Bcl-2, and upregulation of caspase-3 ($P \leq 0.05$). Addition of HIF-1 α or SHH inhibitors partially reversed these effects, confirming pathway involvement. MA enhances PTX antitumor efficacy in LLC-bearing mice by suppressing the HIF-1 α /SHH axis, thereby inhibiting angiogenesis and promoting apoptosis. This combination may represent a potential strategy to improve chemotherapy efficacy in lung cancer.

Keywords: Mulberroside A extract; HIF-1 α /SHH pathway; lung cancer; tumor-bearing mice; paclitaxel

This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0>)

<https://doi.org/10.36899/JAPS.2026.6.0149>

Published first online October 01, 2026

INTRODUCTION

Lung cancer remains a formidable clinical challenge, primarily due to acquired therapeutic resistance (Zhao *et al.*, 2023). Paclitaxel (PTX), a first-line microtubule-stabilizing agent, inhibits tumor cell mitosis by promoting tubulin polymerization. However, despite initial responses, approximately 60% of tumors develop resistance within 12 months, resulting in a five-year survival rate below 45% (Lan *et al.*, 2024). The hypoxic tumor microenvironment (TME) plays a pivotal role in this process by stabilizing hypoxia-inducible factor 1- α (HIF-1 α), upregulating drug efflux pumps and induces aberrant angiogenesis, creating physical and biochemical barriers that severely compromise drug delivery (Zhang *et al.*, 2023; Blanco-Fernandez *et al.*, 2021).

Emerging evidence indicates that HIF-1 α interacts closely with the Sonic Hedgehog (SHH) pathway to potentiate resistance. Under hypoxia, HIF-1 α transcriptionally activated SHH to relieve the suppression of Smoothened (SMO) by Patched-1 and drive the movement of glioma-associated oncogene (GLI) transcription factors into the nucleus. The "hypoxia-HIF-1 α -SHH" cascade enhanced tumor stemness and blocked apoptosis through elevating the downstream targets Bcl-2 and Cyclin D1 (Lu *et al.*, 2025; Zhao *et al.*, 2022). Although this pathway was already connected to drug resistance in various solid tumors, including breast cancer (Xie *et al.*, 2025), its involvement in lung cancer together with its viability as a target for therapy still stayed poorly investigated.

Mulberroside A (MA) is a bioactive flavonoid derived from *Morus alba* L., which possesses a broad range of pharmacological actions, covering anti-inflammatory, anti-angiogenic, and anti-tumor effects (Zhang *et al.*, 2025; Zhao *et al.*, 2024). MA was shown to regulate several critical oncogenic signaling routes, including PI3K/Akt and NF- κ B, to suppress melanoma cell growth, and to alleviate liver fibrosis—a well-recognized antecedent of hepatocellular carcinoma (Lu *et al.*, 2023; Shi *et al.*, 2023; Zheng *et al.*, 2023). In spite of these encouraging biological activities, no previous investigations have examined whether MA could potentiate PTX sensitivity in lung cancer through disruption of the HIF-1 α /SHH signaling axis.

Consequently, the present work was the first to evaluate the chemosensitizing action brought about by MA when administered together with PTX in Lewis lung carcinoma (LLC)-bearing mice model, as well as to clarify the

mechanistic contribution of the HIF-1 α /SHH pathway. This study was intended to offer a mechanistic foundation for a novel, natural-product-derived approach to improve chemotherapy efficacy.

MATERIALS AND METHODS

Animals: Specific pathogen-free male C57BL/6 mice (6-8 weeks old, 18-22g; Beijing SPF Biotechnology Co., Ltd., China) were housed in controlled conditions (22 $\pm$ 2°C, 50 $\pm$ 5% humidity, 12-hour light/dark cycle) and provided unrestricted access to sterilized feed and drinking water throughout the acclimatization phase. Approval for this work was granted by the hospital's Ethics Committee, and all procedures adhered to the ARRIVE 2.0 standards as well as the *Guide for the Care and Use of Laboratory Animals*.

Establishment of LLC tumor-bearing model: Establishment of LLC tumor-bearing model: The LLC cell line (American Type Culture Collection, USA) was cultured in RPMI-1640 medium (HyClone, USA) supplemented with 10% FBS (Gibco, USA) at 37°C inside a 5% CO₂ incubator. Cells in the logarithmic growth phase were detached using trypsin, washed three times with PBS, then re-suspended to reach a final concentration of 5 $\times$ 10⁶ cells/mL (viability $\geq$ 95% confirmed by hemocytometer counting (Improved Neubauer, Marienfeld, Germany)). A volume of 0.1 mL of the single-cell suspension containing 5 $\times$ 10⁵ cells was given subcutaneously in the right anterior axilla region of each mouse. Vevo 3100 is a relatively high-frequency ultrasound imaging device (VisualSonics, Canada), and it will be periodically applied to monitor changes in the tumour. Tumour size refers to the largest and smallest dimensions (L) and (W) of the lesion, and its volume (V) was calculated using the formula $V=0.52\times L\times W^2$ (Liu *et al.*, 2015). Three independent measurements were taken for each animal to reduce operational variability, and a tumour model was deemed established successfully when the volume reached $\geq 50\text{ mm}^3$.

Grouping and administration plan: A total of 76 C57BL/6 mice were used in this study. Among them, ten mice served as a blank control group and were not inoculated with tumor cells. The remaining 66 mice were used to establish a LLC xenograft tumor model, of which 6 mice were excluded from subsequent experiments due to unsuccessful modeling, ultimately resulting in 60 successfully modeled mice. These 60 tumor-bearing mice were randomly divided into 6 groups, with 10 mice in each group, as follows:

The control group (Control) received intragastric administration once daily, with 0.9% normal saline given each time at a dose volume of 10 mL per kilogram of body weight per day.

In the model group (Model), tumor-bearing mice received daily oral gavage of 0.9% saline (10 mL/kg, once daily).

In the PTX monotherapy group (PTX), tumor-bearing mice were administered PTX (10 mg/kg, *i.p.*, twice weekly).

In the MA monotherapy group (MA), tumor-bearing mice received oral gavage of MA (MA, 100 mg/kg, once daily; Wuhan Kemike Biochemical Co., Ltd, China).

In the combination therapy group (PTX+MA), tumor-bearing mice received both PTX (10 mg/kg, *i.p.*) and MA (100 mg/kg).

In Inhibitor control group 1 (Control-1), tumor-bearing mice received PTX+MA combined with YC-1 (HIF-1 α inhibitor, 5 mg/kg, *i.p.*, once daily), administered concurrently with MA.

In Inhibitor control group 2 (Control-2), tumor-bearing mice received PTX+MA combined with Cyclopamine (SHH inhibitor, 10 mg/kg, *i.p.*, once daily), administered concurrently with MA.

All groups underwent a three-day pretreatment period before chemotherapy initiation. Continue to apply the treatment continuously for 21 days, and then 24 hours after the last time it was applied, both the tumour and the surrounding tissue were removed.

In vivo efficacy evaluation

(1) General state observation of mice: Daily monitoring of body weight changes and activity status in mice, including feeding behavior, fur gloss, and locomotor ability, was conducted.

Feeding ability assessment: at a fixed time each day, the remaining feed was weighed to compute daily mean food intake per mouse. Feeding behaviour was assessed with a score according to the method in Luise *et al.* (2019); the four categories were: 0 (normal feeding), 1 (mild appetite reduction), 2 (moderate anorexia), and 3 (severe refusal to eat).

For gloss assessment: Two independent experimenters performed a double-blind test of fur gloss in natural light (Zou *et al.*, 2019), and the following scoring system was adopted: 0 points for smooth and shiny; 1 point for local dullness; 2 points for widespread fluffiness; 3 points for hair loss with scabbing, and score consistency was guaranteed.

Locomotor ability assessment: An open-field test was adopted to assess the mice's locomotor ability. A 50 $\times$ 50 cm open field was used, and each mouse was introduced to the center of this area at the start of the trial. Over a span of 5 minutes, EthoVision (Noldus Information Technology, Netherlands) recorded both the total distance travelled and the

percentage of time spent without movement. All behavioral tests were conducted in a soundproof, light-avoidant environment.

(2) Tumor growth monitoring: Every three days, tumor size was measured using ultrasound, recording the longest diameter (*L*) and shortest diameter (*W*), and calculating the tumor volume. Finally, mice were euthanized by cervical dislocation under anesthesia (50 mg/kg pentobarbital sodium intraperitoneal injection). Tumor tissue was excised and weighed. Tumor inhibition rate (%) = [(model group average tumor weight-treatment group tumor weight)/model group tumor weight] × 100% (Gong *et al.*, 2015).

Sample processing: Following weighing of the harvested tumor specimens, a portion of the tissue samples was fixed in 10% neutral formalin solution, subsequently embedded in paraffin, and then sectioned for immunohistochemical staining analysis. The remaining tumor tissue was rapidly frozen and stored in liquid nitrogen for subsequent experiments.

Pathological evaluation of tumor tissue

(1) H&E staining: Collected tumor specimens were immersed in a 4% paraformaldehyde solution obtained from Solarbio (China) and kept there for a full 24 hours to complete the fixation process. Then, according to the method of Sinopharm, the samples underwent a stepwise ethanol dehydration procedure using reagents from Sinopharm, after which they were embedded and cut into uninterrupted tissue sections measuring 4 μm in thickness. The resulting sections were stained with hematoxylin from Solarbio for 5 minutes, followed by differentiation in a 1% hydrochloric acid ethanol solution supplied by Sinopharm, and finally stained with eosin for 2 minutes. After mounting with neutral gum (Solarbio, China), slides were visualized under an optical microscope (200×/400×, BX53, Olympus, Japan) to examine the structure of tumor cells, necrotic regions, and infiltrating inflammatory cells. Histopathological changes underwent evaluation through the application of a semi-quantitative scoring framework (0-3 points) (Wu *et al.*, 2024).

(2) TUNEL apoptosis detection: Employing Roche in Situ Cell Death Detection Kit (Roche Diagnostics, Switzerland), sections were deparaffinized with xylene (Sinopharm, China) and digested with proteinase K (Sinopharm, China). The sections were then incubated with TdT reaction solution (Thermo Fisher, USA) at 37 °C for 1 h. After DAPI counterstaining of the nuclei (Solarbio, China), apoptotic cells were visualized under a fluorescence microscope (488 nm excitation wavelength, Axio Imager M2, Zeiss, Oberkochen, Germany). Five random fields (400× magnification) were selected to count the green fluorescent-labeled apoptotic cells. Apoptosis rate = (Apoptotic cell number /Cell total number) ×100%.

(3) CD31 immunofluorescence staining: Following antigen retrieval using sodium citrate solution provided by Solarbio (China), tissue slices were placed at 4 °C for the entire night together with rabbit anti-mouse CD31 monoclonal antibody diluted at 1:200 from Abcam (UK). The slices were further incubated with Cy3-labeled secondary antibody sourced from Jackson ImmunoResearch (USA) for one hour under dark conditions, and DAPI staining was finally applied for nuclear counterstaining. Observations were made employing a laser confocal microscope (LSM 880, Zeiss, Germany, 200× magnification). The microvascular density (MVD) was calculated by selecting three regions with the highest vascularization (hotspots). A vessel was defined as a CD31-positive lumen structure with a diameter exceeding 8 μm and containing intact endothelial cells.

Real-time quantitative PCR (RT-qPCR): Collect tumor tissue samples, and then use an RNA rapid extraction reagent (TianGen Biotech, China) to extract total RNA. RNA concentration and purity were assessed using a microvolume spectrophotometer sourced from Thermo Fisher Scientific (USA). One μg of total RNA was collected from each sample, and using Oligo dT primers and reverse transcriptase reagents from the Takara (Japan) reverse transcription kit, complementary DNA was produced. Sangon Bioengineering in China created and produced the target primer sequences for VEGF, Bcl-2, Caspase-3, HIF-1α, SHH, SMO, and GLI1, as well as the internal reference β-actin, with the details shown in Table 1. SYBR Green Premix Pro Taq HS Kit for qPCR was used from Yisheng Biotech (China). Each reaction mixture had a final volume of 20 μL, and it contained 2 μL of cDNA template, 0.4 μM of each primer, and 10 μL of SYBR Green mix. Amplification was conducted on a Bio-Rad CFX96 Touch™ real-time PCR instrument (USA): The first denaturation step was carried out at 95 °C for 3 seconds, and then 40 cycles of 95 °C for 5 seconds and 60 °C for 30 seconds were performed. A melting curve analysis was performed to verify the specificity of the amplified fragments. The $2^{-\Delta\Delta C_t}$ method was used for relative quantification of the target genes, and β-actin served as the internal control for data normalization. To guarantee experimental dependability, every specimen was tested in triplicate.

Table 1. Primers for qRT-PCR amplification of *HIF-1α*/*SHH* pathway-related genes

Gene	Upstream (5'-3')	Downstream (5'-3')
<i>VEGF</i>	GAGGTCAAGGCTTTGAAGGC	CTGTCCTGGTATTGAGGGTGG
<i>Bcl-2</i>	AGTGACGGCCCTTCTTACG	CCGAGGGGTAGTAGCCATC
<i>Caspase-3</i>	TGGTGATGAAGGGGTCATTTATG	TTCGGCTTTCCAGTCAGACTC
<i>HIF-1α</i>	CAGCTATTTGCGTGTGAGGA	TGGGTAGGAGATGGAGATGC
<i>SHH</i>	TGACCCCAATTACAACCCCT	CGGAGTTCTCTGCTTGCGTA
<i>SMO</i>	CTGGCTCTGCCTCATCTTCG	GCTCCAGGTAGTCGTTGCTG
<i>GLI1</i>	AGCCAAGAACAACCGCATCT	TGCAGACCCACTTTCTTGCG
<i>β-actin</i>	GGCTGTATCCCTCCATCG	CCAGTTGGTAACAATGCCATGT

Note: These primers served to measure mRNA levels of *VEGF*, *Bcl-2*, *Caspase-3*, *HIF-1α*, *SHH*, *SMO*, *GLI1*, and the housekeeping gene *β-actin*. All primers shared an annealing temperature of 60 °C, and the resulting amplified fragments fell between 100 and 200 bp.

Western blotting: Tumor specimens were first measured for their weight and subsequently broken down on ice with chilled RIPA buffer (Beyotime, China) that included 1% protease inhibitor mix (Sigma-Aldrich, USA) along with phosphatase inhibitors (Roche, Switzerland). Each sample was then homogenized and subjected to centrifugation at 12,000×g for a duration of 15 min at 4 °C to collect the clear upper liquid. The BCA method (Thermo Fisher, USA) was employed to determine protein concentrations. Following this, 30 µg of protein taken from every sample were separated on 10% SDS-PAGE gels (Bio-Rad Laboratories, USA) and subsequently transferred onto PVDF membranes (Merck Millipore, USA). These membranes were initially treated with 5% non-fat milk (BD Biosciences, USA) for one hour at ambient temperature for blocking purposes, and afterward kept overnight at 4 °C together with primary antibodies directed against *HIF-1α* (1:1000; Cell Signaling Technology, USA), *SHH* (1:80; Abcam, UK), *SMO* (1:500; Proteintech Group, China), and *GLI1* (1:80; Abcam, China). After washing with TBST, the membranes were left for one hour at 25°C with the HRP-linked secondary antibodies (1:500, Cell Signaling Technology, USA). Enhanced chemiluminescence substrates (Merck Millipore, USA) were used for signal detection, and ChemiDoc™ MP systems (Bio-Rad Laboratories, USA) were employed to take pictures. Analysis of the acquired images was performed with Image Lab 6.1 (Bio-Rad Laboratories, USA). Expression levels of the target proteins were adjusted relative to *β-actin* (1:2000; Proteintech Group, China) serving as the internal reference. The data were repeated thrice for each group, and the average value was taken.

Statistical analysis: Continuous data were presented as mean ± SD through the application of SPSS 26.0. Before performing one-way ANOVA, both normality (Shapiro-Wilk) and homogeneity of variance (Levene's test) had been verified. Once those prerequisites were satisfied, group comparisons were undertaken. For multiple comparisons between groups, Tukey's honestly significant difference (HSD) test was used for post hoc pairwise comparisons when one-way ANOVA showed a significant overall effect ($P \leq 0.05$). Every test was run as two-tailed, and a P value below 0.05 was considered to reflect statistical significance.

RESULTS

General state of mice: Throughout the entire treatment period (21 d), all eight groups of mice (10 mice per group) survived without significant toxicity or mortality. Weight changes indicated that prior to treatment, slight differences existed in body weight among the Control, Model, PTX, MA, PTX+MA, Control-1, and Control-2 groups ($P > 0.05$). After 21 days of treatment, the body weight decreased by 1.7 ± 0.3 g, 2.9 ± 0.4 g, and 1.9 ± 0.2 g in the Model group, PTX group, and PTX+MA group, respectively ($P \leq 0.05$); additionally, the PTX group had a greatly lower body weight than both the Model and PTX+MA groups ($P \leq 0.05$) (Figure 1A). Regarding feeding behavior, coat glossiness, and motor ability, after 21 d of treatment, relative to the control group, the other groups showed markedly higher feeding scores and coat glossiness scores, as well as a prominent reduction in total movement distance ($P \leq 0.05$) (Figure 1B–D). Compared with the Model and PTX+MA groups, the PTX group exhibited higher feeding scores and hair glossiness scores but lower total movement distance ($P \leq 0.05$ for all comparisons).

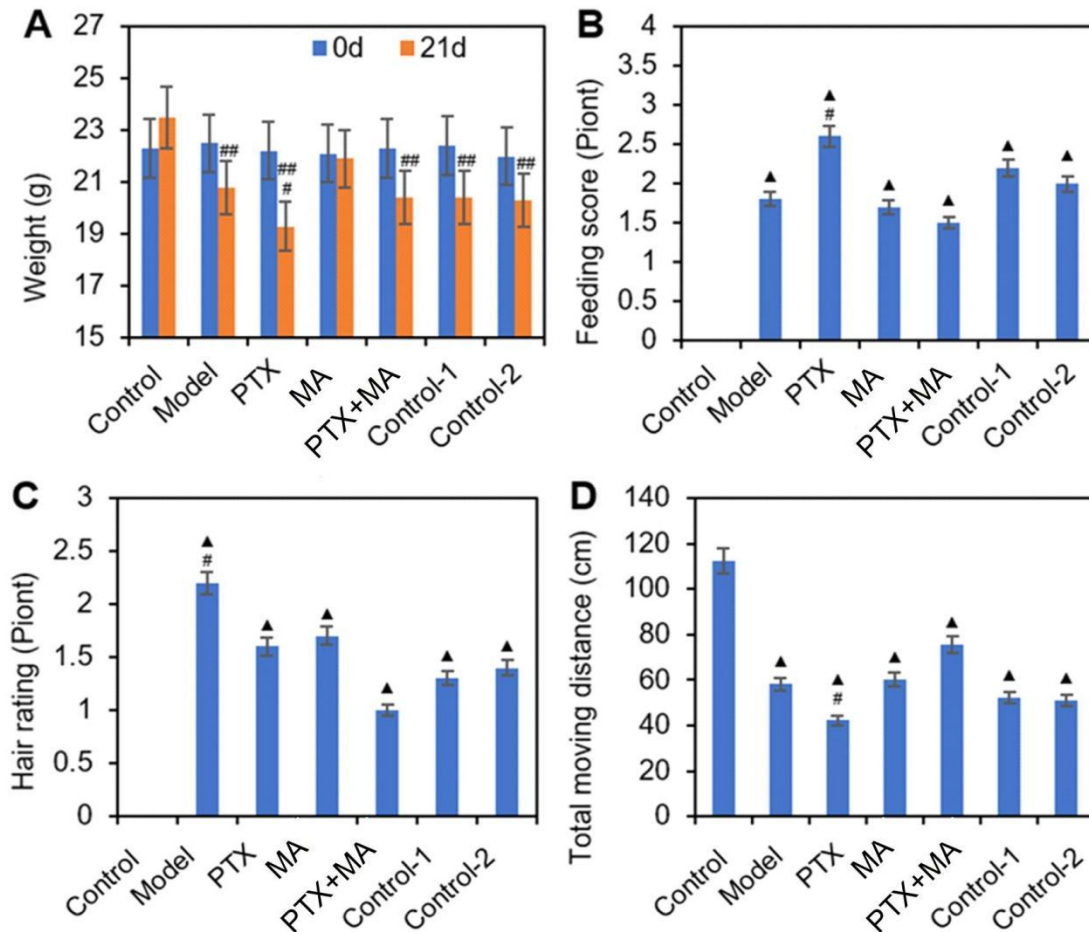


Figure 1. General condition comparison among mouse groups. A: body weight changes; B: feeding score; C: coat glossiness score; D: motor ability. ## $P \leq 0.05$ compared with pre-treatment status; # $P \leq 0.05$ vs. both Model and PTX+MA groups; ▲ $P \leq 0.05$ vs. Control group.

Tumor growth inhibition effect: Comparison of pre-treatment showed no statistically significant differences in tumor volume among groups ($P > 0.05$). After 21 days of treatment, compared with the Model group, the PTX group showed significant reductions in tumor volume (decreased by $164.2 \pm 15.6 \text{ mm}^3$) and weight (decreased by $0.62 \pm 0.08 \text{ g}$) ($P \leq 0.05$), whereas the MA group exhibited no significant changes (decreased by $22.8 \pm 12.3 \text{ mm}^3$, decreased by $0.07 \pm 0.04 \text{ g}$; $P > 0.05$). Relative to the PTX group, the PTX+MA group demonstrated further decreases in tumor volume (decreased by $138.9 \pm 10.7 \text{ mm}^3$) and weight (decreased by $0.54 \pm 0.06 \text{ g}$) ($P \leq 0.05$). In contrast, both Control-1 and Control-2 groups displayed increased tumor volumes (increased by $123.2 \pm 14.1 \text{ mm}^3$ and $115.5 \pm 13.9 \text{ mm}^3$, respectively) and weights (increased by $0.49 \pm 0.07 \text{ g}$ and $0.46 \pm 0.06 \text{ g}$, respectively) compared to the PTX+MA group. ($P \leq 0.05$). Tumor inhibition rate analysis revealed that the PTX, Control-1, and Control-2 groups showed $28.6\% \pm 3.1\%$, $25.3\% \pm 2.7\%$, and $23.7\% \pm 2.9\%$ lower inhibition rates than the PTX+MA group ($P \leq 0.05$). Notably, the MA group's inhibition rate was significantly inferior to the PTX group ($29.2\% \pm 3.4\%$, $P \leq 0.05$) (Figure 2).

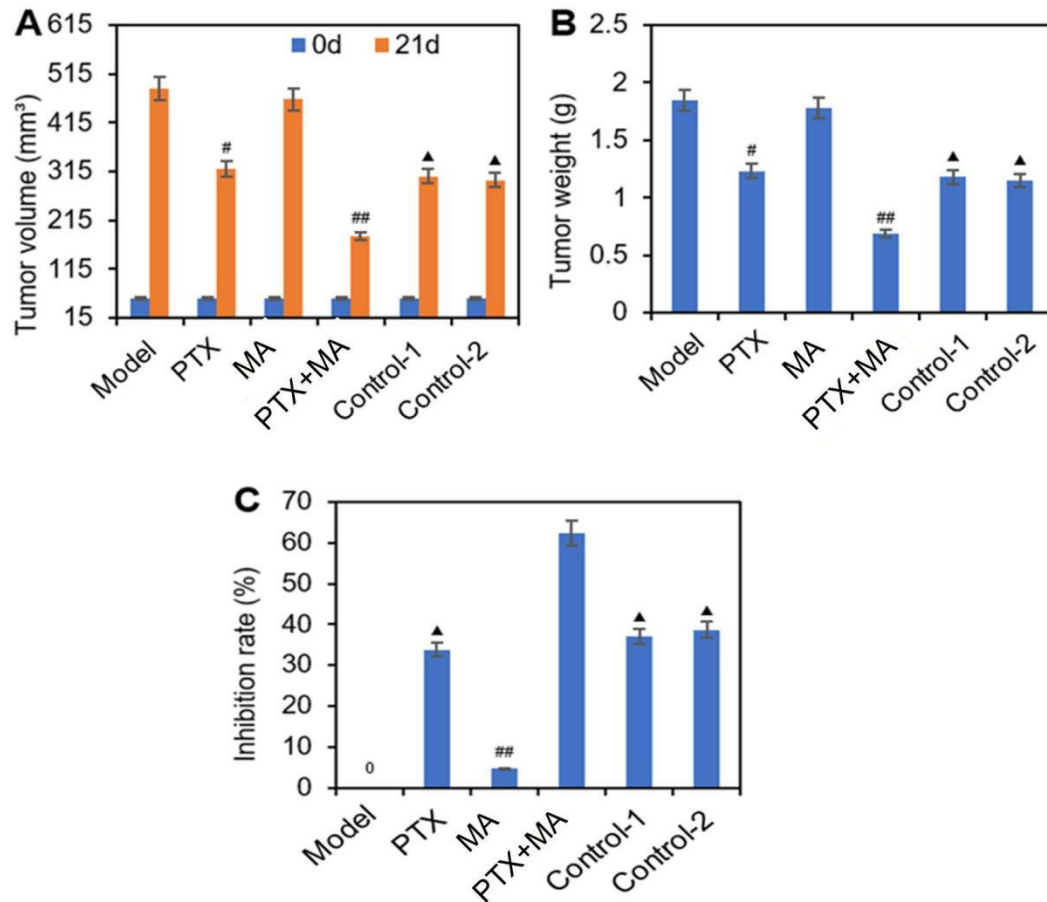


Figure 2. Comparison of tumor proliferation suppression among different groups. **A** represents tumor volume; **B** denotes tumor weight; **C** indicates tumor inhibition ratio. [#] $P \leq 0.05$ vs. Model group and PTX+MA group; ^{##} $P \leq 0.05$ vs. PTX group; [▲] $P \leq 0.05$ vs. PTX+MA group.

HE staining results analysis: The HE staining results are shown in Figure 3. Model group displayed disorganized tumor cell architecture, extensive necrotic regions, and prominent inflammatory cell infiltration, with a histological score of 2.8 ± 0.4 . In PTX group, the disordered cell arrangement, necrosis, and inflammatory cell infiltration were reduced, with a histological score of 2.2 ± 0.4 . PTX+MA group showed further improvement compared to PTX group, with a histological score of 1.2 ± 0.3 , significantly inferior to PTX group ($P \leq 0.05$). MA group (2.6 ± 0.3), Control-1 group (2.4 ± 0.5), and Control-2 group (2.5 ± 0.2) showed negligible differences in HE staining and histological scores versus Model group ($P > 0.05$).

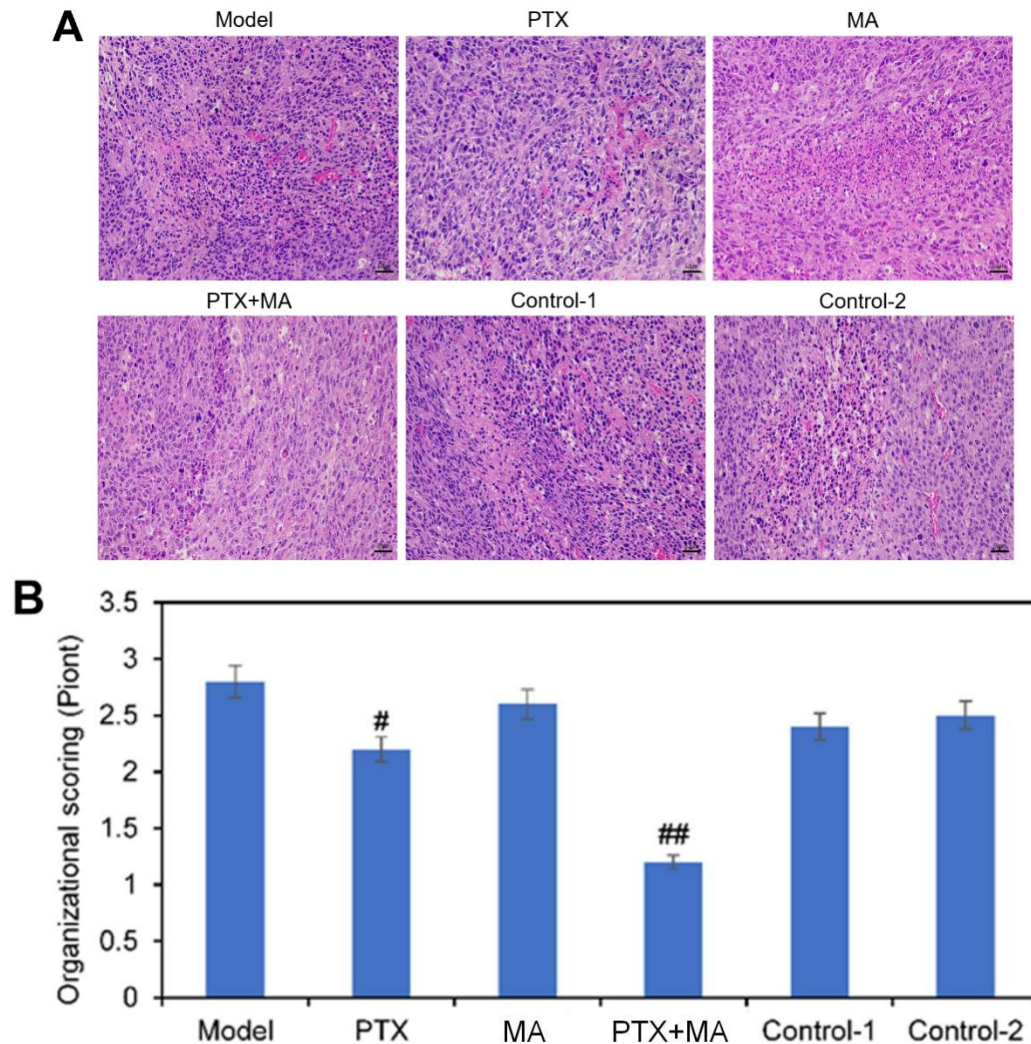


Figure 3. Comparison of HE staining results in groups. **A:** Representative H&E staining images (original magnification 20×, scale bar=100 μm) demonstrated distinct histological patterns across groups. The model group exhibited a disorganized cellular arrangement and prominent necrosis. Both PTX and PTX+MA groups showed progressively reduced necrotic areas and inflammatory infiltration, with the PTX+MA group displaying tissue architecture most closely resembling normal morphology; **B:** histological scores. [#] $P \leq 0.05$ vs. Model and PTX+MA groups; ^{##} $P \leq 0.05$ vs. PTX group.

Changes in cell apoptosis and MVD: TUNEL apoptosis assay results showed that the apoptosis rate in Model group was $5.2 \pm 1.1\%$, while in PTX group, the apoptosis rate increased to $18.4 \pm 2.5\%$ ($P \leq 0.001$). In the combined treatment group, the apoptosis rate further increased to $32.7 \pm 3.1\%$ ($P \leq 0.001$ vs. PTX group) (Figure 4A, C). In the inhibitor control groups, the apoptosis rates were substantially lower than in the combined treatment group: Control-1 ($20.1 \pm 2.8\%$) and Control-2 ($19.6 \pm 2.7\%$) (both $P \leq 0.05$), suggesting that inhibition of *HIF-1α/SHH* pathway partially reversed the pro-apoptotic effect of the combined treatment. MA group had an apoptosis rate of $6.8 \pm 1.3\%$, which differed slightly from Model group ($P > 0.05$).

CD31 immunofluorescence staining implied that the MVD in Model group was $25.6 \pm 2.8/\text{field}$, while the MVD in PTX group decreased to $20.4 \pm 2.1/\text{field}$ ($P \leq 0.01$). The combined treatment group showed the lowest MVD ($12.3 \pm 1.5/\text{field}$, $P \leq 0.001$ vs. PTX group). The MVD in the inhibitor control groups (Control-1: $18.7 \pm 2.0/\text{field}$; Control-2: $19.2 \pm 1.9/\text{field}$) greatly surpassed that in combined treatment group (both $P \leq 0.01$), indicating that inhibition of *HIF-1α/SHH* pathway weakened the anti-angiogenic effect of the combined treatment (Figure 4B, D). The MVD in MA group ($24.1 \pm 2.5/\text{field}$) differed slightly from Model group ($P > 0.05$).

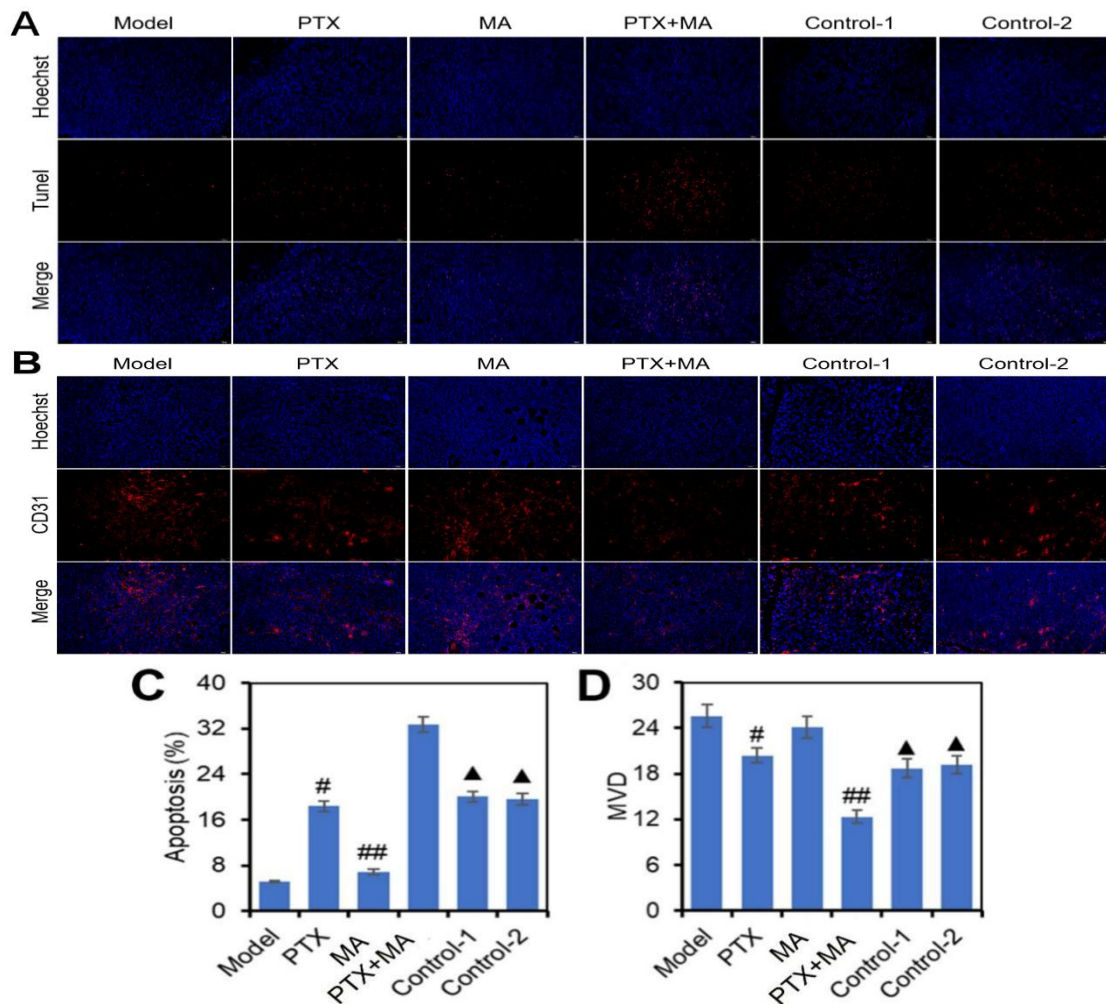


Figure 4. Cell apoptosis and MVD changes across groups. A: TUNEL staining of apoptotic nuclei (blue; 20×, scale bar=100 μm); **B:** CD31 immunofluorescence of microvessels (red; 20×, scale bar=100 μm); **C:** quantified apoptosis rate; **D:** MVD. [#] $P \leq 0.05$ vs. Model & PTX+MA groups; ^{##} $P \leq 0.05$ vs. PTX group; [▲] $P \leq 0.05$ vs. PTX+MA group.

Western blotting and qPCR results: The measured mRNA levels of VEGF and Bcl-2 turned out to be notably lower in the PTX group than those seen in the Model group, whereas Caspase-3 mRNA showed a marked rise compared to the Model group ($P \leq 0.05$); however, no meaningful difference was observed between the PTX and MA groups ($P > 0.05$). When set against the PTX group, the combination therapy group (PTX + MA) exhibited a more pronounced drop in VEGF and Bcl-2 mRNA levels, along with a clear elevation in Caspase-3 mRNA. Specifically, the relative amounts of VEGF and Bcl-2 transcripts in the PTX+MA group stood substantially below the corresponding values in the PTX group, while Caspase-3 transcripts were significantly elevated in the PTX+MA group relative to the PTX group ($P \leq 0.05$). Both Control-1 and Control-2 groups displayed markedly higher VEGF and Bcl-2 mRNA levels than those found in the PTX+MA group, and their Caspase-3 levels were notably lower than those in the PTX+MA group ($P \leq 0.05$). Regarding the Hedgehog pathway components, the PTX group showed considerable reductions in both protein and transcript levels of HIF-1 α , SHH, SMO, and GLI1 when compared with the Model group ($P \leq 0.05$), whereas only minor changes were detected in the MA group ($P > 0.05$). The PTX+MA group gave rise to a striking decrease in the relative protein and mRNA abundances of HIF-1 α , SHH, SMO, and GLI1 relative to the PTX group ($P \leq 0.05$). In the Control-1 group, the relative protein and mRNA expression of HIF-1 α dropped substantially compared to the PTX+MA group, but the levels of SHH, SMO, and GLI1 did not show any meaningful differences ($P > 0.05$). As for the Control-2 group, the relative protein and transcript levels of SHH, SMO, and GLI1 were markedly diminished ($P \leq 0.05$), yet HIF-1 α expression remained unchanged (Figure 5).

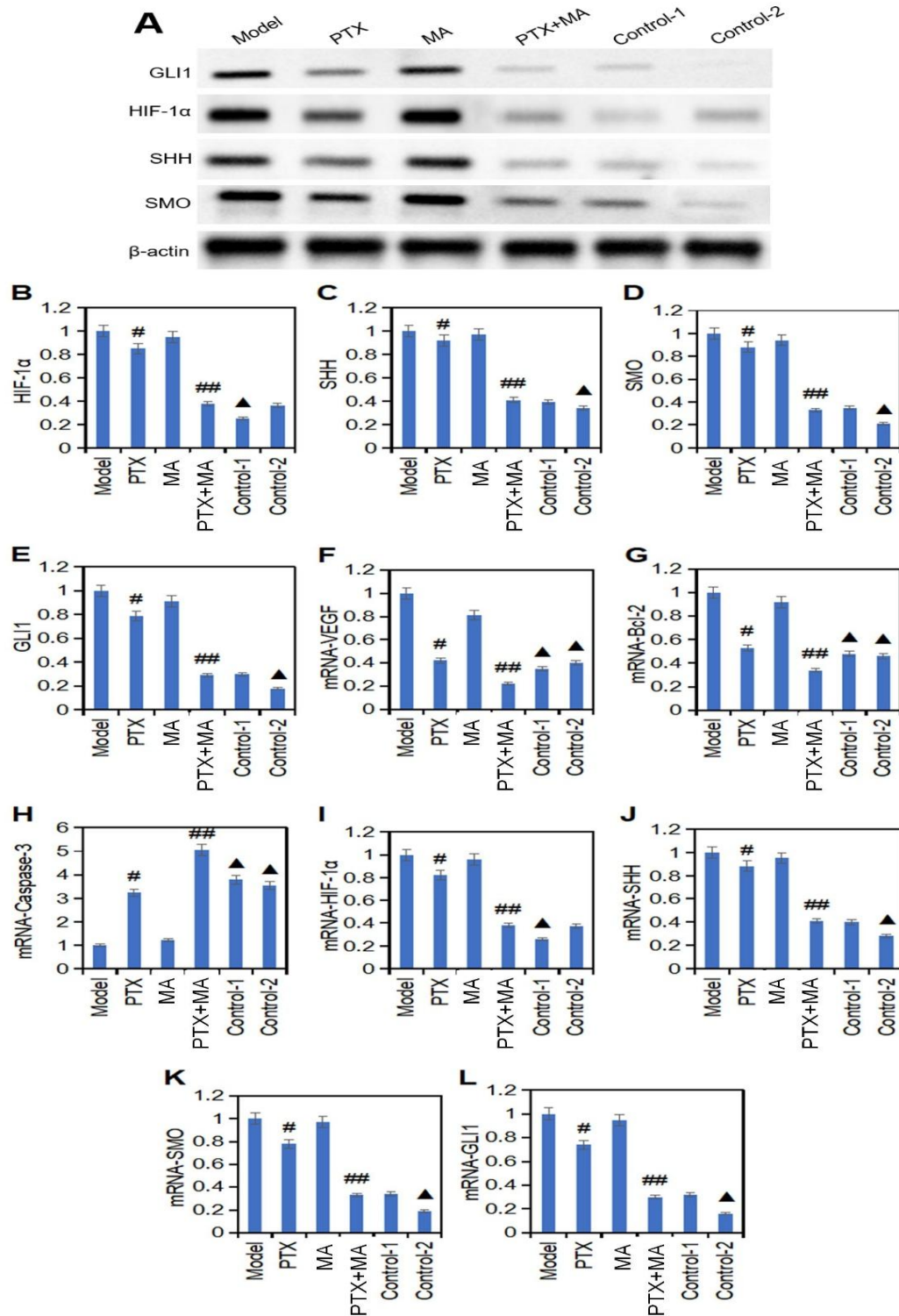


Figure 5. Comparison of Western blot and quantitative polymerase chain reaction results. A: western blot images; B: *HIF-1α* relative protein expression; C: *SHH* relative protein expression; D: *SMO* relative protein expression; E: *GLI1* relative protein expression; F: *VEGF* relative mRNA expression; G: *Bcl-2* relative mRNA expression; H: *Caspase-3* relative mRNA expression; I: *HIF-1α* relative mRNA expression; J: *SHH* relative mRNA expression; K: *SMO* relative mRNA expression; L: *GLI1* relative mRNA expression. # $P \leq 0.05$ vs. Model and PTX groups; ## $P \leq 0.05$ vs. PTX group; ▲ $P \leq 0.05$ vs. PTX+MA group.

DISCUSSION

This research firstly uncovered the molecular mechanism whereby MA improves the chemotherapy sensitivity of LLC tumor-bearing mice to PTX via targeting the HIF-1 α /SHH signaling pathway. Experimental findings demonstrated that the combined application of MA and PTX could markedly inhibit tumor proliferation, with an inhibitory rate reaching 32.7%, and simultaneously produce a synergistic effect to reduce the expression levels of *HIF-1 α* , *SHH*, *SMO* and *GLI1*. Furthermore, this combined treatment concurrently inhibited angiogenesis and promoted tumor cell apoptosis. These results suggest that MA may enhance antitumor efficacy through multiple mechanisms: (1) downregulating the *HIF-1 α /SHH* signaling pathway to suppress tumor stemness maintenance and angiogenesis; (2) modulating apoptosis- and angiogenesis-related factors (*VEGF*, *Bcl-2*, and *Caspase-3*) to promote tumor cell apoptosis and reduce MVD, thereby improving the TME.

Some studies suggested that combining natural products with conventional chemotherapeutic agents demonstrates a promising synergistic effect. For example, the combination of *Mahonia aquifolium* extract with doxorubicin is proved to enhance cytotoxicity, induce cell cycle arrest, reduce migration ability, and improve chemotherapy efficacy. Likewise, the combined application of Fei-Liu-Ping ointment and cyclophosphamide was found to more effectively suppress tumor growth, reduce inflammatory cytokine levels, remodel the microenvironment surrounding the tumor, and enhance the efficacy of chemotherapy (Yang *et al.*, 2021). Related work by (Hao *et al.* 2019) further demonstrated that matrine could halt the progression of lung cancer cells through modulation of the PI3K/Akt signaling pathway, thereby strengthening the effectiveness of chemotherapeutic drugs. Furthermore, Islam *et al.* (2019), reported that *M. latifolia* plant extracts contain several potentially bioactive compounds that effectively inhibit cancer cell growth and exhibit anticancer properties. Building upon these findings, this study further demonstrated that MA synergistically enhanced PTX-mediated tumor growth inhibition and histological improvement *in vivo*, suggesting its dual role in both synergistic cytotoxicity and long-term therapeutic potentiation via TME modulation. Compared to PTX monotherapy, the MA+PTX combination group exhibited significantly superior outcomes in tumor volume, weight, and histological scores in LLC tumor-bearing mice. Histological examination showed more organised tumour cell arrangement and reduced necrosis in the combination group; it was believed that MA could suppress necrosis-related pro-inflammatory responses and alleviate chronic inflammation in the TME to improve drug delivery and apoptotic signalling. These observations provide support for the concept of natural product-mediated multi-mechanistic enhancement of PTX efficacy and thus prompt further mechanistic studies.

MA has been shown *in vivo* to enhance the antitumor efficacy of LLC tumors by targeting the HIF-1 α /SHH signaling axis, and this is the first such experiment. Combined with PTX, there was a reduction in the tumour and improved histopathological outcomes; thus, a synergistic effect rather than simple addition had occurred. PTX+MA reduced HIF-1 α and its downstream targets SHH, SMO, and GLI1 through a specific mechanism. HIF-1 α is enhanced under hypoxic conditions in the TME; therefore, it promotes both angiogenesis and tumor stemness via the SHH pathway to help the cancer evade treatment (Chen *et al.*, 2015; Dai *et al.*, 2021). Our results indicate that MA interrupts the "hypoxia-stemness-resistance" continuum at a node. Add the specific inhibitor and then show the impact on this path. MA was shown to enhance the antitumor efficacy of LLC to PTX, and the underlying reason was a downregulation of the HIF-1 α /SHH signalling pathway; consequently, angiogenesis was inhibited and apoptosis was promoted. Notably, when specific inhibitors (YC-1 or Cyclopamine) were used to pharmacologically suppress HIF-1 α or SHH, the synergistic effect of PTX+MA was partially inhibited. This did not reduce the significance of this path; rather, it strongly indicated that a dynamic equilibrium or a particular inhibitory state of this path was required to achieve the best synergistic effect of PTX+MA. MA probably operated via this route through an improved regulatory mode that differed from strong inhibitors, and over-inhibition of the pathway may have induced adaptive resistance in cancer cells. Therefore, we can inhibit this signalling pathway to regulate the necessary components of the TME. It downregulates the pro-survival factor Bcl-2 and the angiogenesis-related VEGF protein, and at the same time increases the expression of caspase-3 to trigger cell death. In combination, the above molecular modifications induce apoptosis in cancer cells and reconstruct the normal structure of the tumor's blood vessels to improve the delivery efficiency and therapeutic effect of drugs. This research is innovative in confirming that the HIF-1 α /SHH signaling pathway serves as a direct molecular target for MA to enhance tumor drug sensitivity. Unlike most investigations into other natural compounds that only report general and non-specific regulatory mechanisms, this study offers a clear and targeted mechanistic explanation (Li *et al.*, 2024). From a translational perspective, our results suggest that combining standard chemotherapy like PTX with a natural compound such as MA, could be a viable strategy to improve patient outcomes. More experiments need to be conducted on cell lines and improved delivery methods (such as nanoformulations for enhanced MA bioavailability), but our work has provided strong support for the improving chemotherapy efficacy of MA for PTX in lung cancer.

TUNEL and CD31 assay results show that the combination therapy group had an increased rate of apoptosis and a lower MVD. qPCR data show that MA reduces the expression of both VEGF and Bcl-2, while increasing the

expression of Caspase-3; therefore, it likely promotes apoptosis in cancer cells and restricts angiogenesis through this mechanism. Therefore, MA can inhibit the stemness of cancer cells via signalling pathways and simultaneously exert multiple effects to enhance the anti-cancer action of PTX through regulation of the apoptosis and angiogenesis pathways inside and outside the cell. Some naturally occurring products have been found by researchers that can regulate the TME and enhance the effect of chemotherapy. These natural substances can promote the death of cancer cells and the development of blood vessels to improve the effect of cancer therapy via multiple biological pathways. Zhang (W) and others (2022) have found that curcumin triggers fibroblast ROS stress and induces apoptosis via the PERK-eIF2 α -ATF4 pathway, and Huo *et al.* (2022) have shown that flavonoids such as quercetin can strengthen the tumor cell death response by altering calcium ion homeostasis. These findings provide literature support for the mechanisms underlying MA-induced *Caspase-3* upregulation and *VEGF* suppression observed in the present study. Natural products also act critically in inhibiting tumor angiogenesis. They regulate angiogenic factors such as *VEGF* and *VEGFR2* expression, thus inhibiting tumor blood vessel formation. For instance, flavonoids and calcipotriol exhibit anti-angiogenic activity (Lu *et al.*, 2025). Furthermore, natural products like quercetin and protocatechuic acid exert anti-angiogenic effects by regulating endothelial cell apoptosis, *etc.* (Li *et al.*, 2021). Therefore, in this study, MA inhibited tumor angiogenesis by reducing *VEGF* levels, reflecting the universal mechanism characteristics of natural products' anti angiogenic effects. In this study, MA inhibited *HIF-1 α /SHH* pathway, reduced *VEGF* expression, and suppressed tumor angiogenesis, potentially enhancing the anti-tumor effects of PTX. Finally, the *HIF-1 α* and *SHH* pathways play crucial roles in tumor hypoxic adaptation, invasion, and metastasis. Natural compounds block these routes, which then curbs tumor growth and spread. One case is quercetin, which lowers *HIF-1 α* levels to cut back on tumor cell growth and movement. (Fakhri *et al.*, 2024). Additionally, natural products like quercetin and protocatechuic acid exert anti-angiogenic effects (Yang *et al.*, 2021). MA enhanced the anti-tumor effects of PTX by inhibiting *HIF-1 α /SHH* pathway.

Although this study reveals the sensitizing mechanism of MA, there are certain limitations. First, the experiment focused solely on the *HIF-1 α /SHH* axis and did not explore the impact of MA on other related pathways. Second, the effect of MA on PTX-resistant cell lines has not been studied, and more *in vitro* models need to be built for validation. Finally, the long-term toxicity profile and pharmacokinetic characteristics of this treatment scheme have not been fully investigated, and further comprehensive toxicology studies need to be carried out to support clinical application. It should be noted that in recent years, nanodelivery systems (such as liposomal encapsulation of flavonoids) have been used to improve the oral bioavailability of drugs substantially, providing a new path for the clinical application of MA.

Conclusion: In short, MA increases the sensitivity of PTX in LLC-bearing mice by inhibiting the *HIF-1 α /SHH* pathway simultaneously. The underlying mechanism involves promoting apoptosis, normalizing blood vessels, and regulating resistance-related genes. Based on the above results, a new combined-therapy strategy for improving chemotherapy efficacy in lung cancer has been proposed, and a theoretical basis for applying natural compounds to regulate the TME has been laid.

Authors' Contributions: Aiqin Liu and Richeng Jiang designed experiments; Aiqin Liu, Bing Yan and Richeng Jiang analyzed data; Aiqin Liu, Bing Yan and Richeng Jiang collected samples; Aiqin Liu, Bing Yan and Richeng Jiang performed experiments; Aiqin Liu and Richeng Jiang wrote the manuscript. All authors agreed to publish this article.

Animal rights statement: All animal experiments were approved by the Animal Ethics Committee of Tianjin Cancer Hospital Airport Hospital (Tianjin, China), in compliance with Chinese national guidelines for the care and use of animals.

Conflict of interests: None

REFERENCES

- Blanco-Fernandez, B., V. M. Gaspar, E. Engel, and J. F. Mano (2021). Proteinaceous Hydrogels for Bioengineering Advanced 3D Tumor Models. *Adv Sci.* 8(4): 2003129. <https://doi.org/10.1002/advs.202003129>
- Chen, S., M. Zhang, L. Xing, Y. Wang, Y. Xiao, and Y. Wu (2015). HIF-1 α contributes to proliferation and invasiveness of neuroblastoma cells via SHH signaling. *PLoS One.* 10(3): e0121115. <https://doi.org/10.1371/journal.pone.0121115>
- Dai, H., X. Sheng, Y. Wang, L. Zhou, Y. Lin, Y. Du, F. Yang, R. Sha, J. Peng, L. Yao, W. Yin, and J. Lu (2021). HIF1 α Regulates IL17 Signaling Pathway Influencing Sensitivity of Taxane-Based Chemotherapy for Breast Cancer. *Front Cell Dev Biol.* 9: 729965. <https://doi.org/10.3389/fcell.2021.729965>
- Fakhri, S., S. Z. Moradi, F. Faraji, L. Kooshki, K. Webber, and A. Bishayee (2024). Modulation of hypoxia-inducible factor-1 signaling pathways in cancer angiogenesis, invasion, and metastasis by natural compounds: a

- comprehensive and critical review. *Cancer Metastasis Rev.* 43(1): 501-574. <https://doi.org/10.1007/s10555-023-10136-9>
- Gong, T., C. F. Wang, J. R. Yuan, Y. Li, J. F. Gu, B. J. Zhao, L. Zhang, X. B. Jia, L. Feng, and S. L. Liu (2015). Inhibition of Tumor Growth and Immunomodulatory Effects of Flavonoids and Scutebarbatines of *Scutellaria barbata* D. Don in Lewis-Bearing C57BL/6 Mice. *Evid Based Complement Alternat Med.* 2015: 630760. <https://doi.org/10.1155/2015/630760>
- Hao, Y., H. Yin, C. Zhu, F. Li, Y. Zhang, Y. Li, X. Wang, and D. Li (2019). Matrine inhibits proliferation and promotes autophagy and apoptosis in non-small cell lung cancer cells by deactivating *PI3K/AKT/mTOR* pathway. *Nan Fang Yi Ke Da Xue Xue Bao.* 39(7): 760-765. <https://doi.org/10.12122/j.issn.1673-4254.2019.07.02>
- Huo, J. L., W. J. Fu, Z. H. Liu, N. Lu, X. Q. Jia, and Z. S. Liu (2022). Research advance of natural products in tumor immunotherapy. *Front Immunol.* 13: 972345. <https://doi.org/10.3389/fimmu.2022.972345>
- Islam, M. S., C. A. Jahangir, M. S. Rahi, M. M. Hasan, S. A. Sajib, K. M. F. Hoque, and M. A. Reza (2019). In-vivo antiproliferative activity of *Morus latifolia* leaf and bark extracts against Ehrlich's ascites carcinoma. *Toxicol Res.* 36(1): 79-88. <https://doi.org/10.1007/s43188-019-00011-7>
- Lan, N., S. Bai, M. Chen, X. Wang, Z. Feng, Y. Gao, B. Hui, W. Ma, X. Zhang, F. Hu, W. Liu, W. Li, F. Wu, and J. Ren (2024). MECOM Locus classical transcript isoforms affect tumor immune microenvironment and different targets in ovarian cancer. *J Ovarian Res.* 17(1): 207. <https://doi.org/10.1186/s13048-024-01522-0>
- Li, L. B., L. X. Yang, L. Liu, F. R. Liu, A. H. Li, Y. L. Zhu, H. Wen, X. Xue, Z. X. Tian, H. Sun, P. C. Li, and X. G. Zhao (2024). Targeted inhibition of the HNF1A/SHH axis by triptolide overcomes paclitaxel resistance in non-small cell lung cancer. *Acta Pharmacol Sin.* 45(5): 1060-1076. <https://doi.org/10.1038/s41401-023-01219-y>
- Li, R., X. Song, Y. Guo, P. Song, D. Duan, and Z. S. Chen (2021). Natural Products: A Promising Therapeutics for Targeting Tumor Angiogenesis. *Front Oncol.* 11: 772915. <https://doi.org/10.3389/fonc.2021.772915>
- Liu, H., H. W. Xu, Y. Z. Zhang, Y. Huang, G. Q. Han, T. J. Liang, L. L. Wei, C. Y. Qin, and C. K. Qin (2015). Ursodeoxycholic acid induces apoptosis in hepatocellular carcinoma xenografts in mice. *World J Gastroenterol.* 21(36): 10367-10374. <https://doi.org/10.3748/wjg.v21.i36.10367>
- Luise, D., C. Lauridsen, P. Bosi, and P. Trevisi (2019). Methodology and application of *Escherichia coli* F4 and F18 encoding infection models in post-weaning pigs. *J Anim Sci Biotechnol.* 10: 53. <https://doi.org/10.1186/s40104-019-0352-7>
- Lu, L., Y. Ma, Q. Tao, J. Xie, X. Liu, Y. Wu, Y. Zhang, X. Xie, M. Liu, and Y. Jin (2025). Hypoxia-inducible factor-1 alpha (HIF-1 α) inhibitor AMSP-30 m attenuates CCl₄-induced liver fibrosis in mice by inhibiting the sonic hedgehog pathway. *Chem Biol Interact.* 413: 111480. <https://doi.org/10.1016/j.cbi.2025.111480>
- Lu, R., Z. Wei, Z. Wang, S. Xu, K. Sun, P. Cheng, X. Huang, H. You, F. Guo, S. Liang, and A. M. Chen (2023). Mulberroside A alleviates osteoarthritis via restoring impaired autophagy and suppressing MAPK/NF- κ B/PI3K-AKT-mTOR signaling pathways. *iScience.* 26(2): 105936. <https://doi.org/10.1016/j.isci.2023.105936>
- Lu, X., L. J. Friedrich, and T. Efferth (2025). Natural products targeting tumour angiogenesis. *Br J Pharmacol.* 182(10): 2094-2136. <https://doi.org/10.1111/bph.16232>
- Shi, B., J. Qian, H. Miao, S. Zhang, Y. Hu, P. Liu, and L. Xu (2023). Mulberroside A ameliorates CCl₄-induced liver fibrosis in mice via inhibiting pro-inflammatory response. *Food Sci Nutr.* 11(6): 3433-3441. <https://doi.org/10.1002/fsn3.3333>
- Wu, J., P. Wang, X. Xie, X. Yang, S. Tang, J. Zhao, T. Liu, J. Wang, J. Zhang, T. Xia, and X. Feng (2024). Gasdermin D silencing alleviates airway inflammation and remodeling in an ovalbumin-induced asthmatic mouse model. *Cell Death Dis.* 15(6): 400. <https://doi.org/10.1038/s41419-024-06777-5>
- Xie, H., T. Tan, Q. Li, and T. Li (2025). Revolutionizing HER-2 assessment: multidimensional radiomics in breast cancer diagnosis. *BMC Cancer.* 25(1): 265. <https://doi.org/10.1186/s12885-025-13549-7>
- Yang, C., D. Li, C. N. Ko, K. Wang, and H. Wang (2023). Active ingredients of traditional Chinese medicine for enhancing the effect of tumor immunotherapy. *Front Immunol.* 14: 1133050. <https://doi.org/10.3389/fimmu.2023.1133050>
- Yang, M. H., S. H. Baek, A. Chinnathambi, S. A. Alharbi, and K. S. Ahn (2021). Identification of protocatechuic acid as a novel blocker of epithelial-to-mesenchymal transition in lung tumor cells. *Phytother Res.* 35(4): 1953-1966. <https://doi.org/10.1002/ptr.6938>
- Yang, Y., N. Li, T. M. Wang, and L. Di (2021). Natural Products with Activity against Lung Cancer: A Review Focusing on the Tumor Microenvironment. *Int J Mol Sci.* 22(19): 10827. <https://doi.org/10.3390/ijms221910827>
- Zhang, F., T. Zheng, X. Wang, Y. Chen, F. Zhang, X. Liu, S. Wang, G. Yang, S. Xie, Q. Wu, C. Xu, Q. Zhou, D. Wu, H. B. Luo, and Y. Y. Huang (2025). Structure-Based Optimization of Moracin M as Potent and Selective PDE4

- Inhibitors with Antipsoriasis Effects. J Med Chem. 68(6): 6789-6803. <https://doi.org/10.1021/acs.jmedchem.5c00266>
- Zhang, H., L. Hu, W. Xiao, Y. Su, and D. Cao (2023). An injectable, in situ forming and NIR-responsive hydrogel persistently reshaping tumor microenvironment for efficient melanoma therapy. Biomater Res. 27(1): 118. <https://doi.org/10.1186/s40824-023-00462-y>
- Zhang, W., S. Li, C. Li, T. Li, and Y. Huang (2022). Remodeling tumor microenvironment with natural products to overcome drug resistance. Front Immunol. 13:1051998. <https://doi.org/10.3389/fimmu.2022.1051998>
- Zhao, A., J. Su, Q. Xu, J. Zhang, J. Jiang, S. Chen, J. Cheng, C. Chen, L. Wang, J. Di, X. Liu, L. Jiang, L. Liu, Y. Liu, A. Liu, and C. Guo (2024). Elucidation of anti-pneumonia pharmacodynamic material basis and potential mechanisms of Xiebai San by combining spectrum-efficacy relationship and surface plasmon resonance. J Ethnopharmacol. 335: 118609. <https://doi.org/10.1016/j.jep.2024.118609>
- Zhao, D., Y. Zhao, S. He, Z. Liu, K. Li, L. Zhang, X. Zhang, S. Wang, N. Che, and M. Jin (2023). High accuracy epidermal growth factor receptor mutation prediction via histopathological deep learning. BMC Pulm Med. 23(1): 244. <https://doi.org/10.1186/s12890-023-02537-x>
- Zhao, J., G. Zeng, E. Lin, C. Cai, P. Li, B. Zou, and J. Li (2022). Combined HIF-1 α and SHH Up-Regulation Is a Potential Biomarker to Predict Poor Prognosis in Postoperative Hepatocellular Carcinoma. J Invest Surg. 35(8): 1660-1667. <https://doi.org/10.1080/08941939.2022.2090034>
- Zheng, Y., E. H. Lee, S. Y. Lee, Y. Lee, K. O. Shin, K. Park, and I. J. Kang (2023). Morus alba L. root decreases melanin synthesis via sphingosine-1-phosphate signaling in B16F10 cells. J Ethnopharmacol. 301: 115848. <https://doi.org/10.1016/j.jep.2022.115848>
- Zou, W., L. Li, Y. Chen, T. Chen, Z. Yang, J. Wang, D. Liu, G. Lin, and X. Wang (2019). In Vivo Toxicity Evaluation of PEGylated CuInS₂/ZnS Quantum Dots in BALB/c Mice. Front Pharmacol. 10: 437. <https://doi.org/10.3389/fphar.2019.00437>