

Original Article

Dichloromethane fraction from *Ficus hispida* L.f. fruit extract suppresses lung cancer through cell cycle arrest and TNF-induced apoptosis

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Abstract: *Ficus hispida* L.f., a traditional medicinal plant widely distributed in South and Southeast Asia, has long been used in ethnomedicine to treat respiratory disorders, inflammation and tumor-related conditions. Although previous studies have suggested that *F. hispida* possesses cytotoxic and anti-inflammatory activities, the *in vivo* antitumor efficacy and underlying mechanisms of its fruit-derived dichloromethane extract remain unclear. This study aimed to investigate the anti-lung cancer activity of the dichloromethane extract of *F. hispida* fruits (FHF-DE) using integrated phytochemical, cellular, transcriptomic and *in vivo* approaches. FHF-DE inhibited the proliferation of multiple human lung cancer cell lines (including H1299, A549, H1975, H460, and PC9 cells) as well as murine Lewis lung carcinoma (LLC) line. Flow cytometry indicated G0/G1 arrest in LLC cells and S-phase arrest in H1975 cells. FHF-DE also promoted apoptotic cell death, as indicated by increased Bax expression, decreased Bcl-2 expression, and caspase-3 activation. Transcriptome sequencing and reverse transcription-quantitative (RT-q) PCR analyses indicated enrichment of the TNF signaling pathway, with increased TNF- α and related gene expression (e.g., IRF1, BIRC3). In an LLC allograft mouse model, daily oral administration of FHF-DE (200-400 mg/kg) significantly suppressed tumor growth, without evident hepatotoxicity or nephrotoxicity in H&E staining. This *in vivo* finding demonstrates the antitumor efficacy of FHF-DE, although, molecular validation of the proposed mechanism in tumor tissues was not performed. Collectively, our *in vitro* data demonstrate that FHF-DE induces apoptosis and cell cycle arrest in lung cancer cells, with transcriptomic and pharmacological data suggesting a functional association with TNF-related signaling pathways. However, definitive mechanistic proof of direct TNFR1 engagement or extrinsic apoptotic pathway activation, particularly *in vivo*, requires further investigation. The present study provides preclinical evidence supporting further pharmacological and toxicological evaluation of *F. hispida* fruit-derived extracts as natural-product-based candidates for lung cancer intervention.

Keywords: *Ficus hispida* L.f. fruits, lung cancer, TNF signaling pathway, dichloromethane extract

Introduction

Lung cancer is the leading cause of cancer-related deaths globally, thereby imposing a heavy burden on global public health systems [1, 2]. Although molecular targeted therapy and immune checkpoint inhibitors have significantly improved clinical efficacy in specific patient

populations, therapeutic resistance, metastasis, disease recurrence, and adverse reactions continue to limit durable clinical benefit and long-term survival [3]. These challenges highlight the urgent need to identify novel anticancer strategies with improved efficacy, acceptable safety profiles and complementary mechanisms of action.

Natural medicines are increasingly recognized as valuable sources of new anticancer agents. Numerous plant extracts and their bioactive constituents have been shown to suppress the proliferation, survival, invasion, and metastatic potential of lung cancer cells through the modulation of multiple molecular pathways [4]. Among these medicinal resources, *Ficus hispida* L.f., an evergreen tree belonging to the Moraceae family, widely distributed in South and Southeast Asia, has long been used in ethnomedicine for the management of respiratory disorders, inflammation and tumor-related conditions [5, 6]. The fruit of *F. hispida* is particularly rich in flavonoids, triterpenoids, and phenolic compounds, which have been implicated in antiproliferative and pro-apoptotic activities [7, 8]. Although preliminary studies have reported cytotoxic effects of *F. hispida* extracts or constituents against several cancer cell lines, their anti-lung cancer efficacy *in vivo* and the underlying molecular mechanisms remain largely unknown.

Dysregulated cell cycle progression and evasion of apoptosis are fundamental features of malignant cells and key targets for anticancer intervention [9, 10]. Therapeutic strategies capable of inducing cell cycle arrest and restoring apoptosis have been widely exploited in the development of anticancer agents [11]. CDKN1A (p21) can inhibit the formation of the Cyclin D-CDK4/CDK6 complex, thereby inducing cell cycle arrest during the G1/S transition period [12, 13]. Additionally, the Cyclin A-CDK2 complex is crucial for initiating and progressing DNA replication in the S phase, primarily through phosphorylation of key substrates involved in S phase entry and progression [14]. For instance, baicalein upregulates p21 and downregulates CDK4 and cyclin D1, thereby inducing G0/G1 phase arrest in human lung cancer cells [15, 16]. Naringenin and apigenin exert a synergistic anti-proliferative effect on non-small cell lung cancer cells by causing G2/M phase cell cycle arrest and mitochondrial dysfunction [17]. These findings collectively suggest that the flavonoid-rich composition of *F. hispida* may exert anticancer effects, at least in part, through alteration of the cell cycle.

Furthermore, the TNF pathway plays a crucial role in regulating cell apoptosis, inflammation, cell survival and tumor-associated immune

responses, and has been extensively studied [18]. Importantly, TNF- α signaling is pleiotropic, activating both pro-apoptotic and pro-survival cascades. The balance between death and survival is dependent on cellular context, the nature of adaptor recruitment, and the activation of NF- κ B-dependent transcriptional responses [19]. Certain flavonoids present in *F. hispida* fruits have been reported to upregulate TNF- α expression and engage death receptor signaling in cancer cells, suggesting a mechanistic link between the fruit extract and extrinsic apoptotic pathway. For instance, derivatives of *Pseudellone C* have been shown to inhibit glioma progression by modulating the TNF/TNFR signaling pathway, highlighting the therapeutic potential of targeting TNF-mediated apoptotic mechanisms in cancer treatment [20].

Based on the above evidence, we hypothesized that the dichloromethane fraction of *F. hispida* fruit extract (FHF-DE) induces cell cycle arrest and apoptosis in lung cancer cells, and that transcriptomic profiling may reveal signaling pathways associated with these effects, with particular interest in the TNF pathway given its established role in extrinsic apoptosis. The rationale is that the flavonoid-rich composition of FHF-DE may simultaneously perturb the expression of cell cycle regulators and activate the TNF signaling pathway, thereby triggering both cell cycle blockade and extrinsic apoptotic cell death. To test this hypothesis, we employed multiple experimental approaches, including flow cytometry, cell viability assays, WB, transcriptome sequencing, and quantitative PCR, to systematically investigate the anti-proliferative and pro-apoptotic effects of FHF-DE on lung cancer cells. Furthermore, we validated the *in vitro* findings using an immunocompetent C57BL/6 mouse model to assess the translational potential of these extracts *in vivo*. UHPLC-Q-Exactive Orbitrap MS analysis identified the specific bioactive components within the extracts responsible for the observed anticancer activities. The present research not only enhances our understanding of *F. hispida* as a candidate for lung cancer therapy but also underscores the broader significance of ethnomedicine-derived natural products in contemporary oncological research and drug development.

Materials and methods

Plant material and extraction

Fruits of *F. hispida* were collected in July 2023 from Wenchang city, Hainan Province, China, and taxonomically identified by Professor Ye Wang (Hunan University of Medicine). A voucher specimen (J20230701) has been deposited at the School of Pharmaceutical Sciences, Hunan University of Medicine.

Dried fruits (10 kg) were extracted with 10 L of 75% ethanol using ultrasonic assistance at room temperature over four successive cycles, each lasting three days. The combined ethanol extracts were concentrated under reduced pressure, suspended in 3 L of water, and partitioned with dichloromethane. The dichloromethane layer was collected and evaporated to yield 80 g of crude extract.

Cell culture

H1299, H460, LLC, H1975, PC9 and A549 cells were granted by the Penghui Zhou lab at Sun Yat-sen University. H1299, H460, H1975 and PC9 cells were cultured in RPMI-1640 medium, while A549 and LLC cells were maintained in DMEM. All cells were cultured in a humidified incubator at 37°C with 5% CO₂.

Cell viability assay

Cells were inoculated into a 96-well plate at a density of 3×10³ cells per well in 100 µL of culture medium and allowed to adhere overnight. After attachment, cells were treated with various concentrations of FHF-DE (1-625 µg/mL) or an equivalent volume of DMSO (vehicle control, final DMSO concentration < 0.5%) in triplicate wells. Following 48 hours of incubation at 37°C in a 5% CO₂ humidified atmosphere, 10 µL of CCK-8 solution was added to each well, and plates were incubated for an additional 2 hours. Absorbance was measured at 450 nm using a microplate reader, with a reference wavelength of 650 nm. Blank wells containing culture medium without cells were used for background subtraction. Preliminary experiments confirmed a linear correlation between cell number (0.5-8×10³ cells/well) and absorbance (R² > 0.99) under the assay conditions. All experiments were performed in triplicate and repeated at least three times independently.

Allograft mouse model

C57BL/6 mice were purchased from Hunan Silaikejingda Experimental Animal Co., Ltd. and raised under the SPF condition. C57BL/6 mice were subcutaneously injected with LLC cells in the right lower abdomen (3×10⁵ cells per mouse). Seven days later, all animals were randomly divided into 4 groups: Control (saline, administered intragastrically daily), FHF-DE I (200 mg/kg in 20% corn oil and 80% saline, administered intragastrically daily), FHF-DE II (400 mg/kg in 20% corn oil and 80% saline, administered intragastrically daily) and ADM (2 mg/kg doxorubicin, administered at day 7, 10 and 13 through the tail vein). Starting from approximately the 7th day after tumor formation, the tumor volume was measured using an electronic caliper every third day. After 19 days, the mice were euthanized, and the tumor tissues were removed for further analysis. The liver and kidneys were fixed with 4% paraformaldehyde, and then embedded in paraffin. The tissues were cut to 10 micrometers. Subsequently, they were stained with hematoxylin-eosin (H&E) and photographed under a microscope. Mice were euthanized by cervical dislocation without prior anesthesia. This method was chosen because anesthetic agents might interfere with immune cell function, cytokine profiles, and tumor progression in murine models, which could confound the interpretation of the antitumor efficacy data. The procedure was performed by trained personnel, and all animal procedures were approved by the Ethics Committee of Hunan University of Medicine (approval no. 2024-A09099), which specifically reviewed and approved the euthanasia protocol as consistent with institutional guidelines for humane animal care.

Flow cytometry analysis of cell cycle and apoptosis

H1975 and LLC cells were inoculated into 6-well plates and treated with 5 µg/ml extract or an equivalent volume of DMSO (solvent control) [21]. After 48 h, the cell cycle was analyzed using the Beckman Cytoflex flow cytometer following the protocol provided in the Cell Cycle Assay Kit (Elabscience® Bionovation Inc.). Apoptosis was assessed using the Annexin V-APC/PI Apoptosis Kit (Elabscience® Bionovation Inc.).

Table 1. Primer design

Gene	Primer	Sequence of primer (5'-3')
TNFAIP3	Forward	CTCAACTGGTGTGAGAAGTCC
	Reverse	TTCCTTGAGCGTGCTGAACAGC
IL6	Forward	AGACAGCCACTCACCTCTTCAG
	Reverse	TTCTGCCAGTGCCTCTTTGCTG
MAP3K14	Forward	GGAATACCTCCACTCACGAAGG
	Reverse	CTGTGAGCAAGGACTTTCCAG
TRAF1	Forward	CGATGGCACTTTCTGTGGAAG
	Reverse	TACAGCCGAGGCACAACCTTGT
TNF	Forward	CTCTTCTGCCTGCTGCACTTTG
	Reverse	ATGGGCTACAGGCTTGCTACTC
JUN	Forward	CCTTGAAAGCTCAGAACTCGGAG
	Reverse	TGCTGCGTTAGCATGAGTTGGC
CREB5	Forward	GTCAGTGAAGTCCAGCATCATGG
	Reverse	GTGGTGAGTCAATGCAGCCTTC
BIRC3	Forward	GCTTTTGCTGTGATGGTGGACTC
	Reverse	CTTGACGGATGAACTCCTGTCC
IRF1	Forward	GAGGAGGTGAAAGACCAGAGCA
	Reverse	TAGCATCTCGGCTGGACTTCGA
GAPDH	Forward	GTCTCCTCTGACTTCAACAGCG
	Reverse	ACCACCCTGTTGCTGTAGCCAA

Western blot analysis

H1975 and LLC cells were treated with 5 µg/ml extract for 24 hours and 48 hours respectively, and then prepared by lysing with RIPA lysis buffer containing phosphatase inhibitors and protease. Western blot analysis was performed using primary antibodies including anti-p21 CDKN1A (Abways Technology; Cat. no. CY5543), anti-Cyclin D1 (Abways Technology; Cat. no. CY5404), anti-Cyclin A2 (Huabio; Cat. no. ET1612), anti-CDK4 (Abways Technology; Cat. no. CY5836), anti-CDK6 (Proteintech Group, Inc.; Cat. no. 14052-1-AP), anti-Bax (Abways Technology; Cat. no. CY5059), anti-Bcl2 (Zen-Bio, Inc.; Cat. no. 381702), anti-Caspase-3 (Cell signaling; Cat. no. 9662) and anti-β-actin (ABclonal Technology).

Transcriptome sequencing

H1975 cells were incubated with 5 µg/ml extract for 48 h. To construct the sequencing library, the quality of the total RNA was tested (with an RNA integrity number (RIN) of ≥ 9.5). The second-generation sequencing was carried out according to the standard procedures of Illumina. Expression levels of mRNA quantified

in fragments per kb of transcript per million mapped reads. Differentially expressed genes (DEGs) were identified using the following criteria $|\log_2(\text{fold change})| \geq 1$, corresponding to a ≥ 2-fold change (FC), and adjusted $P < 0.05$. The false discovery rate (FDR) was controlled using the Benjamini-Hochberg method to correct for multiple hypothesis testing. Only genes meeting both criteria were considered statistically significant DEGs. The sequencing results were further analyzed through the soft GSEA 4.1. The heatmap was generated using the R package.

Reverse transcription-quantitative (RT-q) PCR

H1975 cells were incubated with 2.5 and 5 µg/ml extracts for 48 hours. Extract RNA using the RNA extraction kit (Beijing Genenode Biotech Co., Ltd.) and reverse transcribed using the SureScript™ First-Strand cDNA Synthesis kit (GeneCopeia, Inc.; Cat. no. QP057). The cDNA products were amplified using the SYBR Green qPCR mixture (GeneCopeia, Inc.). The reaction mixture consists of 4 µL of qPCR Mix, 2 µL of forward PCR primer, 2 µL of reverse PCR primer, 2 µL of template, and 10 µL of water. The reaction conditions are 95°C for 30 seconds and 40 cycles (95°C for 10 seconds, 60°C for 30 seconds). The specific primer sequences used in this study are presented in **Table 1**.

Ultra high performance liquid chromatography-mass spectrometer (UHPLC-MS) analysis

For sample preparation, 100 mg of FHF-DE was accurately weighed and dissolved in 1 milliliter of methanol. Centrifuge at 12,000 revolutions per minute for 20 minutes, then take 5 microliters of the supernatant for UHPLC-MS/MS analysis. UHPLC separation was carried out using the Ultimate 3000 system (Thermo Fisher Scientific, Inc.). A InfinityLab Poroshell 120 EC-C18 chromatographic column (Agilent Technologies; Thermo Fisher Scientific, Inc.; 2.1×150 mm, 2.7 µm) was used, and the column temperature was maintained at 45°C. The mobile phase consisted of 0.1% (by volume) aqueous solution of formic acid (A) and acetonitrile (B). The separation is carried out using gradient elution with a flow rate of 0.3 ml/min: 0-2 min, 95% A; 2-5 min, 95-85% A; 5-20 min, 85-65% A; 20-30 min, 65-45% A; 30-50 min,

45-20% A; 50-60 min, 20-5% A; 60-62 min, 5-95% A; 62-65 min, 95% A.

A Q-Exactive Orbitrap mass spectrometer fitted with a heated electrospray ionization (HESI) source was used for the mass spectrometry analysis. The instrument can operate in both negative and positive ion modes, with the m/z range being 100-1500. A large amount of data was analyzed with Xcalibur 4.1 software (Thermo Fisher Scientific, Inc.).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 8 software. For comparisons between two groups, a two-tailed unpaired Student's t -test was used. For multiple group comparisons, one-way or two-way analysis of variance (ANOVA) was applied as appropriate. For one-way ANOVA, Tukey's post hoc test was used for pairwise comparisons among all groups. For two-way ANOVA, Dunnett's post hoc test was applied to compare each treatment group with the control group. The P value is indicated by an asterisk, as follows: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and n.s. = nonsignificant.

Results

FHF-DE inhibits the proliferation of lung carcinoma cells in vitro and vivo

The cytotoxicity of FHF-DE against lung carcinoma cells was evaluated using the CCK-8 kit. As stated in the hypothesis, FHF-DE significantly inhibited the proliferation of multiple human lung carcinoma cell lines (H1299, A549, H460, H1975, and PC9) and the murine Lewis lung carcinoma (LLC) line in a dose-dependent manner (**Figure 1A**). Among them, H1975 and LLC cell lines, which exhibited lower IC_{50} values, were selected for further mechanistic studies.

To further evaluate the anti-tumor property of FHF-DE in vivo, an LLC allograft model was established. Tumor-bearing mice received oral FHF-DE at 200 or 400 mg/kg/day, whereas the positive control group received doxorubicin (ADM) via tail vein injection once every 3 days [22]. FHF-DE treatment significantly reduced both tumor volumes and weights (**Figure 1B**). The Doxorubicin-treated group, also showed significant tumor growth inhibition. No direct statistical comparison was made between the

FHF-DE and doxorubicin groups due to differences in administration route, dosing schedule, and mechanism of action. The H&E staining results indicated that oral gavage of FHF-DE did not induce obvious hepatotoxicity (up) or nephrotoxicity (bottom) in mice (**Figure 1C**). Collectively, these results demonstrate that oral administration of FHF-DE significantly suppresses tumor growth in the LLC allograft model with no apparent hepatotoxicity or nephrotoxicity. However, as the tumor tissues were not examined for molecular markers of apoptosis or proliferation, this in vivo finding should be interpreted as evidence of antitumor efficacy rather than mechanistic validation of the in vitro observations.

FHF-DE interferes with G1/S phase in lung carcinoma cells

Cell cycle plays a central role in cell proliferation and tumor progression, and its disruption can effectively suppress cancer cell survival [12, 23]. To assess the effect of FHF-DE on cell cycle distribution, H1975 and LLC lung carcinoma cells were cultured with 5 μ g/ml FHF-DE for 48 h and analyzed via flow cytometry. In LLC cells, FHF-DE treatment markedly upregulated the proportion of cells in the G0/G1 phase, while downregulating the proportion of S phase cells (**Figure 2A**). Western blotting confirmed reduced expression of Cyclin D1, along with elevated levels of p21 and Cyclin A2 (**Figure 2C**). By contrast, a significant decrease in the G0/G1 phase and an increase in the S phase were observed in H1975 cells after exposure to FHF-DE (**Figure 2B**). Western blot analysis revealed decreased levels of Cyclin D1 and Cyclin A2, a gradual increase in p21, and no significant change in CDK4 and CDK6 expression (**Figure 2D**). Overall, these results indicated that FHF-DE induces G0/G1 phase arrest in LLC cells and S phase arrest in H1975 cells, by regulating corresponding cyclins. Together, the data suggested that FHF-DE primarily targets the G0/G1 and S phases to inhibit lung carcinoma cell proliferation.

FHF-DE induces apoptosis in lung carcinoma cells

Apoptosis, as a form of programmed cell death, death, plays a critical role in tumor suppression [24]. This study utilized the Annexin V/PI detection assay to investigate the potential of FHF-

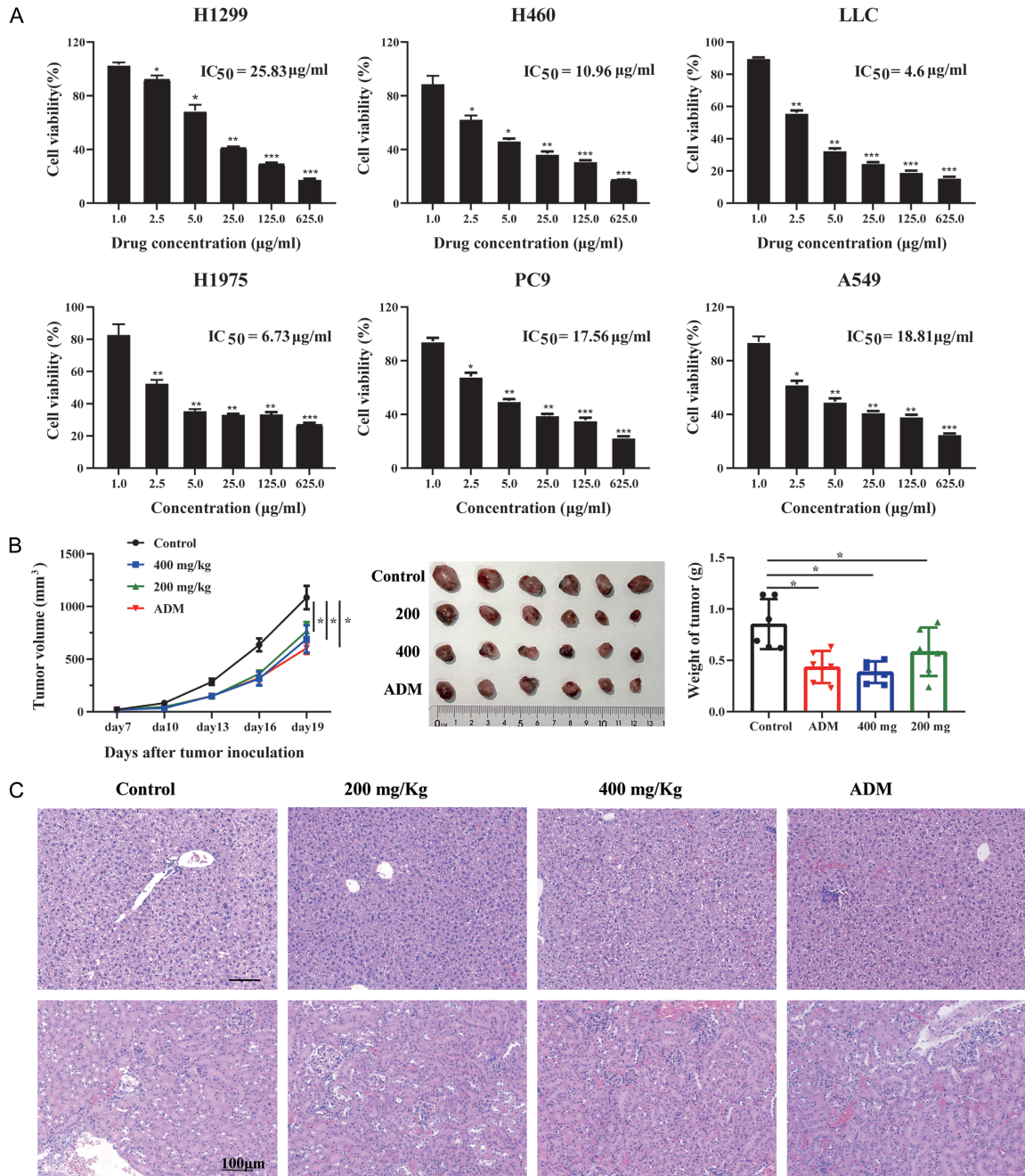


Figure 1. Effect of FHF-DE on the proliferation and tumor growth of lung carcinoma cells. A. Cell viability of lung carcinoma cells treated with different concentrations (1-625 $\mu\text{g/ml}$) of FHF-DE, CCK8 assay was performed at 48 h. Controls treated with DMSO. Data are representative of three independent experiments. B, C. Antitumor effect and safety of FHF-DE. After subcutaneously inoculating with LLC cells for 7 days, mice were administered 200 and 400 mg/kg FHF-DE orally once a day for 12 days by gavage. ADM, 2 mg/kg doxorubicin was administered on day 7, 10, and 13 through the tail vein. B. Tumor volumes recorded at the indicated times, tumor images and weights of harvested tumors on day 19 are shown, $n = 6$ mice per group. Doxorubicin was included as a positive control. No direct comparison was made between the FHF-DE and doxorubicin groups due to differences in administration routes and dosing schedules. C. The histopathological images of liver (up) and kidney (bottom) by H&E staining (400 $\times$). Data are representative of two independent experiments. Error bars, mean $\pm$ SEM. Statistical significance was determined using two-tailed unpaired Student's t-test for two-group comparisons, and one-way or two-way ANOVA for multiple group comparisons. For multiple group comparisons, two-way repeated-measures ANOVA with Dunnett's post hoc test was performed for comparisons with the control group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

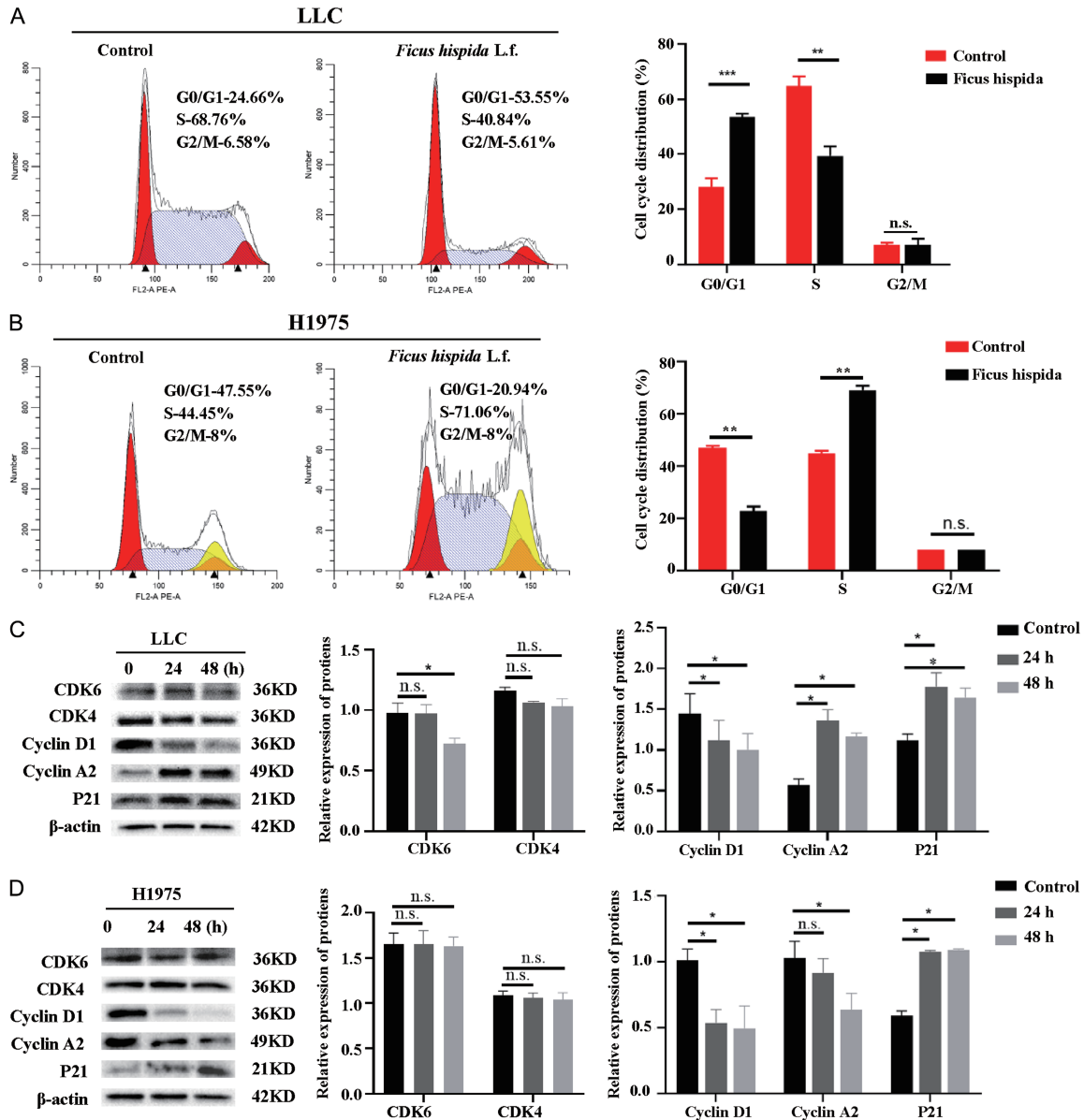


Figure 2. Cell cycle analysis of lung carcinoma cells treated with FHF-DE. A, B. PI staining assay of LLC and H1975 cells treated with FHF-DE. C, D. Western blot analysis was performed to examine cell cycle-related protein expression in LLC and H1975 cells treated with FHF-DE. Data are representative of three independent experiments. Error bars, mean $\pm$ SEM. Statistical significance was determined using two-tailed unpaired Student's t-test for two-group comparisons, and one-way ANOVA for multiple group comparisons. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

DE in inducing apoptosis in LLC and H1975 cells. As shown in **Figure 3A**, the percentage of late apoptosis (Annexin V⁺/PI⁺) LLC cells significantly increased in a time-dependent manner. Similarly, the early apoptosis rate (Annexin V⁺/PI⁻) was significantly elevated in the FHF-DE group of H1975 cells (**Figure 3B**). Consistently, western blotting revealed elevated levels of Bax and cleaved caspase-3, with concomitant reductions in Bcl-2 and full-length (pro)-caspase-3 in both cell lines relative to controls

(**Figure 3C, 3D**). Together, these results indicated that FHF-DE triggers apoptosis in LLC and H1975 cells.

Transcriptomic profiling reveals FHF-DE-mediated activation of TNF signaling in lung carcinoma cells

To further elucidate the regulatory network through which FHF-DE influences lung carcinoma cells, RNA sequencing analysis was con-

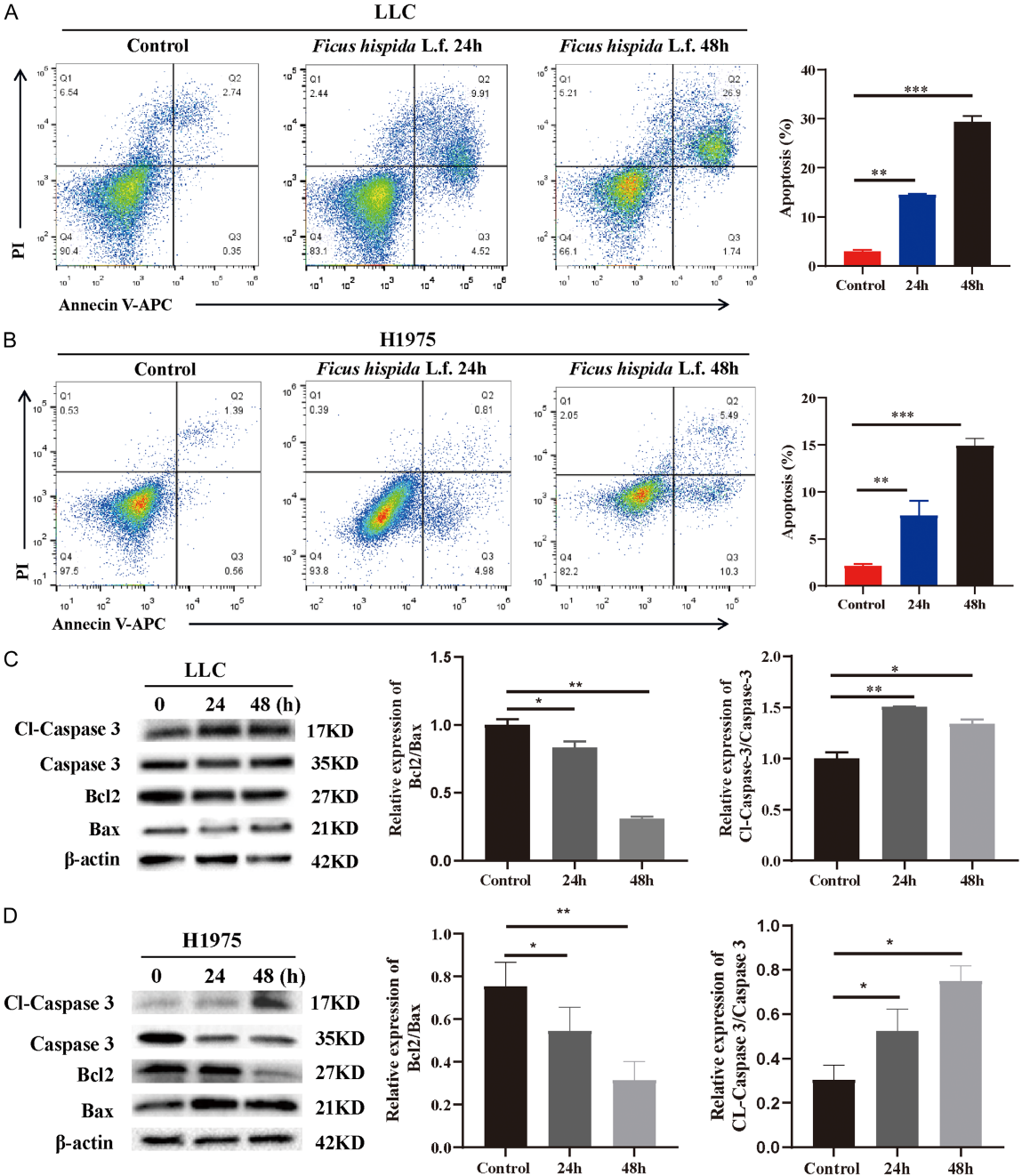


Figure 3. FHF-DE induced lung carcinoma cells apoptosis. A, B. Flow cytometry detected apoptosis of LLC and H1975 cells treated with FHF-DE at 24 and 48 h by Annexin V-APC/PI staining. C, D. LLC and H1975 cells treated with FHF-DE for 24 and 48 h, the expression levels of the apoptosis protein markers were analyzed by Western blot. Data are representative of three independent experiments. Error bars, mean $\pm$ SEM. Statistical significance was determined using two-tailed unpaired Student's t-test for two-group comparisons, and one-way ANOVA for multiple group comparisons. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

ducted using H1975 cells. As illustrated in the volcano plot, this analysis identified 385 upregulated and 375 downregulated genes ($|\log FC| > 1$, $P < 0.05$) in H1975 cells upon exposure to FHF-DE (Figure 4A). Differentially expressed

genes are listed in Table S1. KEGG pathway enrichment revealed that the pro-inflammatory pathways were significantly enriched, including rheumatoid arthritis, IL-17 signaling and NF- κ B signaling (Figure 4B). Among these, the TNF sig-

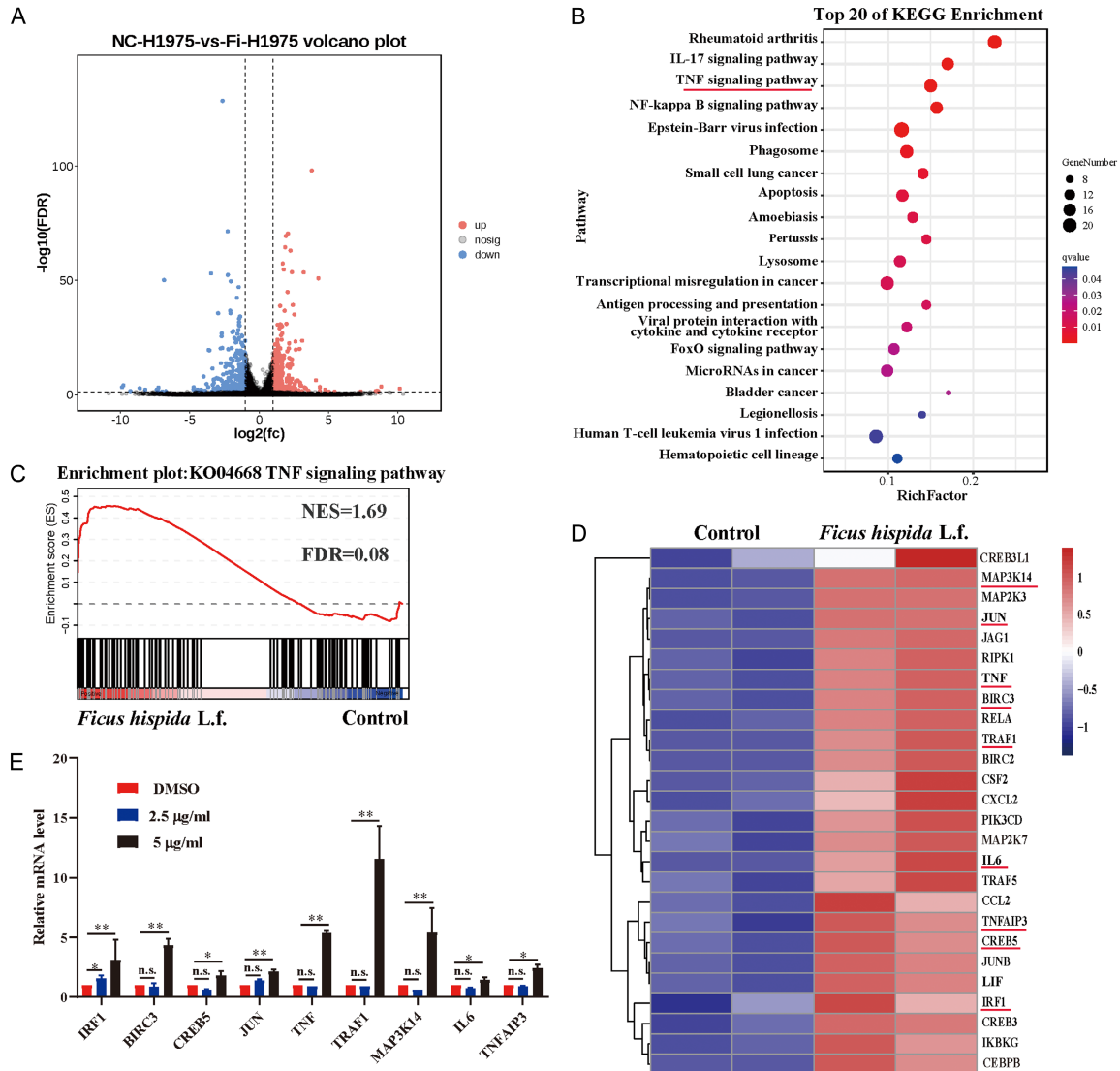


Figure 4. Transcriptomic results of H1975 cells treated with FHF-DE. A. Volcano plot of differentially expressed mRNAs upon exposure to FHF-DE for 48 h in H1975 cells. B. KEGG enrichment analysis illustrating the signaling pathways of differentially expressed genes between FHF-DE and control groups. C. Enrichment of genes in the KEGG TNF signaling pathway by GSEA. D. Heatmap of FHF-DE regulated genes involved in the TNF signaling pathway. E. RT-qPCR analysis of the mRNA expression of the indicated genes in FHF-DE treated and controlled H1975 cells that are associated with TNF signaling pathway. Data are representative of three independent experiments. Statistical significance was determined using two-tailed unpaired Student's t-test for two-group comparisons, and one-way ANOVA for multiple group comparisons. Error bars, mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, n.s. = nonsignificant.

naling pathway is a key driver of rheumatoid arthritis [25] and can initiate apoptosis through the extrinsic pathway. Consistently, gene set enrichment analysis (GSEA) showed that the TNF signaling pathway was positively enriched in FHF-DE-treated cells (Figure 4C). Core genes of this pathway are displayed in the heatmap of Figure 4D, including IRF1, BIRC3, CREB5, JUN, TNF, TRAF1, MAP3K14, IL6 and TNFAIP3 were

further confirmed through RT-qPCR analysis (Figure 4E).

FHF-DE induces apoptosis associated with the TNF signaling pathway

We subsequently investigated whether FHF-DE induces apoptosis in lung carcinoma cells through the TNF signaling pathway. As depicted

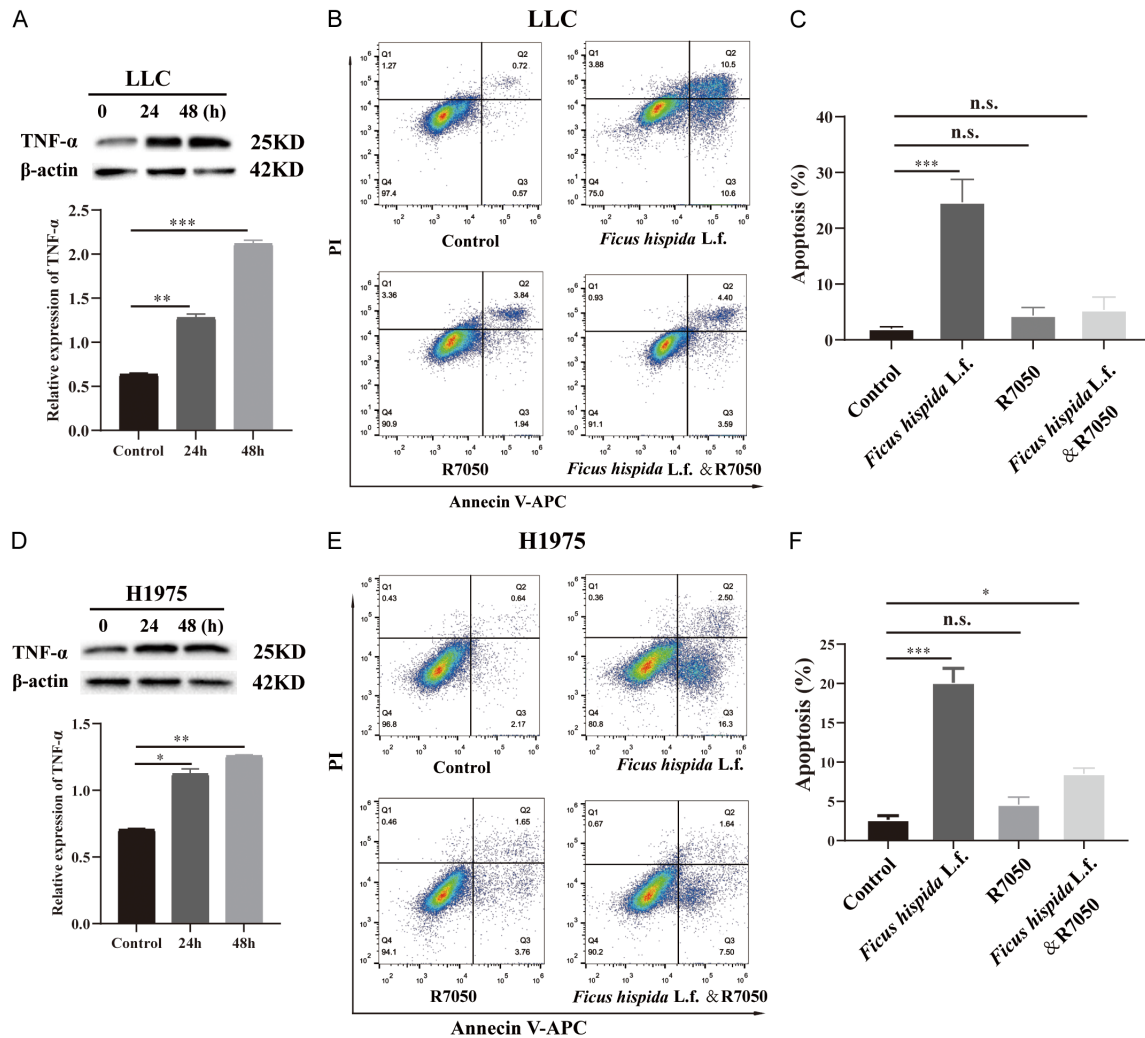


Figure 5. FHF-DE induces apoptosis associated with the TNF signaling pathway. A. LLC cells treated with FHF-DE for 24 and 48 h, the expression levels of the TNF- α were analyzed by Western blot. B, C. Flow cytometry detected apoptosis of LLC cells treated with FHF-DE at 48 h by Annexin V-APC/PI staining with or without TNFR antagonist R7050 pretreatment. D. H1975 cells treated with FHF-DE for 24 and 48 h, the expression levels of the TNF- α were analyzed by Western blot. E, F. Flow cytometry detected apoptosis of H1975 cells treated with FHF-DE at 48 h by Annexin V-APC/PI staining with or without TNFR antagonist R7050 pretreatment. Data are representative of three independent experiments. Error bars, mean $\pm$ SEM. Statistical significance was determined using two-tailed unpaired Student's t-test for two-group comparisons, and one-way ANOVA for multiple group comparisons. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

in **Figure 4A** and **4B**, treating LLC and H1975 cells with FHF-DE at their respective IC_{50} concentrations for 24 or 48 h resulted in an increased apoptosis rate in LLC and H1975 cells. Consistently, TNF- α protein levels were elevated in FHF-DE-treated cells, indicative of TNF pathway activation (**Figure 5A**, **5D**). To further evaluate the involvement of TNF signaling in FHF-DE-induced apoptosis, cells were pre-treated with the TNFR antagonist R7050 for 4 h prior to FHF-DE exposure. Cells were pretreated with 5 μ M R7050, a concentration previous-

ly reported to selectively inhibit TNFR1 [26]. Pretreatment with 5 μ M R7050 significantly decreased the apoptotic rate induced by FHF-DE in both LLC (**Figure 5B**, **5C**) and H1975 cells (**Figure 5E**, **5F**). This pharmacological inhibition provides supportive, though not definitive, evidence for the functional involvement of TNFR1 signaling in the apoptotic response. However, several interpretations of this finding must be considered. First, R7050 may have off-target effects at the concentration used, and its specificity was not formally verified in our

cellular systems. Second, the partial nature of the reversal indicates that TNFR1-independent pathways, potentially including the intrinsic mitochondrial pathway, as suggested by Bax upregulation and Bcl-2 suppression, also contribute substantially to the overall apoptotic effect. Third, as a complex botanical extract, FHF-DE likely engages multiple signaling cascades concurrently, and the TNF pathway may represent one of several contributing mechanisms rather than a single primary driver. Therefore, these data should be interpreted as pharmacological evidence of functional association, not as mechanistic proof of direct pathway engagement.

Identification of bioactive compounds in FHF-DE by UHPLC-MS analysis

The chemical composition of FHF-DE was profiled by UHPLC-Q-Exactive Orbitrap MS in both positive and negative ion modes. Phytochemicals were identified by comparison with Compound Discoverer 3.3 and reference standards. A total of 37 compounds were identified, including 14 flavonoids, 8 phenolic acids, 4 fatty acids, 3 terpenoids, 2 alkaloids, 2 quinones, 1 diketone, and 3 other types of compounds. The complete list of identified compounds, along with their retention times, molecular formulas, and MS/MS fragmentation data, is presented in **Table 2**.

Among the 14 flavonoids identified, 10 have been previously reported to possess anticancer activities against various cancer cell lines, including lung cancer [24-36]. Several phenolic acids identified in FHF-DE, including gallic acid [37], catechol [38], neochlorogenic acid [39], ferulic acid [40], and isoferulic acid [41], have also been reported to possess antitumor potential. Additionally, compounds such as aloesin [42] and curcumin [43] identified in the extract have documented anticancer activities through diverse signaling pathways. Collectively, these findings suggest that the anti-lung cancer effects of FHF-DE may stem from its abundant flavonoids and other bioactive constituents.

Discussion

Ficus hispida has long been used in Zhuang and Dai ethnomedicine for various diseases [5, 44]. Although its fresh fruits are unsuitable for

direct consumption, proper extraction can unlock their therapeutic potential. Accumulating pharmacological evidence supports its anticancer potential. Stem extracts, for example, suppressed breast cancer cell proliferation *in vitro* [45]. Hispidacine, isolated from the stem bark and leaves, inhibited growth in multiple tumor cell types [46]. Fractionations of fruit extracts using ethyl acetate and *n*-butanol markedly enhanced antiproliferative activity and enabled identification of a key bioactive flavonoid [7]. Aligning with these findings, FHF-DE similarly demonstrated potent anticancer activity in both human and murine lung cancer cells. Notably, this study provides the first *in vivo* validation of the antitumor efficacy of *F. hispida* fruit extracts in a syngeneic lung cancer model, filling the gap between *in vitro* mechanism studies and translational relevance. For safety assessment, we examined liver and kidney tissues via H&E staining and found no significant organ damage after continuous oral administration [47]. A limitation of this study is the lack of biochemical markers for hepatotoxicity or nephrotoxicity, such as alanine aminotransferase, aspartate aminotransferase and creatinine. Before discussing potential mechanisms, it is important to clarify the evidentiary status of our *in vivo* findings. The LLC allograft experiment demonstrates that FHF-DE significantly suppresses tumor growth when administered orally. However, as we did not perform immunohistochemical staining for key molecular markers, including TNF- α , cleaved caspase-3, or Ki-67, in the harvested tumor tissues, *this in vivo* study provides evidence of antitumor efficacy only, not mechanistic validation of the TNF-associated apoptotic pathway identified *in vitro*. The mechanistic link between FHF-DE treatment and *in vivo* tumor suppression therefore remains to be established. This limitation is critical and should be kept in mind when interpreting the translational implications of our findings.

FHF-DE exerted differential cell cycle arrest patterns in the two lung cancer cell lines examined, G0/G1 arrest in murine LLC cells and S-phase arrest in human H1975 cells. In LLC cells, FHF-DE treatment upregulated p21 and downregulated cyclin D1, consistent with G1/S blockade. In H1975 cells, FHF-DE treatment also induced p21 upregulation, but this was accompanied by cyclin A2 downregulation and

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Table 2. Chemical compounds identified in FHF-DE by LC-MS analysis

No.	Compound name	RT (min)	Molecular formula	M/Z	Ionization form	Error (ppm)	MS ₂ fragment
1	Gluconic acid	1.25	C ₆ H ₁₂ O ₇	195.0538	[M-H] ⁻	3.93	177.0427, 159.0318, 148.9114, 129.0205, 99.0092, 87.0089, 75.0087
2	D-(-)-Quinic acid	1.31	C ₇ H ₁₂ O ₆	191.0587	[M-H] ⁻	3.43	166.1085, 157.2604, 129.0868, 105.0200, 85.0296
3	D-(+)-Malic acid	1.38	C ₄ H ₆ O ₅	133.0155	[M-H] ⁻	1.03	115.0045, 89.0246, 72.9930, 71.0137
4	Pyrogallol	2.71	C ₆ H ₆ O ₃	125.0255	[M-H] ⁻	0.27	123.0096, 105.9364, 97.0299, 69.0344
5	Gallic acid	2.86	C ₇ H ₆ O ₅	169.0164	[M-H] ⁻	2.39	151.0416, 125.0254, 97.0299, 81.0346
6	Catechol	5.32	C ₆ H ₆ O ₂	109.0302	[M-H] ⁻	3.10	97.3904, 92.8608, 78.5017
7	Baicalin	5.45	C ₁₅ H ₁₀ O ₅	271.0598	[M+H] ⁺	-0.94	123.008, 95.0134
8	Neochlorogenic acid	9.74	C ₁₆ H ₁₈ O ₉	353.0942	[M-H] ⁻	2.17	191.0587, 161.0263, 148.9817
9	trans-Cinnamic acid	14.92	C ₉ H ₈ O ₂	147.0467	[M-H] ⁻	3.40	118.9672, 102.9493, 89.0246, 87.9255, 74.9619, 61.9881
10	Ferulic acid	15.36	C ₁₀ H ₁₀ O ₄	193.0533	[M-H] ⁻	3.98	178.0294, 149.0623, 134.0385, 61.9881
11	Isoferulic acid	19.08	C ₁₀ H ₁₀ O ₄	193.0533	[M-H] ⁻	3.89	178.0293, 165.0576, 161.0262, 149.0623, 137.0257, 134.0385, 105.0715
12	Rutin	24.12	C ₂₇ H ₃₀ O ₁₆	609.1477	[M-H] ⁻	2.47	301.0359, 271.0253, 243.0299, 151.0027
13	Quercetin	25.94	C ₁₅ H ₁₀ O ₇	301.0407	[M-H] ⁻	1.76	273.0451, 239.0752, 179.0009, 151.0053, 121.03036
14	Schizokinen	27.23	C ₁₆ H ₂₈ N ₄ O ₉	419.1787	[M-H] ⁻	1.68	356.1328, 329.1453, 195.06900, 165.0577, 149.0259, 59.0135
15	Benzyl 4-O-β-D-glucopyranosyl-β-D-glucopyranoside	27.27	C ₁₉ H ₂₈ O ₁₁	431.1538	[M-H] ⁻	-4.73	431.1538
16	Apigenin	27.56	C ₁₅ H ₁₀ O ₅	269.0503	[M-H] ⁻	4.69	250.5929, 225.1149, 181.0685, 176.7585, 160.1887, 145.4020, 91.0556
17	Isoquercitrin	29.24	C ₂₁ H ₂₀ O ₁₂	463.0906	[M-H] ⁻	4.91	331.1769, 301.0356, 300.02800, 271.0253, 255.0301, 243.0303, 161.0447, 151.0027, 101.0231, 71.01247
18	Aloesin	29.64	C ₁₉ H ₂₂ O ₉	395.1371	[M+H] ⁺	1.77	359.1748, 326.2749, 264.3864, 200.3823, 157.7936
19	Catechin-4-ol 3-O-β-D-galactopyranoside	29.66	C ₂₁ H ₂₄ O ₁₂	469.1355	[M+H] ⁺	3.02	469.1355
20	Quinine	32.39	C ₂₀ H ₂₄ N ₂ O ₂	325.1908	[M+H] ⁺	-0.8	324.1786
21	Isowigtheone hydrate	32.53	C ₂₀ H ₂₀ O ₆	355.1249	[M-H] ⁻	3.02	356.1285, 337.1113, 282.0585, 281.0502, 176.7264, 133.0034
22	Corchorifatty acid F	33.34	C ₁₈ H ₃₂ O ₅	327.2235	[M-H] ⁻	0.81	309.2126, 229.1482, 211.1370, 171.1047, 85.0297

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23	Rubiadin	34.39	$C_{15}H_{10}O_4$	253.0550	[M-H] ⁻	4.22	238.0665, 209.1576, 180.2941, 159.3351, 130.1952
24	Curcumin	38.32	$C_{21}H_{20}O_6$	367.1252	[M-H] ⁻	3.75	352.1016, 295.0661, 282.0585, 223.6991, 147.2476
25	Isowighteone	39.93	$C_{20}H_{18}O_5$	337.1141	[M-H] ⁻	4.53	338.1176, 322.6197, 293.0508, 281.0505, 163.2606
26	Genistein	40.74	$C_{15}H_{10}O_5$	269.0504	[M-H] ⁻	3.82	223.0859, 206.0421, 201.2931, 151.2708, 119.4644
27	Alpinumisoflavone	41.72	$C_{20}H_{16}O_5$	335.0982	[M-H] ⁻	2.46	360.1019, 335.0919, 320.0746
28	Calocarpin	44.33	$C_{20}H_{20}O_4$	323.1347	[M-H] ⁻	3.87	305.2173, 279.1443, 254.9207, 241.8489, 204.67878, 170.4344
29	Ficusin A	45.37	$C_{25}H_{24}O_5$	403.1620	[M-H] ⁻	-3.81	404.1655, 403.1620, 357.8457, 335.0984
30	3'-(3-methylbut-2-en-1-yl)biochanin A	46.80	$C_{21}H_{20}O_5$	351.1299	[M-H] ⁻	3.04	352.1333, 336.1036, 335.0988, 307.0663, 281.0502
31	Myrsininone A	48.92	$C_{25}H_{26}O_5$	405.1777	[M-H] ⁻	3.87	406.1815, 389.7852, 361.9356, 229.9373, 151.0790, 93.7252
32	Naringenin	53.93	$C_{15}H_{12}O_5$	271.0660	[M-H] ⁻	3.46	253.2210, 225.2259
33	Oleanolic acid	55.27	$C_{30}H_{48}O_3$	455.3614	[M-H] ⁻	0.37	403.0883, 363.3337, 304.7968, 271.2327, 180.7129, 94.8742
34	Linoelaidic acid	55.96	$C_{18}H_{32}O_2$	279.2378	[M-H] ⁻	0.36	261.2269, 233.6912, 171.7487, 148.4459
35	Asperdemin	56.00	$C_{21}H_{28}O_7$	393.1894	[M-H] ⁺	-3.54	393.1894, 392.30463
36	Oleic acid	57.84	$C_{18}H_{34}O_2$	281.2535	[M-H] ⁻	2.3	209.2853, 203.5021, 164.0675, 127.2997, 88.1070
37	Stearic acid	59.80	$C_{18}H_{36}O_2$	283.2692	[M-H] ⁻	4.48	231.78500, 214.3051, 165.5871, 128.1572

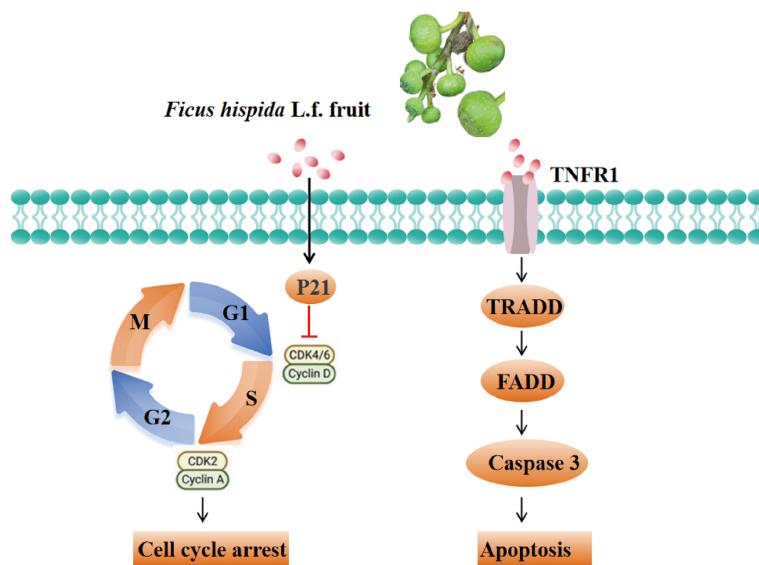


Figure 6. Schematic diagram of FHF-DE against lung cancer.

S-phase accumulation rather than G0/G1 arrest [48-50]. The mechanistic basis for this differential response, despite shared p21 upregulation, remains unknown. While the genetic backgrounds of these cell lines differ including TP53 mutation status in H1975 and Trp53 mutation in LLC, our data do not establish a causal link between any specific genetic alteration and the distinct cell cycle outcomes. Definitive elucidation of the mechanisms underlying the differential cell cycle arrest would require systematic investigation of the specific cell cycle regulators and checkpoints operative in each cellular context.

Our data do not establish direct TNFR1 engagement or extrinsic apoptotic pathway activation. Transcriptomic enrichment, pharmacological attenuation by R7050, and elevations in TNF- α and cleaved caspase-3 are all consistent with, but do not prove, a functional association between FHF-DE and TNF-related signaling. These observations remain correlative and pharmacological, not mechanistic. Crucially, we did not assess caspase-8 cleavage, the definitive hallmark of death receptor-mediated apoptosis. In the absence of this, cleaved caspase-3, a downstream executioner shared by both extrinsic and intrinsic pathways, cannot be attributed specifically to TNFR1-driven apoptosis. Indeed, the concurrent Bax upregulation and Bcl-2 suppression we observed point just as plausibly to a mitochondrial contribution,

which could account for a substantial portion of the apoptotic response. Transcriptome sequencing uncovered that FHF-DE treatment was associated with enrichment of inflammatory pathways, including IL-17 signaling, TNF signaling and NF- κ B signaling pathway. Among these, the TNF/TNF receptor pathway is of particular interest as a classical extrinsic apoptotic route [51]. TNF- α signaling is context-dependent and can trigger both apoptotic and survival-promoting cascades [52]. TNF, a proinflammatory cytokine, can directly kill tumor cells. TNF- α , for instance, binds to TNFR1

and recruits FADD to activate apoptosis mediated by caspase-3 (**Figure 6**). Upon binding to TNFR1, TNF- α can recruit TNFR1-associated DEATH domain protein and FADD to form a death-inducing signaling complex that activates caspase-8 and subsequently caspase-3, executing extrinsic apoptosis. Conversely, TNFR1 can also recruit TNF-associated factor 2 (TRAF2), activating NF- κ B-dependent survival programs that counteract cell death. The balance between TNFR1-mediated survival and apoptosis may depend on the nature and intensity of the survival stimuli. Our observation of increased TNF- α protein and apoptotic markers (Bax upregulation, Bcl-2 suppression, and cleaved caspase-3) is consistent with FHF-DE tilting the TNFR1 signaling balance toward the pro-apoptotic arm. Nevertheless, it is important to note that pathway enrichment and pharmacological inhibition do not constitute proof of direct pathway engagement. As a multi-component extract, FHF-DE likely activates multiple signaling cascades, with TNF signaling representing one of several contributing mechanisms. Furthermore, the TNFR antagonist R7050 attenuated apoptosis, suggesting that FHF-DE promotes apoptosis in lung cancer cells, at least in part, through TNFR1-dependent signaling. However, it should be noted that although 5 μ M R7050 within the reported selective range for TNFR1 inhibition, we did not verify its specificity in our LLC and H1975 systems, and potential off-target effects cannot be

excluded. Therefore, our R7050 data should be interpreted as pharmacological evidence supporting the involvement of TNFR1, rather than definitive proof. Future studies utilizing TNFR1/2-specific knockout or TNF- α neutralizing antibodies would be valuable for conclusive validation.

LC-MS analysis identified 37 compounds in FHF-DE, including 14 flavonoids. Ten of these, including baicalein, rutin, quercetin, apigenin, isoquercitrin, isowighteone hydrate, isowighteone, genistein, alpinumisoflavone and naringenin, have been reported to possess anticancer activities against lung cancer and other malignancies. Rather than attributing the anti-tumor effect to a single constituent, we propose that the bioactivity of FHF-DE arises from additive or synergistic interactions among multiple flavonoids and phenolic acids. This poly-pharmacological mechanism is consistent with the emerging paradigm that complex natural extracts often outperform isolated compounds due to multi-target engagement [40]. In addition to flavonoids, several phenolic acids identified in FHF-DE (gallic acid, catechol, neochlorogenic acid, ferulic acid and isoferulic acid) and other compounds such as curcumin have documented antitumor potential through diverse signaling pathways [41, 42]. The complexity of the extract may explain its efficacy across multiple lung cancer cell lines with different genetic backgrounds.

Several limitations of this study warrant acknowledgment. First, regarding dose translation, the oral doses of 200 and 400 mg/kg used in mice correspond to human equivalent doses (HED) of 16.2 and 32.4 mg/kg, respectively, calculated using the body surface area normalization method recommended by Reagan-Shaw *et al.* [53]. These values are within the range of traditional herbal intake, though direct consumption of the crude dichloromethane fraction is not equivalent to traditional preparation. Furthermore, the absolute oral bioavailability of individual flavonoids such as baicalein and quercetin is generally low (< 10-23%) and plasma concentrations of parent compounds may not reach in vitro IC₅₀ levels [54]. However, the extract is a complex mixture, and additive or synergistic effects among multiple constituents, as well as the formation of active metabolites, may contribute to the observed in vivo efficacy. Second, although our

transcriptomic and R7050 data support the functional involvement of TNF signaling in FHF-DE-induced apoptosis, we acknowledge that these findings do not establish that FHF-DE directly engages TNFR1 as its primary mechanism. These data are correlative and pharmacological rather than mechanistic. As a complex botanical extract containing multiple bioactive flavonoids, FHF-DE likely exerts its effects through a multi-targeted network. The enrichment of additional pathways such as NF- κ B and IL-17 signaling in our transcriptomic analysis further supports this notion. Therefore, our data should be interpreted as demonstrating the association, rather than definitive causation through, the TNF pathway. Third, while our R7050 data indicate that TNF signaling contributes to FHF-DE-induced apoptosis, the reversal was only partial. Future studies using combined inhibition of multiple pathways (e.g., TNFR1 blockade plus caspase-9 inhibition or mitochondrial pathway interference) would be valuable to systematically deconvolute the relative contributions of TNF-dependent versus TNF-independent mechanisms to the overall apoptotic effect. Fourth, although our data show increased TNF- α and cleaved caspase-3 levels, we did not examine the upstream initiator caspase-8, which is the hallmark of extrinsic, death receptor-mediated apoptosis. Therefore, we cannot definitively conclude that the observed apoptosis is mediated through the extrinsic apoptotic cascade. Caspase-3 is a common downstream executioner shared by both extrinsic (caspase-8-dependent) and intrinsic (caspase-9-dependent) pathways. Future studies incorporating caspase-8 cleavage assays or selective inhibitors will be required to fully delineate the relative contributions of extrinsic versus intrinsic pathways to FHF-DE-induced apoptosis. Fifth, the differential cell cycle arrest patterns observed between LLC (G0/G1 arrest) and H1975 (S-phase arrest) cells represent an intriguing but mechanistically unresolved finding. While these two cell lines differ in their genetic backgrounds, including TP53 mutation status, our data do not establish a causal relationship between any specific genetic alteration and the distinct cell cycle outcomes. The mechanistic basis for this differential response requires further investigation, including systematic analysis of the specific cyclin-CDK complexes and checkpoint pathways that are differentially engaged in each cell line.

Conclusion

FHF-DE induces cell cycle arrest and apoptosis in lung carcinoma cells in vitro, with transcriptomic and pharmacological data suggesting the involvement of TNF-associated signaling. In vivo, FHF-DE significantly suppressed tumor growth in an LLC allograft model with no apparent hepatotoxicity or nephrotoxicity. However, as the in vivo study lacked direct molecular validation of the proposed mechanism in tumor tissues, the mechanistic link between FHF-DE treatment and in vivo tumor suppression requires further investigation. Future studies incorporating tissue-level molecular analyses will be required to bridge this gap and establish the translational relevance of the proposed mechanism. The identified bioactive compounds provide a foundation for future development of *F. hispida*-based therapeutics.

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Disclosure of conflict of interest

None.

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