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# The extract, LXB-1, from the barks of *Liriodendron × hybrid*, induced apoptosis via Akt, JNK and ERK1/2 pathways in A549 lung cancer cells

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**Abstract:** The effect of LXB-1, an extract from *Liriodendron × hybrid*, was determined on A549 human lung adenocarcinoma cell lines. Growth inhibition of LXB-1 was analyzed by MTT assay. Cancer cell cycle was measured by flow cytometry. To verify the apoptosis effect of LXB-1 on A549 cells, annexin V/PI double staining assay was performed. The expression levels of proapoptotic proteins were also measured by western blot. The potential mechanisms of LXB-1 inducing apoptosis – the expression and phosphorylation of ERK, p38, JNK and Akt – were investigated by western blot. The IC<sub>50</sub> values of LXB-1 on A549 for 24, 48 and 72 h treatment were determined to be 12.97±1.53 µg/mL, 9.55±1.42 µg/mL, and 5.90±0.74 µg/mL, respectively. LXB-1 induced an obvious G2/M cell cycle arrest in A549 cells and resulted in significant cell apoptosis. LXB-1 also increased the cleavage of both caspase-3 and caspase-9, and greatly decreased the protein levels of Bcl-2. Moreover, LXB-1 increased the expression of phosphorylated JNK but

decreased the levels of phosphorylated ERK1/2 and Akt. These results suggest that that LXB-1 induced apoptosis through JNK, ERK1/2, and Akt pathways in A549 cells.

**Keywords:** A549 cells; apoptosis; *Liriodendron hybrid*; lung cancer.

## 1 Introduction

Lung cancer is responsible for a considerable share of cancer-induced mortalities, which is estimated to exceed 800,000 annual deaths worldwide [1, 2]. Chemotherapy is a major therapeutic option for treatment of lung cancer [2]. However, traditional chemotherapy is no longer appropriate for treatment of human adenocarcinoma because of its poor prognosis, metastasis, and drug resistance [3]. Therefore, there is a renewed interest in natural products because they possess strong biological properties with the added benefit of reduced systemic toxicity [4].

Apoptosis is the major type of cell death that accounts for the mechanisms of most anti-cancer drugs. Apoptosis is characterized by specific morphologic and biochemical changes, including cell shrinkage, nuclear condensation and fragmentation, dynamic membrane blebbing, and loss of adhesion to neighbors [5, 6]. Two core pathways, known as the extrinsic and intrinsic pathways, lead to caspase activation and therefore to apoptosis [7, 8]. Another key regulator of apoptosis is the Bcl-2 family, which comprises a number of pro-apoptotic proteins such as Bax, Bak, and Bad as well as anti-apoptotic proteins members, such as Bcl-2 and Bcl-xL [9]. Other apoptotic pathways in cancer include PI3K/Akt, MAPK, p53 and microRNAs (e.g. miR29 and Let7) [10]. These deficiencies in apoptotic signaling and the resulting loss of apoptosis in cancer cells provide the rationale for cancer therapeutics targeting the apoptotic mechanism.

There two distinct species of *Liriodendron* genus (Magnoliaceae family), *L. tulipifera* from North America and *L. chinense* from East Asia, both of which produce valuable hardwood with great ecological and economic values. As an endangered deciduous tree species, *L. chinense*

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(Hemsl.) Sarg. has been listed as a protected Chinese plant [11, 12]. *L. tulipifera* is also a protected species and has shown antiplasmodial [13], antioxidant and anti-cancer properties [14]. As these two species are listed as endangered plants in China, it has become necessary to search for new medicinal plants. In 1963, a new hybrid strain, *L. chinense* × *L. tulipifera* (referred to as *L. hybrid* here), was successfully cultivated by Ye [13, 15–19]. We previously reported that extracts from *L. hybrid* showed potent cytotoxic effects on cancer cells [20]; in this study we further investigated the effect of LXB-1, an extract from *L. hybrid* on A549 cells. We found that LXB-1 inhibited A549 cell growth, induced G2/M cell cycle arrest, and induced A549 cell apoptosis. We further showed that LXB-1 affected the activity of apoptosis-related pathways JNK, ERK and Akt. Our data suggested a potential therapeutic use of *L. hybrid* in the treatment of human lung cancer.

## 2 Materials and methods

### 2.1 Cell culture and reagents

LXB-1 was prepared according to the described method in literature [20]. Dried barks powder (ca 350 g) of *L. hybrid* was extracted with 95% EtOH (500 mL × 3) and the solvent was removed *in vacuo*. The dried residue was re-dissolved in 500 mL MeOH and filtrated, and the filtrate was evaporated under vacuum to get dried LXB-1 (25.3 g) for bioassay. A549 human lung adenocarcinoma cell lines were purchased from the Shanghai Institute of Life Science (CAS) and cultured in F12K medium (Invitrogen, Calsbad, CA, USA) supplemented with 10% fetal calf serum (FCS, Gibco, Brooklyn, NY, USA), penicillin (100 U/mL), and streptomycin (100 µg/mL; Beyotime, Shanghai, China) in a humidified atmosphere containing 5% CO<sub>2</sub>. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from Sigma (St Louis, MO, USA). The Annexin V-PI Apoptosis Detection Kit was obtained from BD (San Diego, CA, USA). Enhanced Chemiluminescence (ECL) western blotting kit was obtained from Millipore (Bedford, MA, USA). Antibodies (anti-caspase-3, anti-cleaved caspase-3, anti-caspase-9, anti-cleaved caspase-9, anti-Bax, anti-Bcl2, anti-p-p38, anti-p38, anti-p-JNK, anti-JNK, anti-p-ERK, anti-ERK, anti-p-Akt, anti-Akt, anti-β-actin) were purchased from Santa Cruz (Santa Cruz, CA, USA). All other chemicals and solvents used were of analytical grade.

### 2.2 Cell proliferation assay

The cytotoxic activity of LXB-1 was determined by standard MTT assay [21]. Briefly, A549 cells were seeded in 96-well plates (Costar, Cambridge, MA, USA) at 37°C and kept overnight for attachment. LXB-1 was weighed and diluted with DMSO to make the stock solution. The stock solution was further diluted with culture media to make a series of final concentrations. A549 cells were treated with fresh medium containing different concentrations of LXB-1 for 24, 48,

and 72 h. Cells treated with 0.1% DMSO were used as the negative control. After incubation, 20 µL of 5 mg/mL MTT were added to each well and the cells were further incubated for 4 h. After removal of the MTT solution, purple formazan crystals were dissolved in 150 µL DMSO. The absorbance was measured at 570 nm using a colorimetric plate reader (Bio-Rad Laboratories, Hercules, CA, USA).

### 2.3 Cell cycle analysis

A549 cells were seeded in six-well plates by the density of  $6 \times 10^5$  per well and kept overnight at 37°C for attachment. After treating with 0.1% DMSO or different concentrations of LXB-1 for 24 h, cells were harvested and washed twice with phosphate buffer solution (PBS), and fixed in 70% ethanol for 1 h. Fixed cells were washed with PBS before incubation with 0.5 mL PBS containing 0.05% RNase and 0.5% Triton X-100 for 30 min. The cells were then stained with 0.1 mg/mL propidium iodide (PI) and DNA content and cell cycle were determined using a FACScan-laser flow cytometer (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ, USA). The data were analyzed using the software MODFIT and CELLQUEST (Version 2.2, BD Biosciences, Franklin Lakes, NJ, USA).

### 2.4 Annexin-V-FITC/PI double staining assay

Apoptotic cell death was assessed using the annexin-V-FITC and PI staining kit according to the manufacturer's recommendations. Briefly, A549 cells were seeded on six-well plates ( $6 \times 10^5$  per well) and allowed to attach overnight, followed by treatment with fresh medium containing LXB-1 for 48 h. Cells treated with 0.1% DMSO were used as the negative control; the positive control was 10 µM taxol. After 48 h of incubation, cells were trypsinized, washed twice with sterilized PBS, resuspended, and incubated with the annexin-V-FITC labeling solution and PI solution for 15 min at room temperature. Flow cytometric analysis was performed immediately after supravital staining. Data acquisition and analysis were performed in the flow cytometer using CellQuest software (version 2.2). Cells in early stages of apoptosis were annexin-V positive; cells that were both annexin-V and PI positive were in the late stage of apoptosis.

### 2.5 Western blot analysis

Western blot analysis was performed as described previously [22]. Briefly, cells were treated with 0.1% DMSO or different concentrations of LXB-1, were washed twice with ice-cold PBS, scraped off the plate, and resuspended in ice-cold RIPA lysis buffer. Cell lysates were incubated at 4°C for 15 min and cellular debris was pelleted by centrifugation at  $15\,000 \times g$  for 15 min at 4°C. Cell lysates were boiled 10 min before loading for analysis. Protein concentrations in the cleared lysate were quantified using BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA). 50 µg total proteins were loaded on SDS-PAGE gels, and then transferred to nitrocellulose membranes. The membranes were first rinsed with TBST buffer and then blocked with 5% (w/v) skim milk in TBST for 1 h at room temperature. The membranes were then incubated with the indicated primary antibodies (diluted 1:1000) and shaken gently at 4°C overnight. After washing, horseradish peroxidase-linked

anti-mouse IgG (Sigma, St Louis, MO, USA) was used as a secondary antibody and then incubated with the membrane for 1 h at room temperature. The signals were detected using ECL western blotting detection reagents (Millipore, Bedford, MA, USA).

## 2.6 Statistical analysis

Samples were analyzed in triplicate, and data were presented as the mean ± standard deviation. Statistical analysis of the data was performed using one way ANOVA analysis and Student's t-test. P-values were two-sided and a value of less than 0.05 was considered statistically significant. IC<sub>50</sub> values were determined by the Graphpad Prism 5 software package (La Jolla, CA, USA).

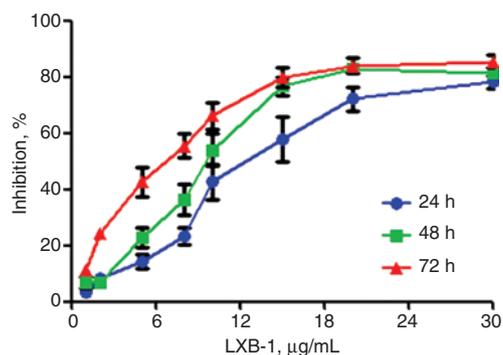
# 3 Results

## 3.1 Effect of LXB-1 on A549 cell proliferation

To investigate the effect of LXB-1 on the viability of A549 cells, cell proliferation was measured using the MTT assay performed with logarithmically growing cells. A549 cells were cultured in the absence or presence of various concentrations of LXB-1 for 24, 48, and 72 h. As shown in Figure 1, LXB-1 induced significant cytotoxicity in A549 cells in a time and dose-dependent manner. The IC<sub>50</sub> values for 24, 48 and 72 h treatment were 12.97 ± 1.53 µg/mL, 9.55 ± 1.42 µg/mL, and 5.90 ± 0.74 µg/mL, respectively.

## 3.2 LXB-1 induced G2/M cell cycle arrest in A549 cells

To test whether LXB-1 could affect the cell cycle of A549 cells, synchronized cells treated with different



**Figure 1:** LXB-1 inhibited A549 cell proliferation. A549 cells were treated with increasing concentrations of LXB-1 for 24, 48 and 72 h, and cell viability was determined by MTT assay.

concentrations of LXB-1 (1 µg/mL, 5 µg/mL and 10 µg/mL) for 48 h were subjected to flow cytometric analysis after DNA staining. 10 µM taxol served as the positive control. Representative histograms for cell cycle distribution in A549 cells are shown in Figure 2A. We found that LXB-1 treatment resulted in an obvious increase in G2/M fraction, which was accompanied by a decrease in G0/G1 cells. Cell cycle distribution are also summarized in histograms in Figure 2B. These results indicated that the inhibitory effect of LXB-1 against proliferation of A549 cells correlated with G2/M phase cell-cycle arrest.

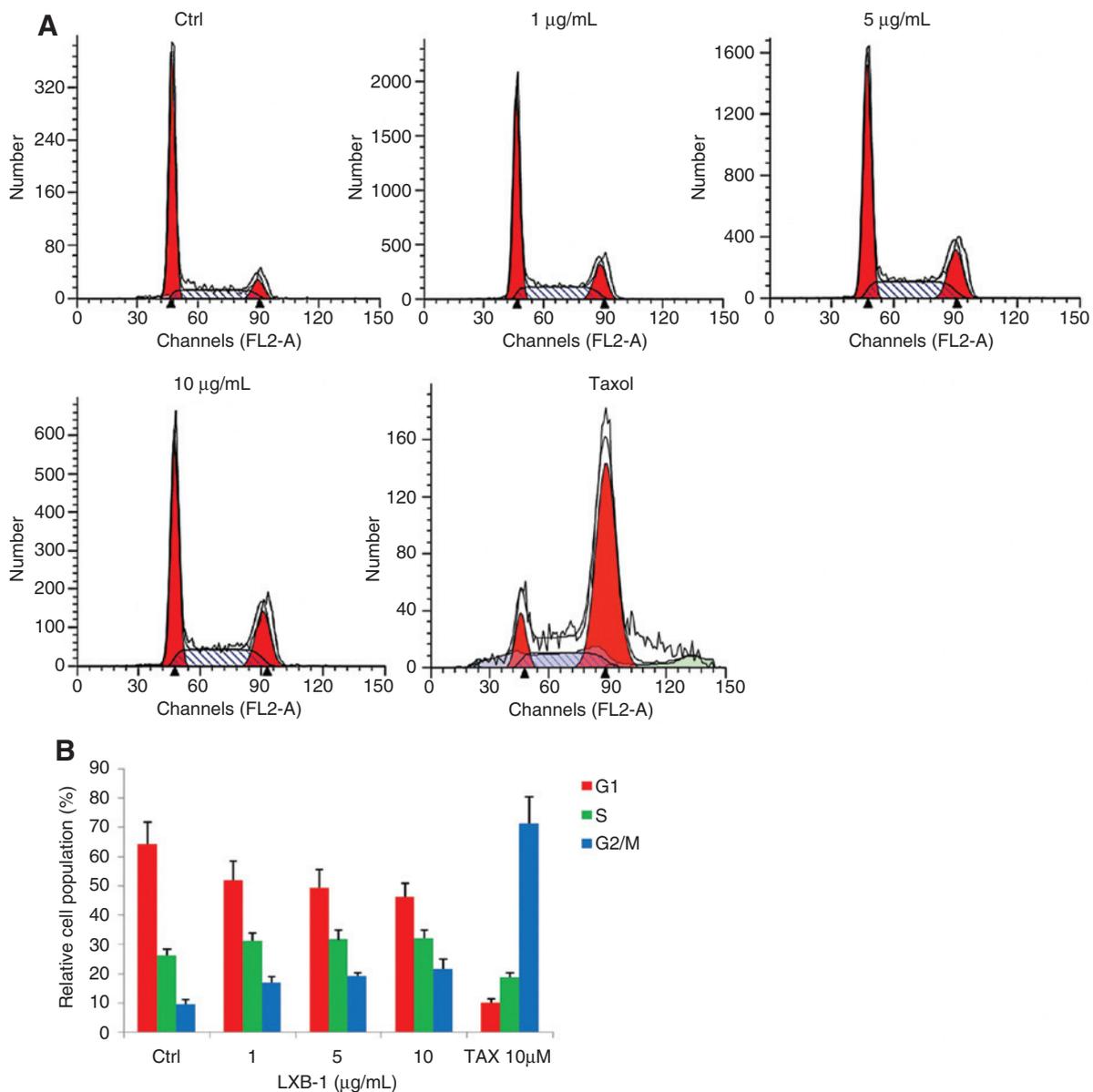
## 3.3 LXB-1 induced apoptosis in A549 cells

To examine the effect of LXB-1 on cell apoptosis, annexin-V-FITC/PI double-staining analysis was performed in A549 cells using flow cytometry. As shown in Figure 3A, LXB-1 induced significant apoptosis in A549 cells. The percentage of apoptotic cells in the control group was 1.85%. After treatment with 1 µg/mL, 5 µg/mL and 10 µg/mL of LXB-1 for 48 h, the early apoptotic cells were increased to 8.14%, 94.53% and 77.55%, respectively. The late apoptotic and necrotic cell population increased slightly in cells treated with 10 µg/mL LXB-1, which showed 14.26% dead.

We next determined the effect of LXB-1 on the expression levels of Bcl-2 and Bax as well as the activation of caspase-3 and caspase-9. Bcl-2 protein is a suppressor of apoptosis while Bax is an activator of apoptosis. Accumulated evidence demonstrates that the cysteine-aspartic acid protease (caspase) family plays a pivotal role in the terminal, execution phase of cell apoptosis [7–10]. Since cytosolic cytochrome c induces caspase-9-dependent activation of caspase-3, we investigated whether caspases-3 and-9 were involved in the apoptotic response induced by LXB-1. As shown in Figure 3B, LXB-1 increased the cleavage of both caspase-3 and caspase-9 in a dose-dependent manner. The protein levels of Bcl-2 were also inhibited, while the expression of Bax protein was gently increased after LXB-1 treatment. Collectively, these results demonstrated that LXB-1 caused cell apoptosis in A549 cells.

## 3.4 LXB-1 regulated the levels of p-JNK, p-ERK and p-Akt

The above results demonstrated that LXB-1 had significant antitumor effects on A549 cells *in vitro*. Next we

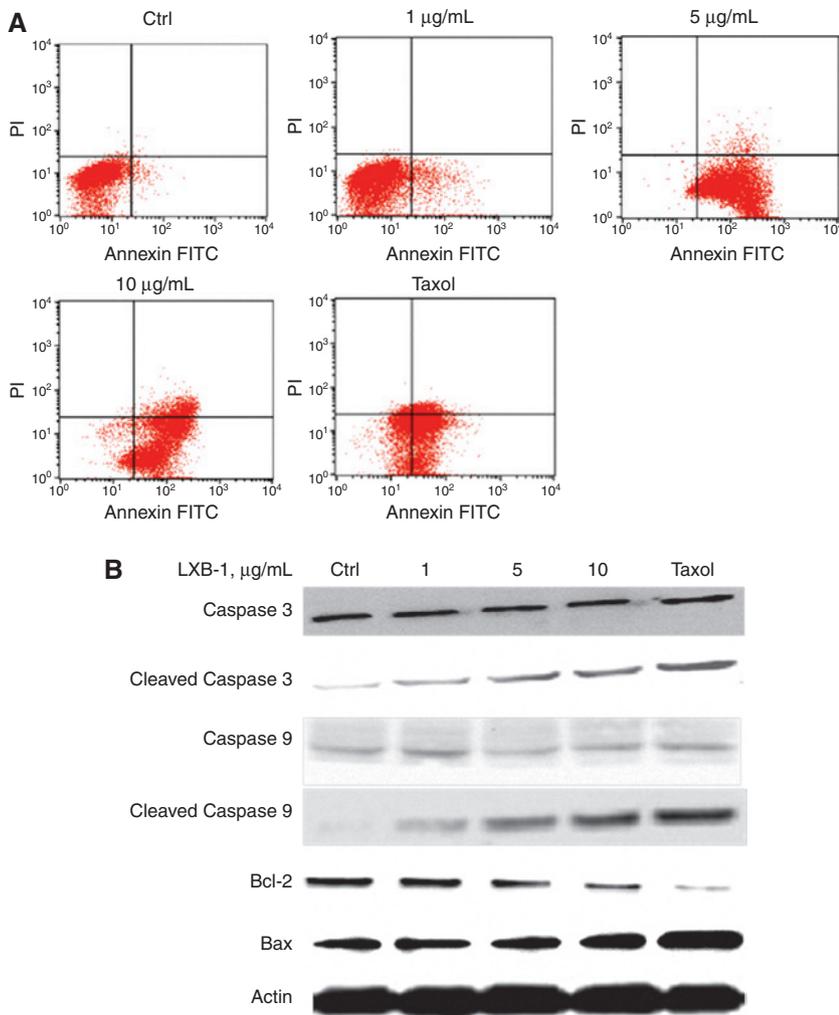


**Figure 2:** LXB-1 induced G2/M cell cycle arrest in A549 cells. (A) After treated with indicated concentrations of LXB-1 for 48 h, A549 cells were stained with PI, and DNA content was analyzed with flow cytometry. (B) Cell cycle distribution was shown. Ten micrometer taxol served as the positive control.

investigated the mechanisms by which LXB-1 induced tumor cell inhibition. Pathways such as PI3K/Akt and the mitogen-activated protein kinase pathway (MAPK) are reported to play a key role in the regulation of diverse cellular events, including cell proliferation, differentiation and apoptosis [23–25]. We found that the exposure of A549 cells to increasing concentrations of LXB-1 for 48 h resulted in an obvious increase of p-JNK (Figure 4). LXB-1 also greatly reduced the phosphorylation of ERK1/2 and Akt in a dose-dependent manner (Figure 4). These data suggest that LXB-1 exerted anti-tumor effects by regulating the levels of p-ERK, p-JNK and p-Akt in A549 cells.

## 4 Discussion

We previously reported the anticancer activities of the extracts prepared from two species of *Liriodendron* genus, and found that the *L. hybrid* extracts (LXB-1) exhibited potent cytotoxic effects on tested cancer cells [20]. These results prompted us to further examine whether LXB-1 exerted anti-cancer effects by inducing cell cycle arrest or apoptosis. This study showed that in A549 cells, LXB-1 induced G2/M cell cycle arrest and that apoptosis was achieved through the mediation on JNK, Akt, and ERK pathways. The data showed herein suggested that LXB-1

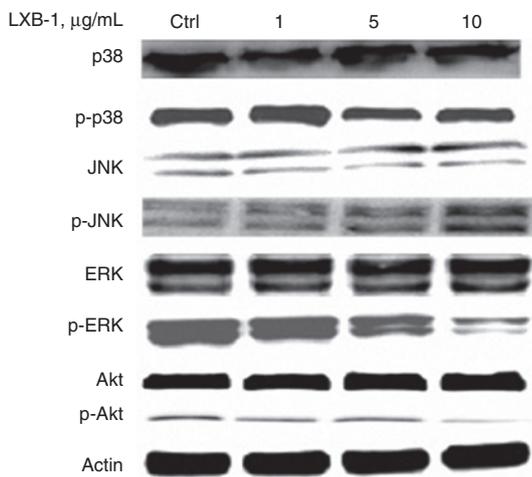


**Figure 3:** LXB-1 induced cell apoptosis in A549 cells. (A) A549 cells were treated with indicated concentrations of LXB-1 for 48 h, and then annexin V/PI double staining assay was performed to measure cell apoptosis. (B) Western blot analysis of expression levels of apoptosis-related proteins.

induced significant cell apoptosis, which was accompanied with marked changes of apoptotic-regulatory proteins, as well as an increased expression of cleaved caspase-3 and cleaved caspase-9. Western blot assay also demonstrated that Bcl-2 protein levels were upregulated and the expression of Bax, an anti-apoptotic member of Bcl-2 family, was decreased.

MAPKs are a well-conserved signaling family of serine/threonine kinases regulating many intracellular processes such as cell proliferation, growth, differentiation, angiogenesis and apoptosis [26, 27]. Three major groups of MAPKs have been well described and include extracellular regulated kinases (ERK1/2), Jun NH<sub>2</sub> terminal kinases (JNK), and p38. ERKs are reported to be proliferative and prosurvival signals in cancer cells and the inhibition of ERK1/2 may enhance cancer cell death [28]. ERK1/2 inhibition leads to the down-regulation of Bcl-2, Bcl-xL,

and Mcl-1 and finally to apoptosis. JNKs, on the contrary, usually induce pro-apoptotic effects. In lymphoid cells, JNK pathway activation by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) contributed to apoptosis activation [29]. p38 MAPK has been demonstrated to predominantly regulate apoptosis, differentiation, growth and inflammatory responses; however, p38 can either suppress or enhance cell apoptosis in a cell type-specific way [30]. We found LXB-1 caused significant changes in MAPK pathways, including a decrease of phosphor-ERK1/2 and an increase of phosphor-JNK. This suggested that LXB-1 could regulate A549 cell apoptosis through MAPK pathways, and that the *L. hybrid* has obtained pharmacologic properties from *L. tulipifera*. However, LXB-1 did not affect phosphor-p38 levels in A549 cells, which probably reflected significant differences among distinct groups of MAPKs.



**Figure 4:** LXB-1 affected JNK, ERK and Akt pathways. A549 cells were treated with increasing concentrations of LXB-1 for 48 h, and the expression and the phosphorylation of p38, JNK, ERK and Akt were shown by western blot. Results were the representatives of at least three independent experiments.

In summary, our results demonstrated that LXB-1 induced apoptosis in A549 cells through Akt, JNK, and ERK1/2 pathways. As there are growing interests in natural medicine, *L. hybrid* should be considered in the future for anticancer drug discovery, as should the analysis of other bioactive phytochemicals.

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