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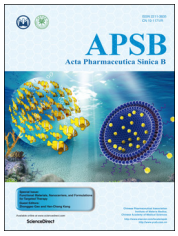

ORIGINAL ARTICLE

Euphorbia factor L2 induces apoptosis in A549 cells through the mitochondrial pathway

Minting Lin[a], Sili Tang[a], Chao Zhang[a], Hubiao Chen[b], Wenjing Huang[a], Yun Liu[a], Jianye Zhang[a,*]

[a]*School of Pharmaceutical Sciences, Guangzhou Medical University, Guangzhou 510182, China*
[b]*School of Chinese Medicine, Hong Kong Baptist University, Hong Kong, China*

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Mitochondrial pathway

Abstract Euphorbia factor L2, a lathyrane diterpenoid isolated from caper euphorbia seed (the seeds of *Euphorbia lathyris* L.), has been traditionally applied to treat cancer. This article focuses on the cytotoxic activity of Euphorbia factor L2 against lung carcinoma A549 cells and the mechanism by which apoptosis is induced. We analyzed the cytotoxicity and related mechanism of Euphorbia factor L2 with an MTT assay, an annexin V-FITC/PI test, a colorimetric assay, and immunoblotting. Euphorbia factor L2 showed potent cytotoxicity to A549 cells. Euphorbia factor L2 led to an increase in reactive oxygen species (ROS) generation, a loss of mitochondrial electrochemical potential, release of cytochrome *c*, activation of caspase-9 and caspase-3, and cleavage of poly(ADP-ribose) polymerase, suggesting that Euphorbia factor L2 induced apoptosis through a mitochondrial pathway. The cytotoxic activity of Euphorbia factor L2 in A549 cells and the related mechanisms of apoptotic induction provide support for the further investigation of caper euphorbia seeds.

*Corresponding author.
E-mail address: jianyez@163.com (Jianye Zhang).

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1. Introduction

Lung cancer is the leading cause of cancer-related mortality in the world and non-small-cell lung cancer (NSCLC) is the most common form of lung cancer[1,2]. Cancer chemotherapy has been regarded as one of the most effective therapy and the development of new anticancer agents is a promising strategy to treat cancer. Indeed, a growing body of evidence suggests that the success of anticancer chemotherapy depends on the discovery of novel anticancer agents[3].

Traditional Chinese medicine provides a rich resource in the search for novel anticancer agents, of which caper euphorbia seed has been recorded for treating cancer[4]. The genus *Euphorbia* (spurges, Euphorbiaceae) has received worldwide attention as its exceptional diversity of growth forms and near-worldwide distribution[5]. It is the largest genus in the spurge family (Euphorbiaceae), containing more than 2000 species. The seeds of *Euphorbia lathyris* L. (caper euphorbia seed) have been used medicinally for the treatment of hydropsy, ascites, terminal schistosomiasis, and snakebites[6]. What's more, the seeds of *E. lathyris* L. can be used for cancer treatment[7].

Multidrug resistance (MDR) is the major impediment to the efficient treatment of tumors. Previous studies revealed that lathyrane diterpenes extracted from the seeds of *E. lathyris L.*, *Euphorbia* factor L1–L11 (EFL1–11), have been considered as promising P-glycoprotein (P-gp) modulators in MDR-mediated resistance[8,9]. We have reported the isolation, identification and anticancer activity of EFL1[10]. On that basis, we further isolated EFL2 (Fig. 1A), and identified the structure using ^{1}H and ^{13}C NMR, DEPT and high resolution electrospray ionization mass spectrometry (HR-EI-MS). The structure was also reported by Appendino and co-workers[11] from the same plant species. In this study, we elucidated the mechanism of EFL2 cytotoxicity against the lung cancer cell line A549. Our results reveal that EFL2 does show potent cytotoxicity and induces apoptosis *via* a mitochondrial pathway in A549 cells.

2. Materials and methods

2.1. Chemicals and reagents

EFL2 was isolated from the seeds of *E. lathyris* L. with a purity of more than 98%. 3-(4,5-Dimethyl-2-thiazolyl)2,5-diphenyl-2*H*-tetrazolium bromide (MTT), 2′,7′-dichlorofluorescin diacetate (DCFH-DA), 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) were obtained from Sigma Chemical Co. (USA). Primary antibodies were obtained as follows: antibodies against GAPDH, anti-mouse IgG-HRP and anti-rabbit IgG-HRP from KangChen Biotechnology Co. (Shanghai, China), antibody against cytochrome *c* from Santa Cruz Biotechnology Co. Colorimetric assay kits for caspase-9 and caspase-3 were purchased from R&D systems (Minneapolis, MN, US). All tissue culture supplies were products of Life Technologies. Other routine laboratory reagents were obtained from commercial sources of analytical or HPLC grade.

2.2. Cell lines and cell culture

Lung cancer A549 cells were maintained in RPMI 1640 medium containing 100 U/mL penicillin, 100 μmol/L streptomycin, and 10% fetal bovine serum (FBS). Cells were cultured in a humidified atmosphere incubator of 5% CO_2 at 37 °C[12].

2.3. Cell viability assay

The effect of EFL2 on cell viability was detected by MTT assay. Cells were harvested and transferred into 96-well plates at a density of 5×10^4 cells/mL. After 24 h incubation, 10 μL EFL2 solution (0, 4.162, 6.243, 9.364, 14.05, 21.07, 31.60, 47.41, 71.11, 106.7, 160 and 240 μmol/L) was added to 96-well plates. After 68 h of treatment, 10 μL MTT (10 mmol/L stock solution in saline) was added to each well and incubated in darkness for 4 h at 37 °C. Subsequently, the supernatant was removed and 100 μL anhydrous dimethyl sulfoxide was added into each well. Cell viability was measured with a Model 550 Microplate reader (BIO-RAD, USA) at 540 nm and 655 nm as reference filter. The growth-inhibitory effect of EFL2 was expressed as IC_{50} estimated from the concentration–response curve (Bliss's software). Cell survival was calculated using the following formula[13]: Survival (%)=(Mean experimental absorbance/Mean control absorbance) × 100.

2.4. Annexin V-FITC/PI assay for apoptosis

Apoptosis was quantified by measuring surface exposure of phosphatidylserine in apoptotic cells using an annexin V-FITC/PI (propidium iodide) apoptosis detection kit (KeyGEN Biotech, Nanjing, China) according to the manufacturer's protocol. Briefly, after A549 cells were treated with the indicated concentrations (40 and 80 μmol/L) of EFL2 for 48 h, the cells were collected and washed twice with ice-cold phosphate-buffered saline (PBS). Then 5×10^5 cells were resuspended with 0.5 mL binding buffer containing Annexin-V (1:50 according to the manufacturer's instruction) and 40 ng/sample of PI for 30 min at 37 °C in the dark. Subsequently, the cells were assayed by flow cytometer

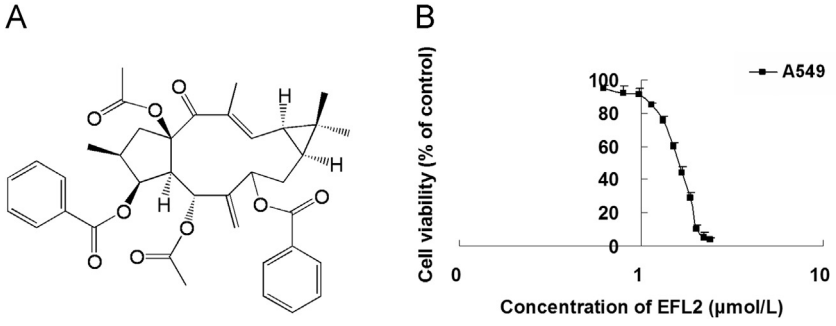

Figure 1 Structure of EFL2 and its cytotoxicity to A549 cells. (A) Chemical structure of Euphorbia factor L2 (EFL2); (B) EFL2 inhibited viability of A549 cells with log scale of concentration.

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