

ORIGINAL ARTICLE

The *Euphorbia lunulata* Bge extract inhibits proliferation of human hepatoma HepG2 cells and induces apoptosis

Feng Gao^{1*}, Zhaoying Fu^{2*}, Hongying Tian², Zhiying He²

¹Department of Clinical Medicine and ²Department of Medical Technology, School of Medicine, Yan'an University, Yan'an, Shaanxi Province, Yan'an, China

*Contributed equally to this work

Summary

Purpose: The purpose of this study was to determine the effect of *Euphorbia lunulata* Bge extract on the proliferation of human hepatoma HepG2 cell line.

Methods: Different dilutions of *Euphorbia lunulata* Bge extract were used to treat human hepatoma HepG2 cells. Hoechst 33258 and PI-staining fluorescence microscopy were utilized to observe the nuclear morphological changes of apoptotic cells. Flow cytometry was used to detect the rates of apoptosis and apoptotic peaks. Western blotting was performed to analyze the subcellular distribution of cytochrome C.

Results: The *Euphorbia lunulata* Bge extract was found to inhibit the proliferation of human hepatoma HepG2 cells via a time and concentration-dependent manner.

Conclusion: Altogether, the results suggest that the *Euphorbia lunulata* Bge extract is effective in inhibiting the proliferation of human hepatoma HepG2 cells and inducing cell apoptosis. The mechanism may be related to the mitochondrial pathways or cellular apoptosis pathways.

Key words: apoptosis, *Euphorbia lunulata* Bge, hepatoma, HepG2 cells

Introduction

Cancers are usually related with abnormal function of cellular processes such as cell proliferation and apoptosis. Induction of apoptosis of tumor cells is an important method for preventing or treating cancers. It is important for anticancer drug research to find active extracts from plants [1-7] that can induce tumor cell apoptosis. The toxic side effects of chemically synthesized drugs are rather frequent and sometimes severe, therefore medicines from natural sources become more and more important due to their relatively low grade side effects. In addition, the cost of research and development of drugs from natural sources are lower than that of the synthesized drugs [8-12]. Therefore, research for medicines from plants are drawing more attention [13].

The chemical composition of the *Euphorbia lunulata* Bge extract include nimbecetin V,

nimbecetin VI, organic acids, kaempferol, quercitrin, kaempferol phenol-3-L-rhamnoside, 6,7-dihydroxy coumarin, sterols, volatile oils, flavonoid glycosides, amino acids and wax [14-16].

In this paper, different dilutions of *Euphorbia lunulata* Bge extract were used to treat human hepatoma HepG2 cells in order to determine its effects on cell proliferation.

Methods

Reagents

Euphorbia lunulata Bge juice was extracted from plant leaves and filtered. The extracted juice was air-dried and weighed, and then prepared to get a concentration of 80 mg/l. Human hepatoma cell line HepG2 was purchased from the Cell Resource Center of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. Dulbecco's Modified Eagle Medium (DMEM)

Table 1. MTT results of HepG2 cells treated with or without *Euphorbia lunulata* Bge extract

Groups	<i>Euphorbia lunulata</i> Bge extract ($\mu\text{g/mL}$)	Mean OD_{490} ($\bar{x} \pm \text{SD}$)		Inhibition rates	
		24 h	48 h	24 h	48 h
Control group (N=5)	0	0.768 \pm 0.022	0.760 \pm 0.023	0	0
Experimental group (N=5)	2.5	0.720 \pm 0.019 ^a	0.704 \pm 0.020 ^a	0.063	0.074
	5	0.603 \pm 0.020 ^{ab}	0.577 \pm 0.018 ^{ab}	0.21	0.24
	10	0.436 \pm 0.019 ^{ab}	0.401 \pm 0.017 ^{ab}	0.43	0.47
	20	0.364 \pm 0.017 ^{ab}	0.303 \pm 0.015 ^{ab}	0.53	0.60
	40	0.297 \pm 0.016 ^{ab}	0.264 \pm 0.016 ^{ab}	0.61	0.68
	80	0.239 \pm 0.015 ^{ab}	0.230 \pm 0.015 ^{ab}	0.69	0.70

^a $p < 0.05$ when compared with the control groups; ^b $p < 0.05$ when compared with the other experimental groups

and trypsin were purchased from Gibco BRL (Grand Island, USA). Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI) and Hoechst33528 (HO) reagents were purchased from Sigma (St.Luis, USA). Flow cytometry equipment was purchased from BD Co (Franklin Lakes, USA), and microtiter detector from Bio-Rad (Hercules, USA).

Cell treatment

Human hepatoma HepG2 cells in the logarithmic phase were treated with the *Euphorbia lunulata* Bge extract (2.5, 5, 10, 20, 40 and 80 $\mu\text{g/mL}$). DMEM without *Euphorbia lunulata* Bge extract was used as control.

Thiazolyl blue tests

The human hepatoma HepG2 cells were treated with various dilutions of *Euphorbia lunulata* Bge and incubated at 37°C in 5% CO_2 atmosphere for 24h, and washed 3 times with phosphate buffered saline (PBS); then 5% thiazolyl blue solution was added. Four hours after incubation the supernatant containing thiazole blue was removed. Optical density (OD) values at 570 nm were detected with a microplate reader. The OD values of the untreated control were accepted as 100%.

Fluorescence microscopy

HO was used to stain the cells according to the manufacturer's instructions. Morphological nuclear alterations were observed by fluorescence microscopy. Cells were also stained with 1ml of 50 mg/ml PI dye at room temperature and incubated in the dark for 30 min. Argon ion laser excitation fluorescence microscopy was used to observe the morphological nuclear changes.

Flow cytometry

Human hepatoma HepG2 cells were seeded in 6-well plates with a cell density of $1 \times 10^5/\text{ml}$. The cells were collected and washed twice with PBS. After addition of 1 ml of PI solution, followed by incubation at

room temperature in the dark for 30 min, flow cytometry was carried out to detect the apoptotic rate.

Western blotting

The mitochondrial isolation kit (Pierce, Rockford, IL, USA) was used to isolate mitochondria from cells. The mitochondrial and cytosolic proteins were extracted and were then separated by SDS-polyacrylamide gel electrophoresis for Western blot analysis. The primary antibodies against cytochrome C (cat# sc-13156; 1:200), VDAC (cat# sc-98708; 1:200), and β -actin (cat# sc-130301; 1:10,000) were purchased from the Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA). Secondary antibodies were horseradish-peroxidase-conjugated secondary anti-mouse IgG (cat # 31430; 1:10,000; Pierce Biotechnology, Rockford, USA) or anti-rabbit IgG (cat # 31460; 1:5,000; Pierce Biotechnology). Bound antibodies were detected using the ECL system (Pierce Biotechnology). Image quantifications were performed using Image Quant software.

Statistics

The data were expressed as mean \pm standard deviation ($\bar{x} \pm \text{SD}$).

For independent samples, t-test followed by One-Way Analysis of Variance (ANOVA) were performed. The statistical package for Social Sciences, version 17.0, software was used for analysis of data. A p value < 0.05 indicated a statistically significant difference.

Results

Euphorbia lunulata Bge extract inhibits proliferation of HepG2 cells

To determine if *Euphorbia lunulata* Bge extract affects the proliferation of human hepatoma HepG2 cells, the cells were treated with DMEM or the extract for 24 and 48h. The MTT test results (Table 1) showed that the proliferation of human hepatoma HepG2 cells was significantly inhibited

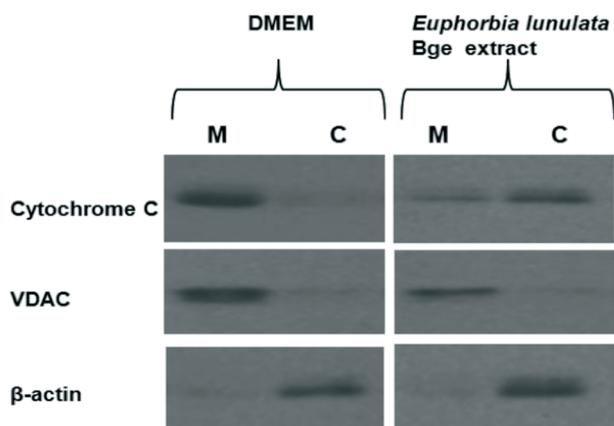


Figure 2. Western blotting analysis of the mitochondrial and cytosolic proteins extracted from HepG2 cells treated with or without *Euphorbia lunulata* Bge extract. The mitochondrial and cytosolic proteins were extracted using the mitochondrial isolation kit for cultured cells (PIERCE, Rockford, IL) from HepG2 cells treated with or without *Euphorbia lunulata* Bge extract (20 $\mu\text{g}/\text{mL}$) for 24 h. Mitochondrial (M) and cytosolic (C) fractions were analyzed via Western blot for cytochrome C, voltage-dependent anion channel (VDAC), or β -actin. The experiments were performed at least in triplicate.

by the extract when compared with the DMEM control ($p < 0.05$). Moreover, when the concentrations of the extract were increased (2.5, 5, 10, 20, 40, and 80 $\mu\text{g}/\text{mL}$), the inhibitory effects on cell proliferation were also increased.

Euphorbia lunulata Bge extract induces HepG2 cell apoptosis

After the HepG2 cells were treated with *Euphorbia lunulata* Bge extract for 24 h, the cells were stained by HO and observed under argon ion laser-excited fluorescence microscopy. The nucleus expressed bright blue. The morphological

changes of apoptotic changes such as nuclear condensation, uneven staining, marginalization and nuclear debris, and enhancement of blue fluorescence were visible. As shown in Figure 1A, the significant difference compared with the control group showed that the *Euphorbia lunulata* Bge extract induced apoptosis of HepG2 cells.

The human hepatoma HepG2 cells were treated with *Euphorbia lunulata* Bge extract for 48 h. Then, the cells were stained with PI. The nuclear morphological changes were observed under argon ion laser excitation fluorescence microscopy. As shown in Figure 1B, when were compared with cells treated with DMEM, cells treated with the extract showed cell shrinkage, hyperchromatic nuclei, chromosome condensation and fragmentation, suggesting that the *Euphorbia lunulata* Bge extract induced apoptosis in HepG2 cells.

Detection of the apoptosis rates of HepG2 cells treated with *Euphorbia lunulata* Bge extract

The HepG2 cells were treated with different concentrations of *Euphorbia lunulata* Bge extract (5, 10, 20 $\mu\text{g}/\text{mL}$) and detected by flow cytometry after 24, 48 and 72 h. The apoptotic rates of HepG2 cells at different concentrations of the drug group were significantly different ($p < 0.05$) in comparison with the control group (Table 2) showing that the *Euphorbia lunulata* Bge extract induced apoptosis of HepG2 cells.

Changes of cytochrome C subcellular distributions of HepG2 cells in the presence of *Euphorbia lunulata* Bge

HepG2 cells were treated with *Euphorbia lunulata* Bge extract (20 $\mu\text{g}/\text{mL}$) for 24h. The untreated

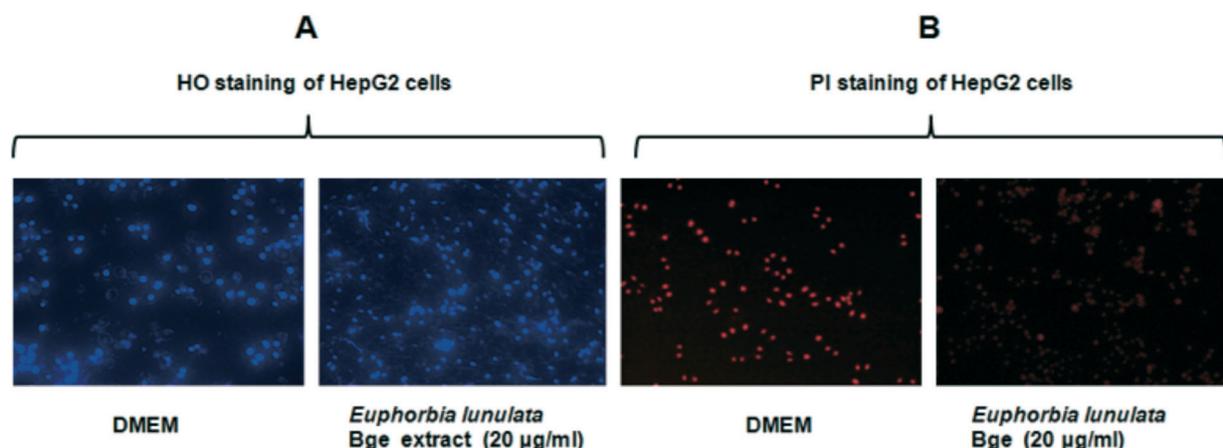


Figure 1. 1 Microscopy detection of HepG2 cells treated with or without *Euphorbia lunulata* Bge extract (20 $\mu\text{g}/\text{ml}$). The cells treated with DMEM served as controls. **(A)** Hochest33528 (HO) staining of HepG2 cells treated with or without *Euphorbia lunulata* Bge extract. **(B)** Propidium iodide (PI) staining of HepG2 cells treated with or without *Euphorbia lunulata* Bge extract

Table 2. Apoptotic rates ($\bar{x} \pm SD$) of HepG2 cells treated with or without *Euphorbia lunulata* Bge extract.

Treatment <i>Euphorbia lunulata</i> Bge extract ($\mu\text{g/ml}$)	Apoptotic rates (%)		
	24 h	48 h	72 h
20	51.32 \pm 2.66*	60.62 \pm 2.57*	62.37 \pm 2.98*
10	42.49 \pm 2.72*	52.38 \pm 2.74*	53.63 \pm 3.11*
5	19.11 \pm 2.59*	31.47 \pm 3.06*	40.45 \pm 3.87*
0	4.92 \pm 2.09	5.21 \pm 2.30	5.99 \pm 2.72

* $p < 0.05$ when compared with the control groups

cells served as negative controls. The mitochondrial and cytosolic proteins were extracted and the mitochondrial and cytosolic fractions were analyzed by Western blotting for cytochrome C, voltage-dependent anion channel (VDAC), or β -actin.

As shown in Figure 2, the mitochondrial protein cytochrome C was easily detected in the untreated control groups, but not in the cytosolic fraction. When the HepG2 cells were treated with *Euphorbia lunulata* Bge extract (20 $\mu\text{g/ml}$) for 24 h, the levels of mitochondrial cytochrome C decreased, while they increased in the cytosolic fraction, suggesting that the cytochrome C translocated from the mitochondria into the cytoplasm, possibly via a mechanism related to initiation of cell apoptosis. In these experiments, VDAC was used as an internal mitochondrial protein control. β -actin was used as an internal cytosolic protein control.

Discussion

Liver cancer is a serious health problem [17]. In this paper, the extract of *Euphorbia lunulata* Bge was found to exert strong inhibition of cell proliferation and induction of apoptosis in HepG2 cells. Further experiments revealed that this activity may be through the mitochondrial pathway (also known as the endogenous apoptosis pathway or the cytochrome C-mediated pathway) [18,19] to induce apoptosis of HepG2 cells. The *Euphorbia lunulata* Bge extract promoted the mitochondrial membrane permeability of HepG2 cells, and made the cytochrome C translocate from the mitochondria to the cytoplasm. Thus, the caspase cascade reaction

was activated, eventually leading to apoptosis of HepG2 cells. Inhibition of tumor cell proliferation and induction of apoptosis are hot fields in cancer research.

It was reported that the acetone extract of the whole plant of *Euphorbia lunulata* Bge may be used as a herbal medicine for the treatment of bronchial asthma [20]. Recently, 23 constituents were isolated from *Euphorbia lunulata* Bge extract and their structures were analyzed by spectroscopic analysis [21]. Further study indicated that the isolated hydrolysable tannins, flavonoids, and flavonol galactopyranoside gallates significantly inhibited the differentiation of the 3T3-L1 preadipocytes, the triglyceride accumulation in maturing adipocytes, and the nitric oxide production in RAW 264.7 cells (a macrophage-like, Abelson leukemia virus transformed cell line derived from BALB/C mice) [21].

The results of the present study are encouraging and suggest that further research and development of novel drugs for treating hepatoma patients should continue. In the future, we will isolate the single-molecule constituents from *Euphorbia lunulata* Bge extract and analyze their molecular structures by mass spectrometry. The effects of these single-molecule constituents from *Euphorbia lunulata* Bge extract on the mitochondrial membrane permeability, cell proliferation, and apoptosis of HepG2 cells will be studied.

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