

# Licochalcone C induces apoptosis via B-cell lymphoma 2 family proteins in T24 cells

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**Abstract.** The current study investigated the mechanisms by which licochalcone C induces apoptosis of T24 human malignant bladder cancer cells. Cell viability was evaluated using an MTT assay. Apoptosis was investigated using a morphological assay, flow cytometry and a caspase-3 activity assay. Alterations in the gene expression levels of Bcl-2 family members were measured by semi-quantitative reverse transcription-polymerase chain reaction assays. The protein levels of pro-caspase-3 and cleaved poly(ADP ribose) polymerase were measured using western blotting. The results indicated that licochalcone C induced T24 cell apoptosis in a concentration-dependent manner. Licochalcone C treatment reduced the levels of the anti-apoptotic mRNAs (Bcl-2, Bcl-w and Bcl-XL) and increased expression of the pro-apoptotic mRNAs (Bax and Bim). The Bcl-2 family inhibitor (ABT-737) reduced apoptosis induced by licochalcone C in T24 cells. The current study demonstrated that licochalcone C may be a potential adjuvant therapeutic agent for bladder cancer.

## Introduction

Bladder cancer is one of the most common urogenital cancers worldwide, with a high incidence in developed countries (1,2). Despite advances in cystoscopy in the detection and surveillance of bladder cancer, progress in the development of effective treatments remains limited. Approximately 50-70% of patients treated with endoscopic resection will undergo recurrence and 10-30% will progress to muscle-invasive disease, which has led to the use of adjuvant therapy with intravesical agents (3,4). However, the conventional chemotherapeutic regimens are often poorly tolerated as a result of the associated side effects (5). These factors highlight the requirement for the production of novel adjuvant agents to improve the efficacy of bladder cancer treatment.

Apoptosis serves an important role in the treatment of cancer as it is a common target of numerous treatment strategies (6-9). Caspases are crucial mediators of programmed cell death (apoptosis) (10). Among them, caspase-3 is a frequently activated death protease, catalyzing the specific cleavage of numerous key cellular proteins (11).

There has been an increase in the discovery of relatively non-toxic natural compounds with a wide range of biological activities (12). Chalcones are ubiquitous natural compounds with anticancer potential and relatively few side effects, which have been reported to inhibit cellular proliferation by inducing cell cycle arrest (5,13) and/or apoptosis of cancer cells (14,15). Licochalcone C (LC; Fig. 1), a chalcone isolated and identified from the root of *Glycyrrhiza inflata* (16,17), exhibits antibacterial (18) and anti-inflammatory effects (19), however the antitumoral activity has not been investigated. Therefore, the aim of the current study was to elucidate the inhibitory effects of LC on bladder cancer cells and explore the underlying mechanisms.

## Materials and methods

**Cell lines and cell culture.** T24, MCF7 and A549 cells were purchased from the China Center for Type Culture Collection (Wuhan, China). The T24 cells were cultured in RPMI 1640 medium (Gibco Life Technologies, Carlsbad, CA,

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USA) and both the MCF7 and A549 cells were cultured in Dulbecco's modified Eagle's medium (Gibco Life Technologies) containing 10% fetal bovine serum (FBS; Tianjin Hao Yang Biological Manufacture Co., Ltd., Tianjin, China) at 37°C with 5% CO<sub>2</sub>. The media contained 100 U/ml penicillin (Sigma-Aldrich, St. Louis, MO, USA) and 100 µg/ml streptomycin (Sigma-Aldrich). The cells were passaged every 3 days and were diluted every day prior to each experiment.

**Cell viability assay.** The LC was purchased from Shanghai Lichen Trading Co., Ltd. (Shanghai, China). The MTT (Beyotime Institute of Biotechnology, Haimen, China) assay was used to evaluate viability of cells, which is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases (20). Cells were seeded onto 96-well plates (8x10<sup>4</sup> cells/ml) and incubated overnight in 100 µl of the culture medium. The cells were treated with a range of concentrations of LC (0, 25, 30, 35, 40 and 45 µg/ml) for 24 h. Following incubation, 20 µl MTT (5 mg/ml) was added to each well, which were then incubated for 4 h prior to removal of the supernatant. A total of 150 µl dimethyl sulfoxide (DMSO; Sigma-Aldrich) was added to each well. Absorbance at 570 nm was measured using a fluorescence plate reader (3001; Bio-Rad Laboratories, Inc., Hercules, CA, USA). The data were expressed as the percentage cell viability compared with the control (DMSO). The inhibition rate was quantified using the following formula:

$$\left(1 - \frac{\text{OD of sample}}{\text{OD of control}}\right) \times 100\%$$

**Morphological assessment.** To investigate whether LC induces apoptosis in T24 cells, the cells were plated in a 4-well chamber slide at 2x10<sup>4</sup> cells/slide, and treated with increasing concentrations of LC (0, 25, 30, 35, 40 and 45 µg/ml) for 24 h to examine the apoptosis of T24 cells. The cells were fixed in formaldehyde (40 g/l; Sigma-Aldrich) in phosphate-buffered saline (PBS) for 20 min followed by Hoechst 33258 (10 mg/l; Sigma-Aldrich) staining for 30 min in the dark at 37°C. Cell nuclei were then analyzed under a computer-assisted microscope (459330; Carl Zeiss AG, Oberkochen, Germany) by fluorescence microscopy. Apoptotic cells were characterized by chromatin condensation and multiple chromatin fragments (21).

**Detection of cell apoptotic rates by flow cytometry.** Apoptotic rates were determined by staining cells with annexin V fluorescein isothiocyanate (FITC) and propidium iodide (PI) labeling (22). The Annexin V/PI Apoptosis kit was purchased from Nanjing KeyGen Biotech Co., Ltd. (Nanjing, China). Cells (1.5x10<sup>5</sup> cells/ml) were incubated with LC for 24 h following which they were washed twice with ice-cold PBS, and 5 µl annexin V-FITC and 5 µl PI (1 mg/ml) were added to stain the cells. The cell staining was analyzed using the FACStar Flow Cytometer (BD Biosciences, San Jose, CA, USA). Viable cells were regarded to be negative for PI and annexin V-FITC, apoptotic cells were positive for annexin V-FITC and negative for PI, whereas late apoptotic dead cells displayed clear annexin V-FITC and PI labeling. Non-viable cells, which underwent necrosis, were positive for PI however were negative for annexin V-FITC.

**RNA extraction and semi-quantitative reverse transcription-polymerase chain reaction (RT-qPCR).** Total RNA was extracted using TRIzol (Sangon Biotech Co., Ltd., Shanghai, China) according to the manufacturer's instructions. RNA quality was assessed using the A260/A280 ratio with a Nanodrop Spectrophotometer (ND-2000; Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA) and 1.5% agarose gel electrophoresis (Biodee Biotechnology Co., Ltd., Beijing, China). Following extraction, 3 µl total RNA was reverse-transcribed to cDNA using a RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific) in a 20 µl reaction volume. The reaction conditions of reverse transcription PCR were established using 12.5 µl 2X Taq PCR MasterMix (Tiangen Biotech Co., Ltd., Beijing, China), 3 µl cDNA template and 0.5 µl of each primer synthesized by Sangon Biotech. Thermocycling conditions were as follows: Pre-denaturation at 94°C for 3 min, 30 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec and extension at 72°C, and a final extension at 72°C for 10 min. The RT-qPCR products were quantified using a Bio-Rad gel imaging system (Bio-Rad Laboratories, Inc.) with GelPro analysis software 4.0 (Media Cybernetics, Rockville, MD, USA). The primer sequences are presented in Table I.

**Measurement of caspase-3 activity.** The activity of caspase-3 was assessed using the Caspase-3 Colorimetric Assay kit (R&D Systems, Inc., Minneapolis, MN, USA), which is based on the spectrophotometric detection of the color reporter molecule p-nitroaniline (pNA) following cleavage from the labeled substrate DEVD-pNA (caspase-3) as an index. Cells were incubated with the designated concentrations of LC (0, 25, 35 and 45 µg/ml). The cells were washed with PBS and suspended in 5 volumes lysis buffer (20 mmol/l HEPES, pH 7.9, 20% glycerol, 200 mmol/l KCl, 0.5 mmol/l EDTA, 0.5% NP40, 0.5 mmol/l DTT and 1% protease inhibitor cocktail; Sigma-Aldrich). The lysates were collected and stored at -20°C until use. The protein concentration was determined by the Bradford method as per the manufacturer's instructions of the Caspase-3 Colorimetric Assay kit. Supernatant samples, containing 100 µg total protein, were added to 96-well plates with the DEVD-pNA and LEHD-pNA at 37°C for 1-2 h to determine caspase-3 activity. The optical density of each well was measured at 405 nm using a fluorescence plate reader (3001; Bio-Rad Laboratories, Inc.). Each plate contained three wells of a given experimental condition and three control wells. The activity of caspase-3 was expressed in arbitrary absorbance units (absorbance at a wavelength of 405 nm).

**Western blot analysis.** Cells at a density of 1.5x10<sup>5</sup> cells/ml were incubated with LC for 24 h. The soluble lysates (15 µl per lane) were subjected to 10% SDS-PAGE, then were transferred onto the nitrocellulose membranes (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) and blocked with 5% non-fat milk in Tris-buffered saline with Tween-20 (TBST; Biodee Biotechnology Co., Ltd) for 2 h at room temperature. Membranes were incubated with the respective primary antibody [anti-caspase-3 antibody (1:2,000; cat no. sc-65496), anti-poly(adenosine diphosphate-ribose) polymerase (PARP) antibody (1:2,000; cat no. sc-56196) or anti-β-actin antibody (1:2,000, cat no. sc-47778), all from Santa Cruz Biotechnology, Inc., Dallas, TX, USA] at 4°C overnight and then incubated with

Table I. Primer sequences.

Primer	Forward	Reverse
Bax	TGGAGCTGCAGAGGATGATTG	GAAGTTGCCGTCAGAAAACATG
Bim	CACATGAGCACATTTCCCTCT	AAGGCACAAAACCTGCAGTAA
Bcl-w	CGGAACATGGCTTGTAGCTC	AATCCCATTCTATCTAGTCGAG
Bcl-2	AGTACCTGAACCGGCATCTG	GCTGAGCAGGGTCTTCAGAG
Bcl-XL	ACATCCCAGCTCCACATCAC	CGATCCGACTCACCAATACC
GAPDH	GACATCAAGAAGGTGGTGAAGC	GTCCACCACCCTGTTGCTGTAG

horseradish peroxidase-conjugated bovine anti-mouse immunoglobulin G (1:10,000; cat no. sc-2371; Santa Cruz Biotechnology, Inc.) as the secondary antibody for 1 h at room temperature. Western blots were developed using enhanced chemiluminescence (Thermo Fisher Scientific) and were exposed on Kodak radiographic film (Kodak, Rochester, NY, USA).

**Statistical analysis.** The data were presented as the mean  $\pm$  standard deviation from a minimum of three independent experiments and evaluated through analysis of variance followed by Student's t-test.  $P < 0.05$  was considered to indicate a statistically significant difference. The analyses were performed using the Origin software, version 8.0 (OriginLab, Northampton, MA, USA).

## Results

**LC inhibited proliferation of T24, MCF7 and A549 cells.** Breast, lung and bladder cancer are frequently malignant, thus have a clear effect on health due to high incidence and recurrence rates (23,24). The current study examined the proliferation inhibition of LC (0, 25, 30, 35, 40 and 45  $\mu\text{g/ml}$ ) against T24 (bladder cancer), MCF7 (breast cancer) and A549 (lung cancer) cells. Subsequent to treatment with LC (45  $\mu\text{g/ml}$ ) for 24 h, the rates of proliferation inhibition of T24, MCF7 and A549 cells were 68, 47 and 40% respectively (Fig. 2). As T24 cells were observed to exhibit a greater sensitivity to LC than MCF7 and A549 cells, T24 cells were selected for use in the subsequent experiments.

**LC induces apoptotic cell death and caspase activation in T24 cells.** Morphological assessment with Hoechst staining verified the fact that LC induces T24 cell apoptosis, with LC-treated cells exhibiting typical morphological features of apoptosis, such as nuclear condensation and fragmentation (Fig. 3A). Annexin V-FITC-PI double-staining was used to detect phosphatidyl serine externalization, a hallmark of early apoptosis, to indicate whether LC-induced apoptosis occurred (25). Treatment of T24 cells with LC (0, 25, 35 and 45  $\mu\text{g/ml}$ ) for 24 h led to a significant increase in the percentage of apoptotic cells, from 0.6% in control cells to 30, 64 and 74% respectively (Fig. 3B). In addition, measurement of a key factor in apoptosis, caspase-3 activity, provided further support for the LC-induced apoptotic response (Fig. 3C).

**Bcl-2 family members served a crucial role in LC-induced T24 cell apoptosis.** As Bcl-2 family members serve a critical role in inducing caspase-3 activation, regulating apoptosis and

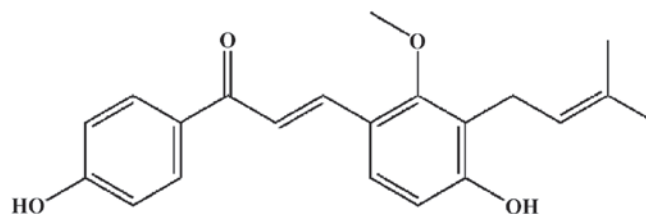


Figure 1. The chemical structure of Licochalcone C.

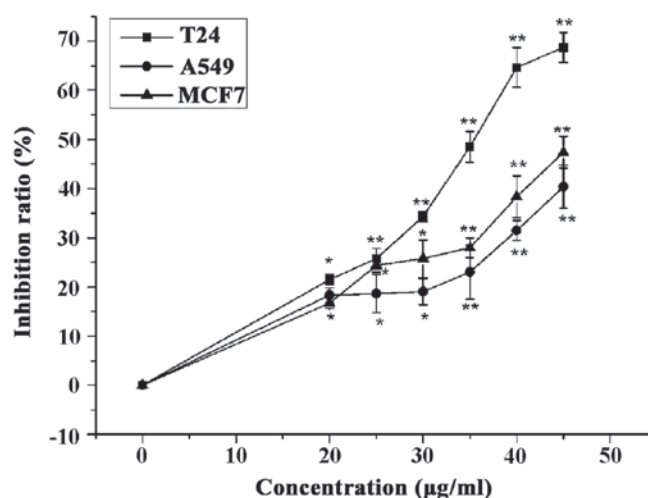
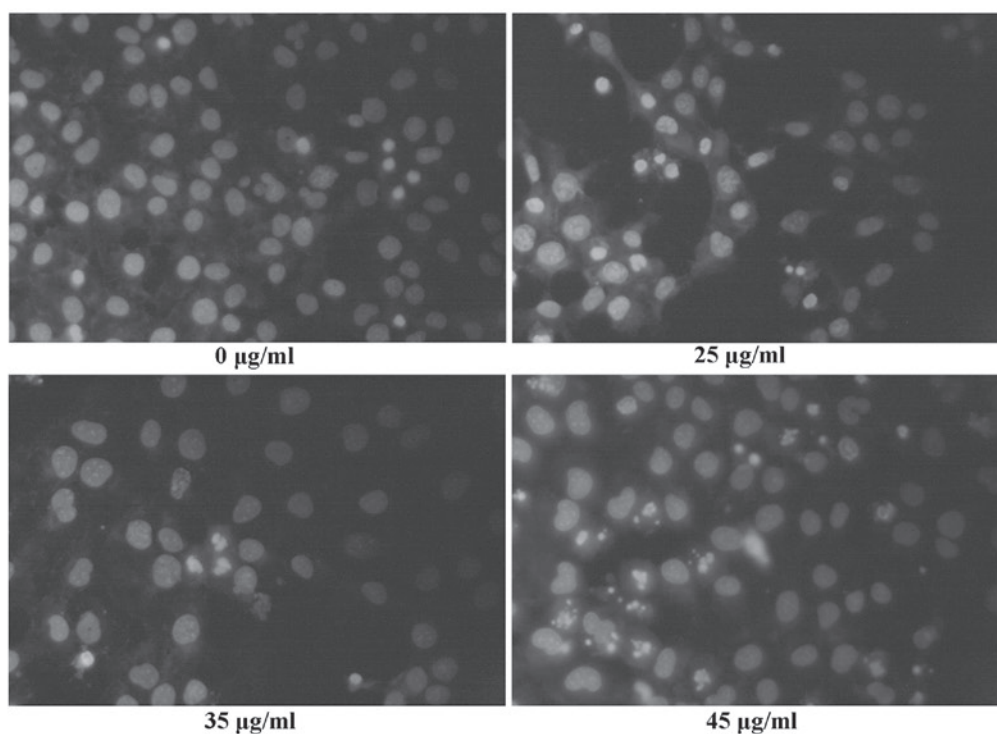


Figure 2. Effect of LC on cell viability of T24, MCF7 and A549 cells. Cell viability was determined using an MTT assay. The cells were treated with LC (0, 25, 30, 35, 40 and 45  $\mu\text{g/ml}$ ) for 24 h. The data represent the mean  $\pm$  standard deviation of three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$  vs. the control group. LC, licochalcone C.

irreversible cellular damage, they are suggested to be important in the determination of cell fate (25). LC-induced caspase-3 activation and apoptosis were observed in the present study. To investigate whether Bcl-2 family members are involved in the apoptosis of T24 cells induced by LC, the expression levels of Bcl-2 family members (Bax, Bim, Bcl-w, Bcl-2 and Bcl-XL) in T24 cells treated with LC were analyzed (Fig. 4A). Compared with the control group, exposure of T24 cells to LC (25, 35 and 45  $\mu\text{g/ml}$ ) resulted in a concentration-dependent reduction in the mRNA level of Bcl-2, Bcl-w and Bcl-XL, with a concomitant increase observed in the levels of Bax and Bim. Based on the importance of Bcl-2 family members in inducing apoptosis, and the alterations in the levels of Bcl-2 mRNA observed in the present study, an inhibitor of the Bcl-2 family (ABT-737) was

A



B

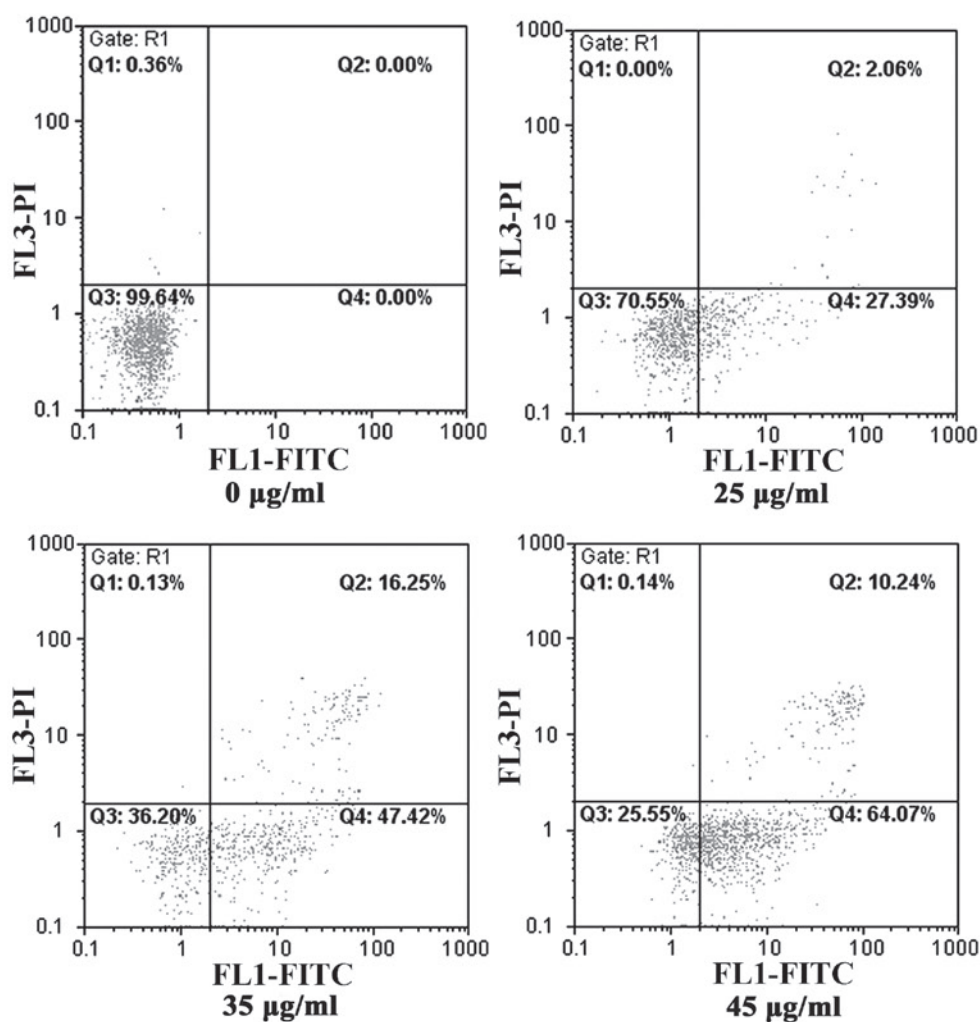


Figure 3. Apoptosis of T24 cells following treatment with LC for 24 h. (A) Morphological alterations in T24 cells examined using Hoechst 33258 staining. Representative images were captured at a magnification of x400. (B) Detection of apoptotic rates conducted via flow cytometry following staining with annexin V-FITC and PI. LC, licochalcone C; FITC, fluorescein isothiocyanate; PI, propidium iodide.



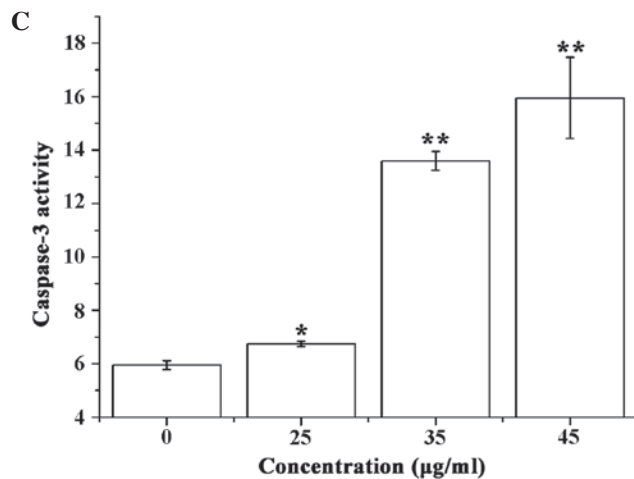


Figure 3. Continued. (C) Caspase-3 activity was examined using the Caspase-3 Colorimetric Assay kit. The data are presented as the mean  $\pm$  standard deviation of three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$  vs. the control group.

used to confirm the role of Bcl-2 family members in LC-treated T24 cell apoptosis. As presented in Figure 4B, the Bcl-2 family inhibitor ABT-737 effectively blocked LC-induced apoptosis associated proteins (pro-caspase-3 and cleaved PARP).

## Discussion

Bladder cancer is a common, however serious, health problem worldwide. Approximately 70-80% of patients with bladder cancer are diagnosed with non-muscle invasive bladder cancer (NMIBC) and may be treated with endoscopic resection (5). Two main problems may occur in the the patients undergoing resection: i) High intravesical recurrence rates; ii) progression to muscle invasive cancer during repeated intravesical recurrence (26). Therefore, the next step is adjuvant intravesical therapy aimed at reducing the risk of tumor recurrence and possibly progression (3). Intravesical Bacillus Calmette-Guérin (BCG) therapy is the most effective and widely used immunotherapeutic method against bladder cancer. However, BCG therapy is associated with clear side effects, and 60-80% patients fail to tolerate the therapy due to the local symptoms of cystitis, including dysuria, pollakisuria, low-grade fever and malaise (27). In addition, mitomycin C, thiotepa, doxorubicin and epirubicin are commonly used to prevent recurrence, however they also have side effects (28). The strong systemic toxicity and incomplete efficacy of the intravesical agents has contributed to the search for novel drugs to reduce the rate of recurrence of bladder cancer. In the present study, it was demonstrated that LC inhibited the growth of several cancer cell lines (T24, MCF7 and A549) with significant growth inhibition against T24 cells thus suggesting that LC has potential to as novel therapeutic agent against various types of human cancer, particularly bladder cancer.

Induction of apoptosis is considered as an important strategy in the treatment of cancer (6), and numerous previous studies have demonstrated the effect of natural products on cancer cell apoptosis (29,30). The results obtained in the present study provide evidence that LC induced significant apoptosis in T24 cells (Fig. 3A), however the mechanism remains to be fully

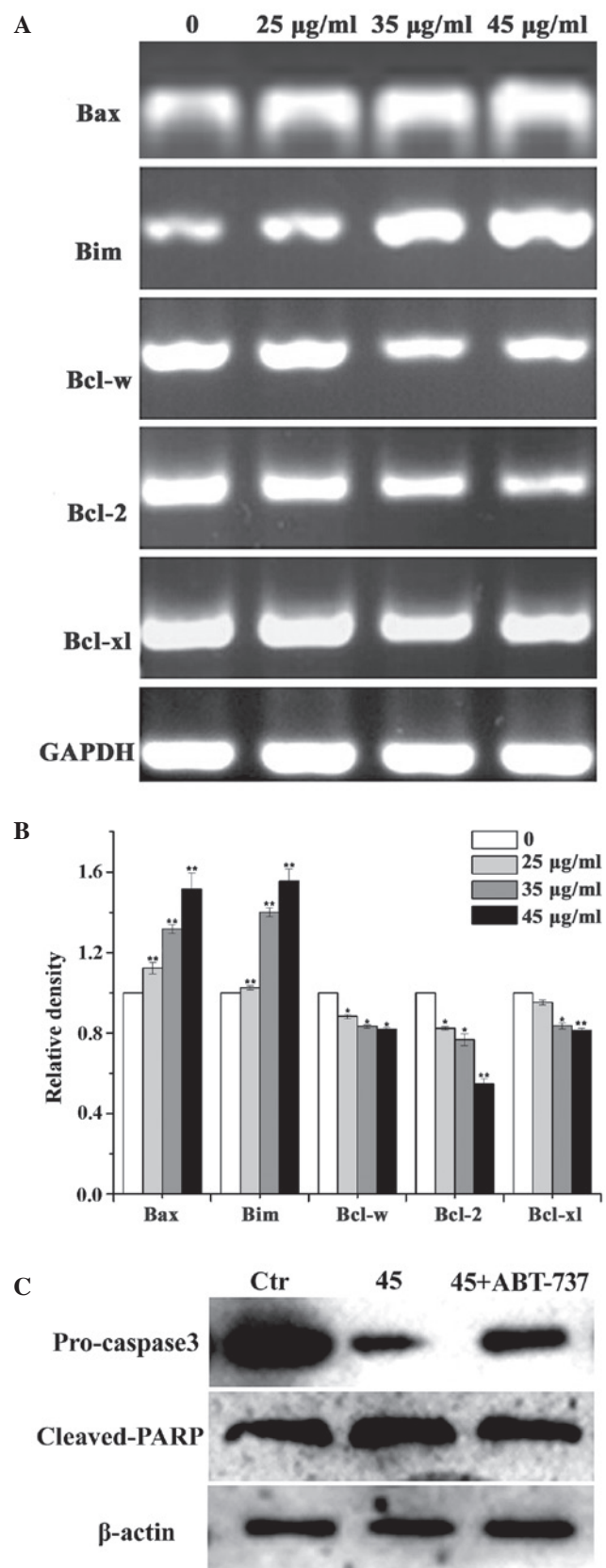


Figure 4. The role of Bcl-2 family members in LC-induced T24 cell apoptosis. (A) mRNA expression of Bcl-2 family members was analyzed by reverse transcription-quantitative polymerase chain reaction then was quantified. Data are presented as the mean  $\pm$  standard deviation of three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$  vs. the control group. (B) The cells were treated with or without the indicated concentrations of LC for 24 h with or without ABT-737 (600 nM). (C) Pro-caspase-3 and PARP cleavage were examined via western blot analysis. LC, licochalcone C; PARP, poly(ADP-ribose) polymerase.

elucidated. Understanding the mechanism by which LC induces apoptosis in T24 cells may aid in the optimization of its anti-cancer activity. In the present study, LC treatment was observed to result in a reduction of anti-apoptotic mRNAs (Bcl-2, Bcl-w and Bcl-XL), and an increase in levels of pro-apoptotic mRNAs (Bax and Bim). Notably, several small molecules have been selected on the basis of their anti-Bcl-2 activity and among them ABT-737 has been previously demonstrated to be a potent inhibitor of Bcl-2/Bcl-w/Bcl-XL (31). The apoptotic response of LC-treated T24 cells was attenuated by ABT-737, supporting a pivotal role of Bcl-2 family members in LC-induced T24 cells apoptosis. Evidence based on these observations supports an important therapeutic effect of LC on bladder cancer. However, the specific role of Bcl-2 family members was only investigated in brief in the current study and considering the fundamental role of Bcl-2 family members in the integration of apoptotic cell stimuli, further investigation is required to fully elucidate this. In conclusion, the evidence of the current study demonstrates that LC led to a concentration-dependent inhibition of bladder cancer cell proliferation, and this antiproliferative effect appears to be due to its ability to promote apoptotic cell death. LC results in alterations in the expression of Bcl-2 family member genes, leading to the cleavage of PARP and the activation of the caspase-mediated cell death signaling pathway. Therefore, LC is suggested to be a promising candidate for further development as a therapeutic agent for bladder cancer.

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