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Antiproliferative activity and apoptosis-inducing mechanism of L-securinine on human breast cancer MCF-7 cells

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Natural products have been discovered to be valuable sources of antitumor drugs. L-Securinine is a natural product extracted from the leaves or roots of *Securinega suffruticosa* Pall Rehd. The current study was done to investigate the molecular mechanisms of antitumor effects of L-securinine. The inhibitory activities of L-securinine on human breast cancer MCF-7 cells were studied *in vitro* by a Cell Counting Kit-8(cck8) assay. Flow cytometry was used to analyze the apoptotic ratio and cell cycle distribution of control and treated MCF-7 cells with L-Securinine. Real-time quantitative PCR was conducted to evaluate expression levels of apoptosis related genes P53, Bax, Bcl-2, Mtor, P70s6k. L-Securinine exhibited remarkable antiproliferation activities on MCF-7 cells in dose- and time-dependent manner (24, 48 and 72 h of incubation). A 48 h exposure to L-securinine at a concentration ranging from 0 to 40 μM resulted in a significant increase in apoptotic ratio. At both low and high concentrations, L-securinine preferably perturbed the cell cycle in MCF-7 cells by arrest of G1 phase. These results were further confirmed by the increased expression of bax, p53 and the decreased expression of bcl-2, mtor, p70s6k in a dose-dependent manner. In summary, these findings suggest that L-securinine has an anti-tumor effect against MCF-7 cells and could be further exploited as a potential lead in antitumor drug development.

1. Introduction

Breast cancer is a common and severe neoplasia in women with increasing incidence. (Forouzanfar et al. 2011). Different treatments such as surgery, radiation therapy, hormone therapy, chemotherapy, and targeted therapy are used, but there are severe problems with side effects and drug resistence, so that improved antitumor agents are urgently needed. Many compounds derived from natural sources possess antitumor effects. (Demain and Vaishnav 2011; Massaoka et al. 2012; Patel et al; 2011; Yang et al. 2013). For example, taxol, camptothecin, vincristine and vinblastine (Heinrich and Bremner 2006; Newman and Cragg 2007) extracted from plants are already used in the treatment of cancer.

The objective of present study was to evaluate the potency of L-securinine as a natural compound for growth inhibiting of human breast cancer line and to unveil the molecular antitumor mechanisms. Securinine which has two optical isomers consisting of L-securinine (Fig. 1) and D-securinine was isolated from plants of the Euphorbiaceae family. Securinine was initially isolated by a Russian scientist in 1956 and structurally characterized by Chinese scientists in 1963. As a classical natural product, securinine plays many roles in a biological system showing GABA receptor antagonistic, antimalarial and antibacterial activities. Studies have shown that securinine is a potential treatment of aplastic anemia (Anon. 1983), and possesses antitumor properties (Xia et al. 2011; Chen et al. 2012).

Apoptosis related to the regulation and development of homeostasis is a physiological mode of cell death. The modulation of programmed cell death (apoptosis) signaling pathways by natural products was a key event in antitumor activities as cancer cells have evolved multiple mechanisms to resist the induction of apoptosis (Fulda 2010). This study was therefore undertaken to examine the antiproliferative and apoptotic effect of L-securinine on breast cancer cells. We have demonstrated the following two points: 1. The inhibitory effect of L-securinine on MCF-7 cells was dose- and time-dependent 2. L-Securinine induced apoptosis in MCF-7 cells by up-regulating the expression of Bax, p53 and down-regulating the expression of Bcl-2, mTOR, P70S6k.

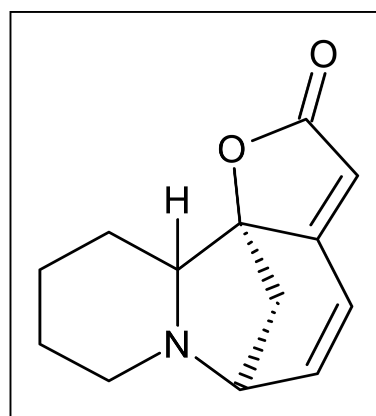

Fig. 1: Structure of L-securinine

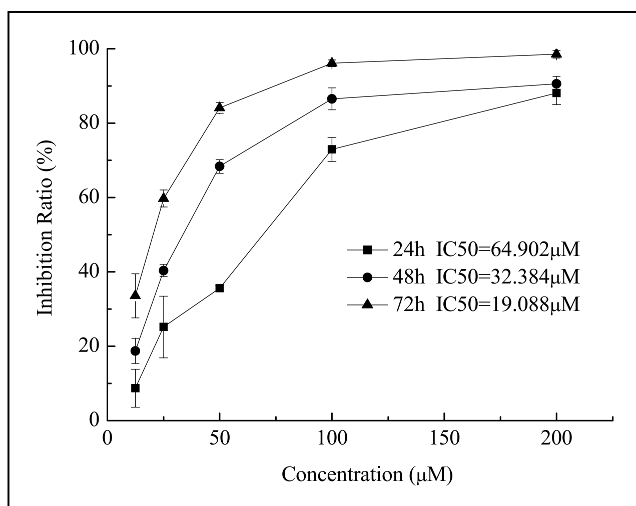

Fig. 2: Growth inhibiting effect of L-securinine on MCF-7 cells. MCF-7 cells were treated with L-securinine(0,5,10,20 and 40) μM for 24, 48, 72 h, respectively. A dosage-time dependent inhibition of cell growth could be observed ($p < 0.05$,ANOVA analysis).

2. Investigations and results

2.1. Effect of l-securinine on the viability of MCF-7 cells

Breast cancer cells were treated with L-securinine at concentrations ranging from 12.5 μM to 200 μM for 24 h, 48 h, 72 h and the inhibitory effect was determined. As shown in Fig. 2, CCK8 assay testified a dose- and time-dependent, and significant inhibitory effect of L-securinine on MCF-7 cells. At 24 h, IC50 was 64.902 μM; at 48 h, IC50 was 32.384 μM; at 72 h, IC50 was 19.088 μM.

2.2. Effect of L-securinine on apoptosis and cell cycle of MCF-7 cells

To determine the effect of L-securinine on apoptosis in MCF-7 cells, we used annexin V-FITC and PI staining through flow cytometry. MCF-7 cells were treated with 5, 10, 20, 40 μM of L-securinine for 48 h for the following research according to the CCK8 results. We observed a marked dose-dependent increase in both the early and late stages apoptosis (Fig. 3). We then tested whether L-securinine affected cell cycle progression. The results showed that L-securinine induced G1 phase with cell cycle arrest in MCF-7 cells. The increase of the treatment concentration caused accumulation of cells in the G1 phase with a concomitant decrease of both S and G2 phases (Fig. 4).

2.3. Effect of l-securinine on the expression of apoptosis related genes

In this study, real-time quantitative PCR was utilized to analyze the mRNA levels of apoptosis related genes (e.g. p53, bax, bcl-2, mtor, p70s6k) in MCF-7 cells exposed to L-securinine at concentrations of 5, 10, 20, 40 μM for 48 h. Results of quantitative real-time PCR are shown in Fig. 5. Increasing concentrations of L-securinine resulted in a dose-dependent increase in p53, bax mRNA expression, whereas a decrease in bcl-2, mtor, p70s6k expression was seen.

3. Discussion

Exploring antitumor agents from plants has become a useful strategy for antitumor drug development. Here, the effect of L-securinine on proliferation of human breast cancer MCF-7 cells was investigated. Our MTT assay results indicated that L-securinine exhibited excellent antiproliferative activity against MCF-7 cells at low concentrations. We have also observed apoptotic response in MCF-7 cells exposed to L-securinine for 48 h. Our results showed that the apoptotic index of MCF-7 cells increased in a dose-dependent manner. An arrest of the cell cycle in G1-phase was found in cell cycle analysis.

We analyzed the mRNA expression levels of five genes: p53, bax, bcl-2, mtor and p70s6k in response to l-securinine exposure in MCF-7 cells for 48 h. Qunatitative real-time PCR results showed that L-securinine induced apoptosis in MCF-7 cells by up-regulating the mRNA level of cell checkpoint protein p53 and pro-apoptotic bax. Expression of anti-apoptotic bcl-2 was down-regulated. Furthermore, both the mRNA of mtor and p70s6k were up-regulated.

Apoptosis involved in programmed cell death (PCD) was demonstrated as the major component in the death of mammalian cells under physiological conditions (Kerr 1965) and is a fast and nonreversible process under the control of several cell intrinsic and extrinsic pathways (Adams 2003; Danial and Korsmeyer 2004; Gelinas and White 2005). The dysregulation of Bcl-2 family may be concerned in the evasion from apoptosis of cancer cells as many solid and hematopoietic tumors rely on elevated the expression of antipoptotic Bcl-2 gene for survival (Galsky and Vogelzang 2010; Oltersdorf et al. 2005; Vogler et al. 2009; Wei et al. 2010). Bax and Bcl-2 are two important members of the Bcl-2 family that is closely associated with cellular fate (Danial and Korsmeyer 2004). Bcl-2 acting as an anti-apoptotic signal is over-expressed while Bax acting as an pro-apoptotic signal is lower expressed in many human cancers (Reed et al. 1996). This results in stimuli including chemotherapeutic drug resistance to a wide variety of agents (Adams and Cory 1998). Hence, it is important to find new cytotoxic agents that are able to increase Bax expression or restore the ability of tumors to undergo apoptosis.Our data demonstrated that L-securinine treatment down-regulated the expression of Bcl-2 and up-regulated the expression of Bax in MCF-7 cells.

P53, the most commonly mutated gene associated with cancer (Johnstone et al. 2002), plays a critical role in making sure that damaged cells are destroyed by apoptosis. P53 blocks the action of cell replication by stopping the cell cycle at G1 or interphase, which enhances cancer cell apoptosis (Takaoka et al. 2003). The data presented in this study indicated that the expression levels of p53 and bax increased while bcl-2 reduced after L-securinine in a concentration-dependent manner. Although the mechanism by which the expression and function of Bcl-2,Bcl- X_L and Bax are activated is not fully understood, p53 molecule is thought to possibly play a role in this process (Strobel et al. 1996). There is evidence that wild-type p53 is able to up-regulate Bax and down-regulate Bcl-2 and proceed to programmed cell death (Miyashita et al. 1994; Miyashita and Reed 1995).

The mammalian target of the rapamycin (mtor) pathway, which drives cell proliferation, is frequently up-regulated in a variety of carcinoma cell lines, as well as in breast carcinomas (van der Hage et al. 2004). Therefore, the mTOR pathway is an attractive target and the inhibition of this pathway holds promise as effective therapy for carcinomas. The mtor pathway is a serine/threonine protein kinase, which has two distinct multiprotein complexes consisting of mTORC1 and mTORC2. The mTORC1 pathway phospharylates downstream proteins p70s6k(s6k), which regulates cell growth by modulating protein synthesis,ribosome biogenesis and autophagy (Wullschleger et al. 2006). The mTORC2 pathway phosphorylates Akt, which is a potential drug target for cancers when there is Akt deregulation. In the present study, the mRNA expression of mtor and p70s6k were investigated in breast cancer MCF-7 cells exposed to L-securinine. The results showed that the mRNA expression of

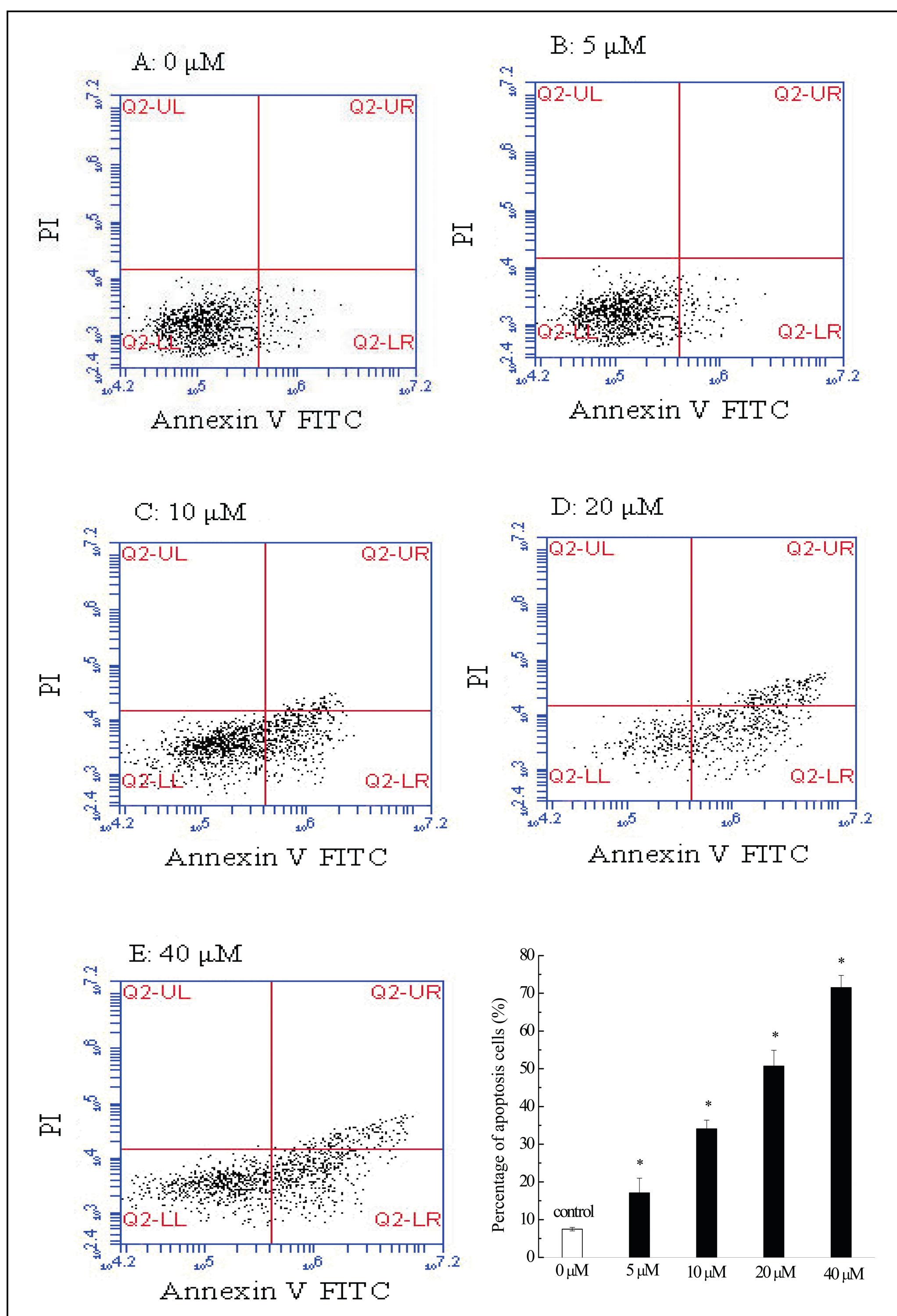

Fig. 3: Apoptosis induced by L-securinine on MCF-7 cells. MCF-7 cells were treated with L-securinine (0,5,10,20 and 40) μM for 48 h. Lower left quadrants, viable cells. Lower right quadrant, early apoptotic cells. Upper right quadrant, late apoptotic or necrotic cells. Upper left quadrant, nonviable necrotic cells. The data showed that L-securinine increased the percentage of early and late apoptotic cells,* $p < 0.05$ *vs.* 0 μM group.

mtor and p70s6k were dose-dependently decreased, suggesting that L-securinine may inhibit MCF-7 cell proliferation through inhibiting the mTOR pathway. Furthermore, the results of this study demonstrate that the expression of mTOR was down-regulated while p53 was up-regulated. It has been demonstrated that p53 can inhibit mTOR *via* activating the AMP-responsive protein kinase (AMPK) (Budanov and Karin 2008). The mTOR pathway after stress was negatively regulated by p53-regulated gene products (Feng et al. 2007). However, what's the detailed mechanism of the interreaction between mTOR and p53 pathways, remains unclear in the present study. Up to now, the p53 and mTOR pathways were individually targeted in many studies, but not targeted together. Thus, there are lots of questions that remain to be solved in future studies. Expounding the connection between mTOR and p53 pathways has implications for effective strategies for cancer therapy.

Taken together, we demonstrated for the first time that L-securinine induced apoptosis in MCF-7 cells by up-regulating the expression of Bax, p53 and down-regulating the expression of Bcl-2, mTOR, P70S6k. Our findings implied that L-securinine

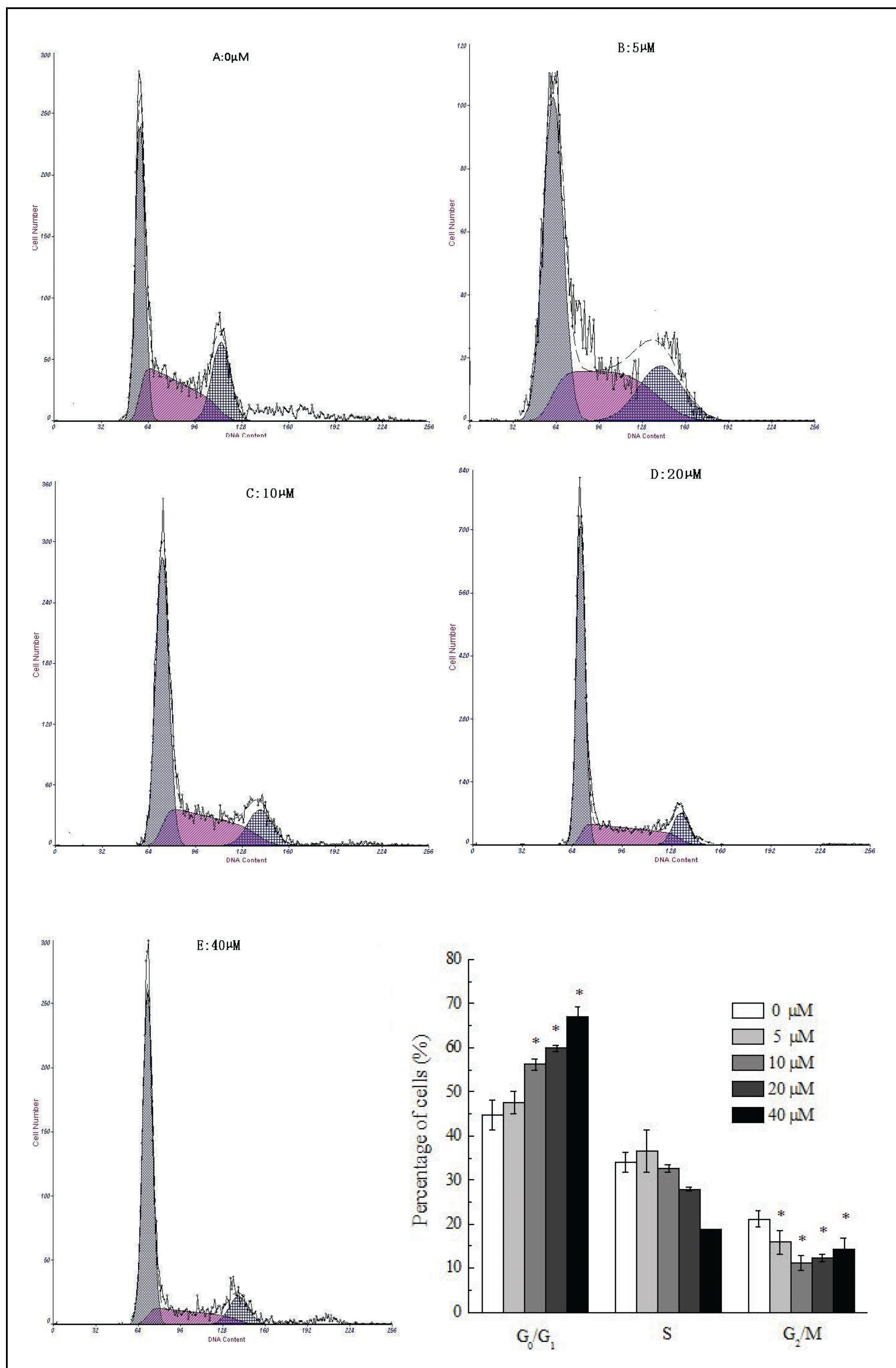

Fig. 4: L-Securinine induced G0/G1 arrest in MCF-7 cells treated for 48 h. MCF-7 cells were treated with L-securinine (0, 5, 10, 20 and 40) μM for 48 h. The results showed that the cell cycle was arrested at the G0/G1 phase when treated with L-securinine, $^{*}p < 0.05$ vs 0 μM group.

may serve as potential lead for future drug development fighting breast cancer. Further studies are required to elucidate the connection between mTOR and p53 pathways and eventually propose mTOR as a target for breast cancer therapy.

4. Experimental

4.1. Reagents

MCF-7 cells were purchased from the Shanghai institute of Cell Biology at the Chinese Academy of Science. DMEM medium (Hoclone, USA); Fetal bovine serum (Gibco, USA); Cell Counting Kit-8 (cck8) (Dojindo, Japan).

4.2. Cell culture

MCF-7 breast cancer cells were inoculated in DMEM medium containing 10% fetal bovine serum and 100 U/ml of both penicillin and streptomycin. Then the cells were cultured in an incubator at 37 °C with a humid atmosphere containing 5% CO_2. The cells were subcultured when entered exponential growth stage.

4.3. Cell viability

The antiproliferative effect of L-securinine on MCF-7 cells was determined by the Cell Counting Kit-8 assay. Exponentially growing cells were seeded into 96-well plates at a density of 3×10^3cells/well in 100 μl culture medium. After 24 h, different doses of L-securinine ranging from 12.5 to

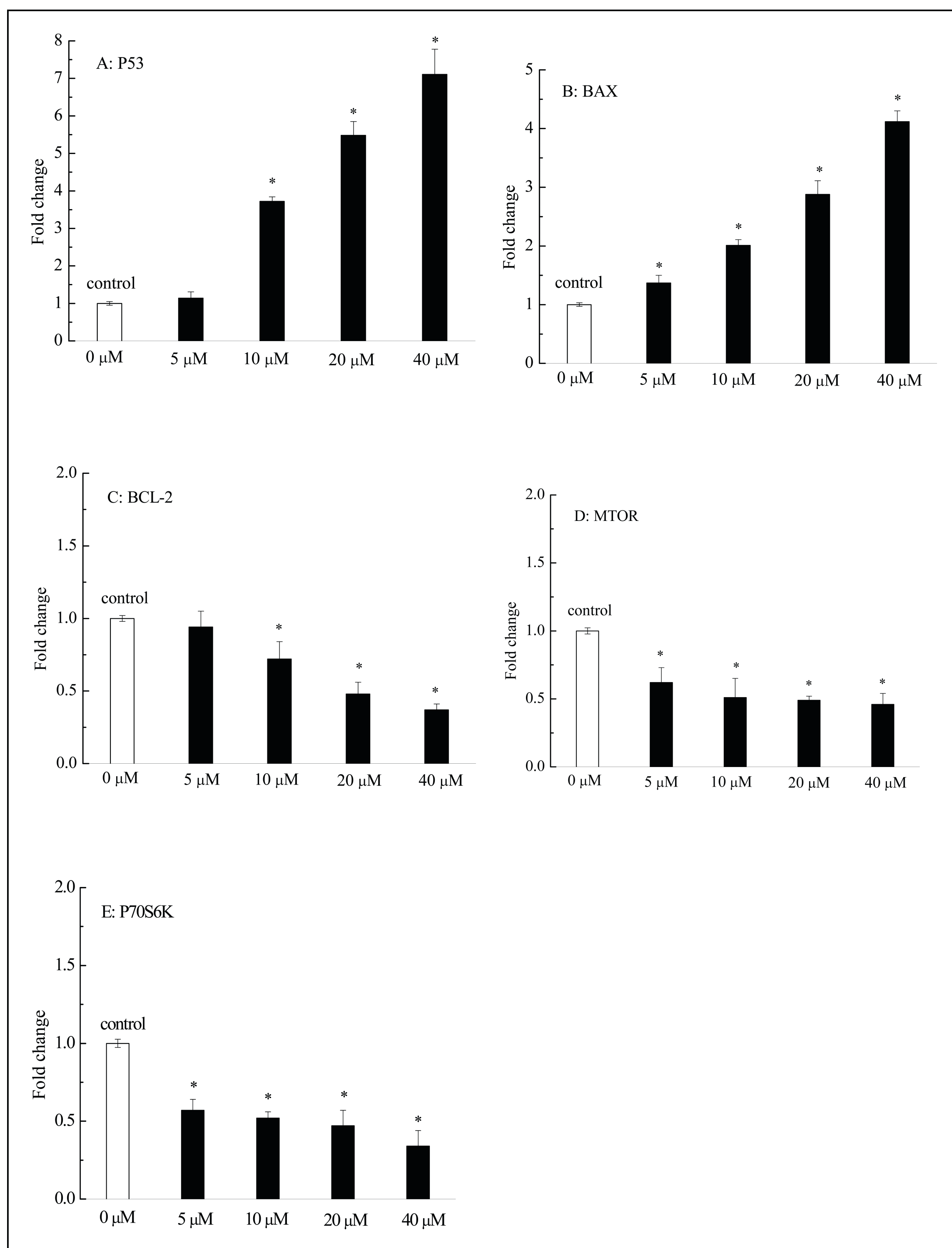

Fig. 5: Relative mRNA expression of p53, bax, bcl-2, mtor, p70s6k, as determined by quantitative real-time PCR. MCF-7 cells were treated with L-securinine (0, 5, 10, 20 and 40) μM for 48 h. Results are representative of three independent experiments and are shown as mean ± SD. The results showed the mRNA expression of p53, bax were up-regulated while bcl-2, mtor, p70s6k down-regulated, $^{*}P < 0.05$ compared with the controls.

200 μmol/l were administered to various groups with 3 duplicate wells for each concentration. A blank control group without tumor cells and a negative control without L-securinine were also created. All cells were cultured for 24, 48, 72 h, and then 10% Cell Counting Kit-8 for 2 h. Absorbance was measured at 450 nm using a microplate reader. Relative cell proliferation inhibition rate (IR) = (absorbance of the control group –absorbance of the experimental group)/(absorbance of the control group- absorbance of the blank control group)*100%.

4.4. Cell apoptosis analysis

Prepared MCF-7 cells ($1*10^6$/ml) treated with different concentrations of L-securinine (5 μM, 10 μM, 20 μM, 40 μM) for 48 h were washed with cold PBS twice and then resuspended gently in 500 μl binding buffer. Thereafter, 5 μl Annexin V-FITC and 5 μl propidium iodide (PI) were added. After 20 min incubation in the dark, the cells were finally analyzed by a flow cytometer (FACSCalibur, Becton Dickinson). The percentage of apoptotic cells was calculated by the internal software system of the FACScan (Becton Dickinson).

4.5. Cell cycle analysis

MCF-7 cells were cultured with different concentrations of L-securinine (5 μM, 10 μM, 20 μM, 40 μM) for 48 h. Then the cells were harvested by trypsinization, washed by PBS, and subjected to flow cytometry analysis according to the manufacture's recommendation. Briefly, cells were fixed

Table: Primer sequences used in real-time PCR

Primer	Structure		Length (bp)
GAPDH	Forward	5' CACCCACTCCTCCACCTTTG 3'	110
	Reverse	5' CCACCACCCTGTTGCTGTAG 3	
P53	Forward	5' GCGTGTGGAGTATTTGGATG 3'	201
	Reverse	5' TTCCGTCCCAGTAGATTACC 3'	
BAX	Forward	5' AGCTGAGCGAGTGTCTCAAG 3'	248
	Reverse	5' TGTCCAGCCCATGATGGTTC 3'	
BCL-2	Forward	5'AGACCGAAGTCCGCAGAACC 3'	113
	Reverse	5' GAGACCACACTGCCCTGTTG 3'	
MTOR	Forward	5' GCCTGGGACCTCTATTATC 3'	176
	Reverse	5' GGTGCTATGGACTGAATGC 3'	
P70s6K	Forward	5' GTGCCAATCAGGTCTTTC 3'	120
	Reverse	5' GTGGGCTGCCAATAAATC 3'	

in 70% ethanol at 4 °C overnight. The cells were washed twice with PBS and then were resuspended in PBS. 20 μg RNase A (10 μg/ml) and 2 μl propidium iodide (2.5 μg/ml) were added.The cells were then incubated for 30 min at 4 °C and were analyzed using a flow cytometer (FACS Calibur, Becton Dickinson).

4.6. *Real-time quantitative PCR analysis*

Total RNA was extracted by the TRIzol method according to the manufacture's instructions and the quality of the RNA was determined by agarose gel electrophoresis. RNA (4 μl) was added to a total volume of 20 μl reaction mixture (4.0 μl of 5*RT buffer, 0.5 μl of oligo(dT), 0.5 μl of dNTPs, 1 μl of M-MLV reverse transcriptase, 10 μl of DEPC treated water) for 1 h at 37 °C to synthesize the first strand cDNA and the reaction was terminated at 95 °C for 5 min to inactivate the M-MLV reverse transcriptase. Following synthesis, 2 μl of cDNA was amplified using the specific primers which were designed and synthesized by Shanghai JRDUN Biotechnology Co. Ltd. China. The sequences of primers are listed in the Table. GAPDH gene expression was used in parallel reactions as an internal PCR control. Real time PCR was performed with the ABI Prism 7500HT Sequence Detection System (Applied Biosystems) and SYBR Green PCR kit (Thermo Fisher Scientific, USA). The following conditions were used: 50 °C for 2 min to permit uracil N-glycosylase cleavage, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 45 s. This was followed by a melt curve analysis to determine the product specificity. The changes in mRNA expression were calculated using the $2^{-\Delta\Delta CT}$ method (CT, the abbreviation of cycle threshold, is the fractional cycle number at which the amount of amplified target reached a fixed threshold), with $\Delta CT = CT_{target\ gene} - CTGAPDH$ and $\Delta\Delta CT = \Delta CT_{treatment} - \Delta CT_{control}$.

4.7. *Statistical analysis*

All statistical analyses were performed using SPSS17.0, and the data were expressed as mean ± SD. Statistical significance of differences in measured variables among groups was analyzed using ANOVA. The null hypothesis was rejected at the 0.05 level.

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