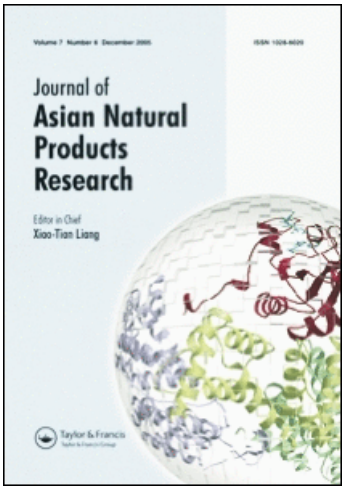


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# Silymarin augments human cervical cancer HeLa cell apoptosis via P38/JNK MAPK pathways in serum-free medium

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Silymarin was proved to have a protective effect of UV-induced A375-S2 cell apoptosis in our previous research. In this study, its pro-apoptotic and anti-apoptotic activities on human cervical cancer (HeLa) cells *in vitro* were investigated. Silymarin induced HeLa cell death through both apoptotic and necrotic pathways. At low doses (below 80 $\mu mol\,l^{-1}$), it induced cell apoptosis, but caused necrosis at high dose (160 $\mu mol\,l^{-1}$). Silymarin induced typical chromatin condensation and nuclear fragmentation as a hallmark of apoptosis. In this case, mitochondrial Bcl-2 family, Bcl-2 and Bax, were not involved in apoptotic effects; however, silymarin-induced cell death was regulated by the activation of p38 and JNK MAPKs. We also found that pan-caspase inhibitor and caspase-3 inhibitor could not antagonise silymarin-induced apoptosis. Therefore, silymarin induced and augmented HeLa cell apoptosis through p38/JNK MAPKs in the serum-free medium.

*Keywords*: Silymarin; Apoptosis; p38 MAPK; JNK MAPK; Caspase

## 1. Introduction

Silymarin (figure 1) was isolated from the seeds of *Silybum marianum*, a tall herb with prickly leaves and a milky sap. It has been widely accepted that silymarin is an effective agent for liver protection and its regeneration [1,2]. It prevents or reverses liver damage caused by alcohol, recreational drugs, pesticides, some poisons, or hepatitis. Recent reports show that silymarin also has therapeutic aspects to reduce biliary cholesterol levels, intervene in hormone refractory human prostate cancer and decrease the nephritic effects of chemical injury [3]. In our previous study, we found that silymarin protected human malignant melanoma cells (A375-S2 cells) against UV irradiation resulting in a reduced apoptotic ratio, but it could not prevent UV-induced human cervical cancer HeLa cell death.

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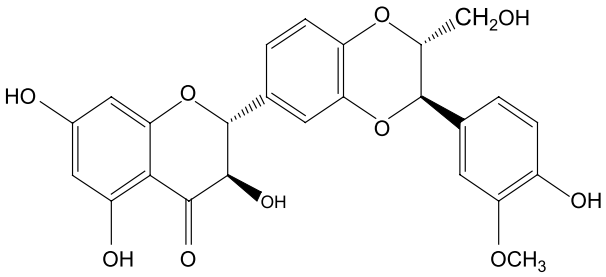


Figure 1. The structure of silymarin.

On the other hand, silymarin has an inhibitory effect on human breast cancer cells MDA-MB 468 [4]. Therefore, silymarin might have bi-directional regulatory effects on the induction of apoptosis.

Apoptotic signals go through many pathways including the caspase family, the mitochondrial pathway and the mitogen-activated protein kinase (MAPK) cascade [5,6]. The MAPK family is an important intermediate in signalling pathways that propagate extracellular stimulation into intracellular responses. MAPKs have been implicated in a wide array of physiological processes including cell growth, differentiation, and apoptosis [7]. There are three major identified subfamilies of MAPKs: (1) p54 and p46 stress-activated protein kinases (SAPKs), also referred to as the c-Jun N-terminal kinase 1 and 2 (JNK1 and 2); (2) p44 and p42 MAPKs, also referred to as extra cellular signal regulated protein kinase 1 and 2 (ERK 1 and 2); (3) p38 MAPK, was a key regulator of cytokine expression. In this research, we investigated silymarin-induced and augmented cell death of human cervical cancer (HeLa) cells.



## 2. Results and discussion

### *2.1 Silymarin-induced cytotoxicity in HeLa cells*

Proliferation of silymarin on HeLa cells was measured by MTT assay. The effects of different concentrations of silymarin were investigated on HeLa cell growth in serum-free and 10% FBS medium (figure 2). The results showed that silymarin had an evident cytotoxic effect on HeLa cells at concentrations ranging from 50 to 500 $\mu mol\,l^{-1}$ in serum-free medium. In figure 2A, the results indicated that silymarin had a cytotoxic effect on HeLa cells at concentrations of more than 300 $\mu mol\,l^{-1}$. In serum-free condition, there were 51.22% viable cells at 48 h, but only 2.02% viable cells after treatment with 100 $\mu mol\,l^{-1}$ silymarin (figure 2B). Therefore, most of the HeLa cell cytotoxicity was attributed to the effects of silymarin, and silymarin induced HeLa cell death in a dose-dependent manner.

### *2.2 Identification of the types of silymarin-induced cell death*

The ratio of LDH released from viable cells, the non-adherent dead cells, and the culture medium reflected the proportion of apoptotic and necrotic cells [8]. Thus, we measured LDH release ratio after treatment with different doses of silymarin in serum-free conditions for

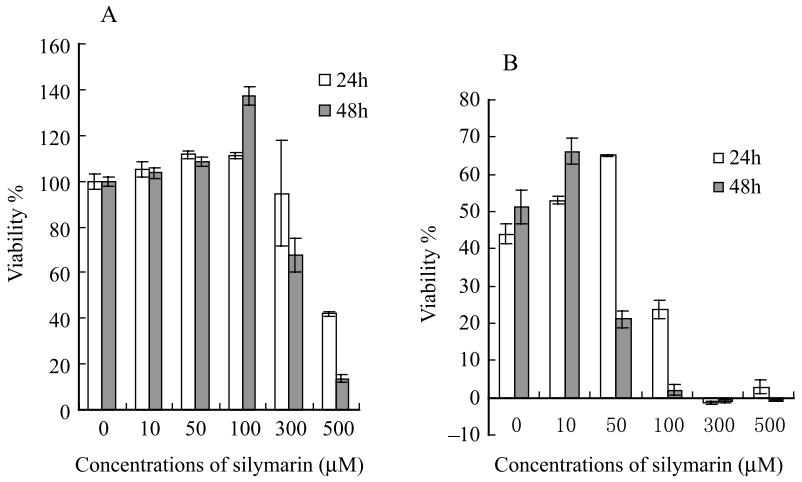


Figure 2. Cytotoxic effects of silymarin on HeLa cell growth. HeLa cells were cultured in different concentrations of silymarin for 24 and 48 h. (A) 10% FBS medium. (B) serum-free medium. $n = 3$, mean ± SD.



24 h (data not shown) and 48 h (figure 3). After treatment with 40 and 80 $\mu$mol l$^{-1}$ silymarin, the apoptotic ratio was higher than the necrotic one. However, in the presence of 160 $\mu$mol l$^{-1}$ silymarin, 80.27% cells died due to necrosis. These results demonstrated that silymarin significantly induced HeLa cell apoptosis at doses below 160 $\mu$mol l$^{-1}$ in the serum-free medium.

Morphological changes were observed by Giemsa staining. As shown in figure 4, integrated cellular membrane and nuclei appeared in the 10% FBS medium group. In the serum-free medium control group, only a few cells showed chromatin condensation. Treated with 80 $\mu$mol l$^{-1}$ silymarin in serum-free conditions, the cells lost their morphological integrity, and nuclei were damaged. To confirm the apoptotic morphological changes, Hoechst 33258 was used to stain cellular nuclei (figure 5). After treatment with 80 $\mu$mol l$^{-1}$ silymarin for 24 h, nuclei of the cells showed granular apoptotic bodies, which was an apoptosis specific morphological change. However, nuclei of normal group were stained

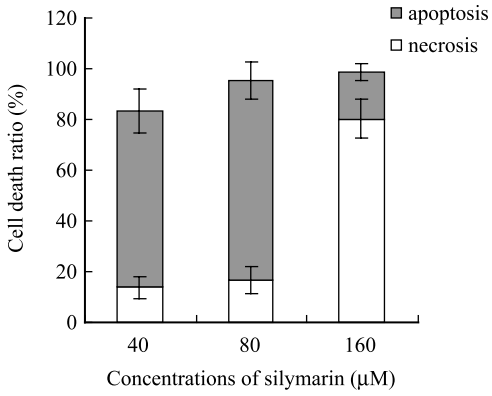


Figure 3. Characterisation of in HeLa cell death. Percentages of apoptosis and necrosis were expressed by LDH activity-based assays after treatment with different concentrations of silymarin for 48 h. $n = 3$, mean ± SD.

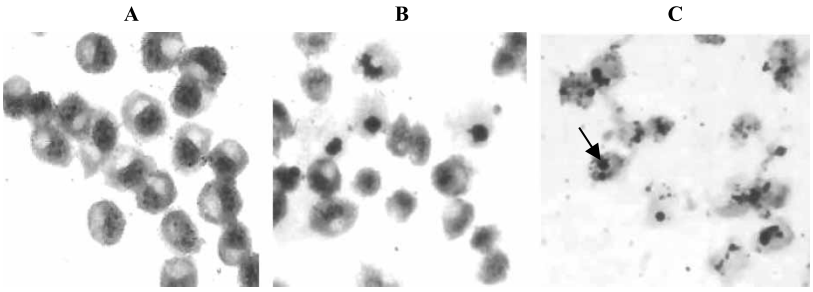


Figure 4. Morphological changes of HeLa cells. Cellular morphology was observed by Giemsa staining at 24 h, in (A) medium containing 10% FBS, (B) serum-free control and (C) medium treated with silymarin (80 $\mu$mol $l^{-1}$) conditions with $\times$ 200 magnifications.

homogeneously. Typical apoptosis of silymarin-induced HeLa cells was also proved by a DNA ladder (figure 6A), a potent hallmark of typical apoptosis.

### *2.3 p38 and JNK MAPKs regulated silymarin-induced cell apoptosis, but not caspase and mitochondrial pathways*

Apoptosis is a complicated process of programmed cell death, in which many molecular signalling mechanisms are involved. Three pathways that regulate cell death have been the subjects of intense research: activated cysteine proteases (caspase), phosphorylated mitogen-activated protein kinase (MAPK) cascades, and changed expression of mitochondrial Bcl-2 family members [9]. Bcl-2 family members modulate mitochondrial function and/or integrity. The loss of mitochondrial integrity leads to the release of agents such as cytochrome *c* and apoptosis-inducing factor (AIF), that can lead to caspase-dependent or independent cell death.

In the MAPK pathway, ERK-activated ribosomal S6 kinase (RSK) enhances the phosphorylation of Bad at S112 [10], a member of the mitochondrial Bcl-2 family. RSK-induced phosphorylation of Bad increases its ability to bind 14-3-3 proteins, family members of dimeric phosphoserine proteins that regulate signal transduction, apoptotic, checkpoint control pathways [11,12], therefore protecting pro-survival Bcl-2 family members Bcl-2 and Bcl-$x_L$. In addition, RSK phosphorylates transcription factor CREB, which can lead to the MEK-dependent transcriptional up-regulation of the pro-survival Bcl-2 family members Bcl-2, Bcl-$x_L$ and Mcl-1 [13]. In this study, we evaluated the effects of those pathways on silymarin-induced HeLa cell death.

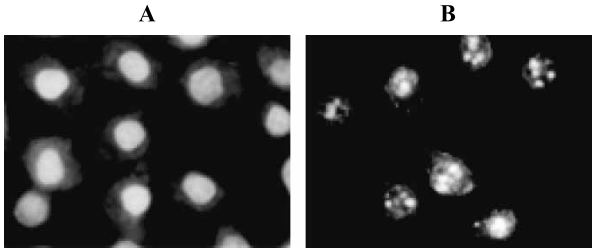


Figure 5. Morphological changes of cellular nuclei. HeLa cells were incubated in the serum-free medium (A) and the medium containing 80 $\mu$mol $l^{-1}$ silymarin (B) for 24 h. Then, cells were stained with Hoechst 33258 with $\times$ 200 magnifications.

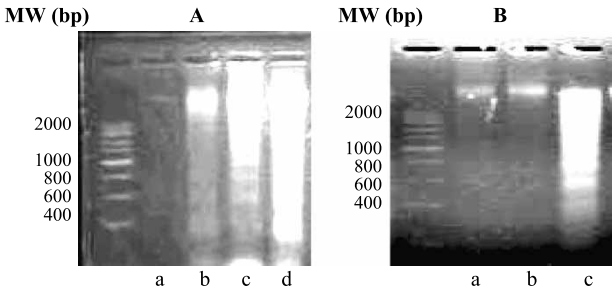


Figure 6. The variation of DNA fragmentation in silymarin-treated HeLa cells. (A) Different doses of silymarin: (a) 0 $\mu$mol l$^{-1}$, (b) 20 $\mu$mol l$^{-1}$, (c) 40 $\mu$mol l$^{-1}$, (d) 80 $\mu$mol l$^{-1}$. (B) the cells were cultured in the absence or presence of p38 inhibitor, 1 h prior to the addition of silymarin (40 $\mu$mol l$^{-1}$): (a) serum-free control group, (b) p38 inhibitor and silymarin (40 $\mu$mol l$^{-1}$) group, (c) silymarin (40 $\mu$mol l$^{-1}$) group. *Hae III*-digested phage $\Phi$x-174 DNA fragments were used as molecular markers.



To assess whether activation of caspase-like proteases is required for silymarin-induced apoptosis, we used a tripeptide or tetrapeptide caspase inhibitor: z-VAD-fmk (pan-caspase inhibitor). The results showed that the specific inhibitors for p38 (SB203580), JNK (SP600125) and ERK (PD98059) were applied to assess the function on MAPKs to silymarin-induced HeLa cell death. After 48 h incubation with 40 $\mu$mol l$^{-1}$ silymarin, 10 $\mu$mol l$^{-1}$ SB203580 effectively blocked apoptosis (figure 7A), and 10 $\mu$mol l$^{-1}$ SP600125 partially inhibited cell death. 10 $\mu$mol l$^{-1}$ PD98059 slightly increased the cell death ratio. Furthermore, 10 $\mu$mol l$^{-1}$ SB203580 attenuated the DNA ladder at 48 h (figure 6B). In addition, in the control medium group, cell death was partially prevented by 20 $\mu$mol l$^{-1}$ z-VAD-fmk at 48 h, but silymarin-induced cell death was not antagonised by pan-caspase and caspase-3 inhibitors in the serum-free medium (figure 7B). The results indicated that silymarin-induced apoptosis was dependent on p38/JNK MAPK activation, but not caspase, in serum-free medium.

The effects of silymarin on p38, JNK and ERK phosphorylation were examined by Western blot analysis. As shown in figure 9, p38 was activated after treatment with 40 $\mu$mol l$^{-1}$ silymarin. Phosphorylation of p38 was increased in HeLa cells from 4 h to 12 h; however, it declined at 16 h. JNK1 was slightly activated at 8 h, and JNK2 appeared to be phosphorylated at 4 h when HeLa cells were treated by 40 $\mu$mol l$^{-1}$ silymarin. But p-ERK expression had no change during this process, whereas p38, ERK and JNK were not phosphorylated in the serum-free medium control group at the same times (data not shown). In figure 8B, p38 and JNK1 inhibitors were applied to examine the influence on p-p38 and p-JNK1 expression. The results indicated that SB203580 could obviously block p38 activation and SP600125 could partially inhibit JNK1 phosphorylation. In addition, the expression of Bax and Bcl-2 proteins did not change in the mitochondrial apoptotic pathway (figure 9). These results showed that they were not involved in silymarin-induced cell death.

Our previous study had shown that silymarin could prevent UV irradiation-induced human malignant melanoma cell (A375-S2) apoptosis by increased phosphorylation of ERK and blocking activities of caspase-3 and caspase-9 in the culture containing 10% FBS; nevertheless, it had no such effect on HeLa cells [14]. Thus, silymarin has the effects of two-way regulation: it induces apoptotic cell death in some conditions and prevents apoptosis in other conditions. In this research, we found that silymarin could induce HeLa cell death via p38/JNK MAPKs pathway in serum-free medium. In particular, activated p38 augments HeLa cell apoptosis.

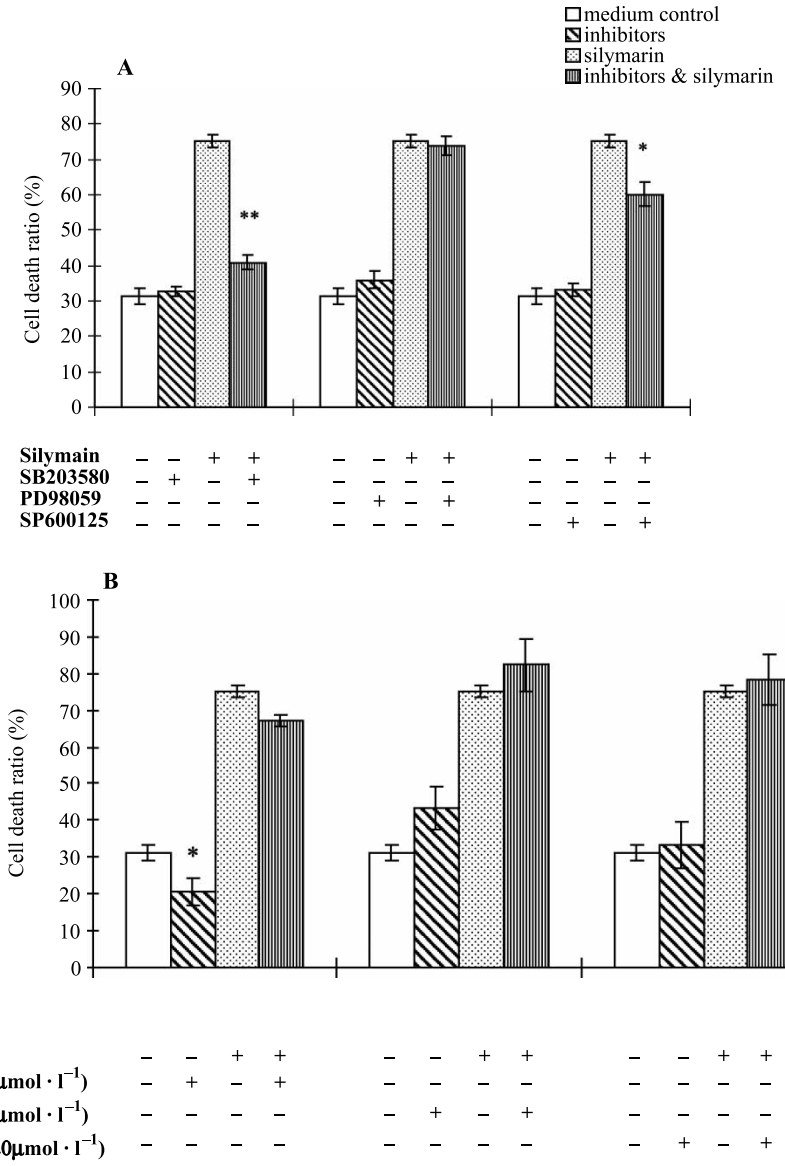


Figure 7. Effect of inhibitors on silymarin-induced HeLa cell death. The cells were pre-treated with (A) MAPK inhibitors (10 $\mu$mol l$^{-1}$ SB203580, 10 $\mu$mol l$^{-1}$ PD98059, 10 $\mu$mol l$^{-1}$ SP600125, respectively) and (B) two caspase inhibitors (20, 40 $\mu$mol l$^{-1}$ z-VAD-fmk, 20 $\mu$mol l$^{-1}$ z-DEVD-fmk) for 1 h, then incubated with or without 40 $\mu$mol l$^{-1}$ silymarin for another 48 h. $n = 3$. mean ± SD. *$p < 0.05$, **$p < 0.01$, vs treatment with silymarin.

## 3. Experimental

### *3.1 Chemicals and reagents*

Silymarin was purchased from Beijing Institute of Biological Products (Beijing, China). The purity of silymarin was measured by HPLC and determined to be about 98%. Silymarin was dissolved in dimethylsulfoxide ($Me_2SO$). The concentration of $Me_2SO$ in the final culture

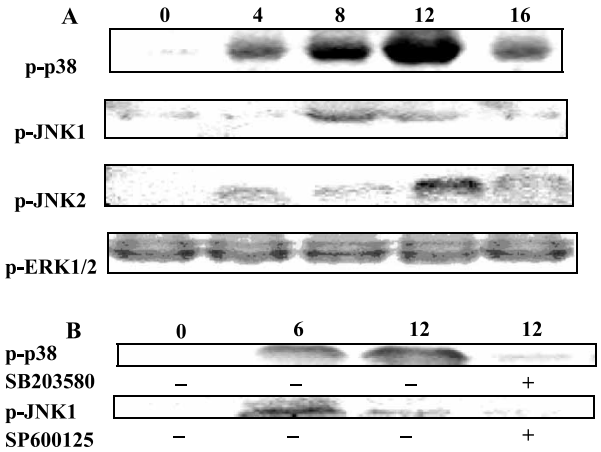


Figure 8. Expression of phosphorylation of p38, JNK, and ERK in silymarin-induced HeLa cells. (A) The cells were treated with silymarin (40 $\mu$mol$l^{-1}$) for 0, 4, 8, 12, and 16 h. (B) The cells were incubated in the medium with or without p38 and JNK inhibitors for 1 h, followed by 40 $\mu$mol$l^{-1}$ silymarin for 0, 6, and 12 h. Protein bands were detected by Western blot analysis.



medium was kept under 0.1%, which had no toxic effect on HeLa cells. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma.

Pan-caspase inhibitor (z-VAD-fmk) and caspase-3 inhibitor (z-DEVD-fmk) were purchased from Enzyme Systems (CA, USA). p38 MAPK inhibitor (SB203580), JNK inhibitor (SP600125), and ERK inhibitor (PD98059) were from Calbiochem (CA, USA). Rabbit polyclonal antibodies against phospho-ERK, phospho-JNK, and horseradish peroxidase-conjugated secondary antibody (goat-anti-rabbit) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal anti-Bcl-2 and Anti-Bax were from Oncogene Research Products (MA, USA). Rabbit polyclonal anti-phospho-p38 was obtained from Techne (CA, USA).

### *3.2 Cell culture*

The human cervical cancer HeLa cells were obtained from the American Type Culture Collection (ATCC, #CCL-2, USA). HeLa cells were cultured in RPMI-1640 medium (GIBCO, USA) supplemented with 10% fetal bovine serum (FBS) (Dalian Biological Reagent Factory, Dalian, China), 0.03% L-glutamine and 2 mmol$l^{-1}$ Hepes (GIBCO, USA) at 37°C in 5% $CO_2$.

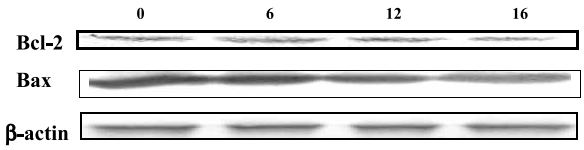


Figure 9. Western blot analyses of Bcl-2 and Bax expression. HeLa cells were treated with silymarin (40 $\mu$mol$l^{-1}$) for 0, 6, 12, and 16 h. Cell lysates were separated by 10% SDS-PAGE electrophoresis.

### 3.3 Bioassays for cytotoxicity

HeLa cells were seeded into 96-well plates (NUNC, Denmark) at $1 \times 10^4$ cells per well in 100 μl RPMI-1640 medium overnight. After pre-incubation with caspase inhibitors z-VAD-fmk, z-DEVE-fmk, p38 inhibitor (SB203580), JNK inhibitor (SP600125) and ERK inhibitor (PD98059) at given concentrations for 1 h, various doses of silymarin were added to serum-free medium, respectively. Cellular death was measured by MTT assay [15] using a Plate Reader (Bio-Rad, USA).

### 3.4 Cellular morphological changes and nuclei damage

HeLa cells ($2 \times 10^5$) were seeded on coverslips in 6-well culture plates (NUNC, Denmark) and cultured overnight. Different doses of silymarin were added to the serum-free medium. After 24 h, all cover slips were fetched from 6-well plates and washed twice with phosphate-buffered saline (PBS). The cells on the cover slips were fixed with acetic acid/chloroform/absolute ethanol (1:3:6) for 15 min, and stained by 0.02% Giemsa. Then the cover slips were sealed by neutral balsam. Cellular morphology was observed by using phase contrast microscopy. Nuclear morphology was observed by Hoechst 33258 staining in situ. HeLa cells were cultured and treated as described above. Then the cells on coverslips were fixed with 3.7% paraformaldehyde at room temperature, then washed, and stained with Hoechst 33258 (167 $\mu mol\,l^{-1}$) at 37°C for 30 min. The coverslips were sealed with the liquid containing 50% glycerol, 20 $mmol\,l^{-1}$ citric acid and 50 $mmol\,l^{-1}$ $NaH_2PO_4$. The photographs were taken by a fluorescence microscope (Leica, Wetzlar, Germany).



### 3.5 LDH activity-based cytotoxicity assays [8,16]

The cells were cultured with silymarin in serum deprivation medium for 24 h. Floating dead cells were collected from the medium by centrifugation (240*g* at 4°C for 10 min), and the lactate dehydrogenase (LDH) content from pellets lysed in 0.1% NP-40 for 15 min was used as an index of cell death via apoptotic pathway ($LDH_A$). The LDH released in the culture medium was used as an index of cell death via necrotic pathway ($LDH_N$). The adherent (viable) cells were lysed in 0.1% NP-40 for 15 min to release HDH ($LDH_V$). The substrate reaction buffer of LDH [L(+)-lactic acid 0.5 $mmol\,l^{-1}$, INT 0.66 $mmol\,l^{-1}$, PMS 0.28 $mmol\,l^{-1}$, $NAD^+$ 1.3 $mmol\,l^{-1}$ in Tris PH 8.2] was added. The A value at $\lambda =$ 490 nm of reaction for 1 and 5 min were assayed. LDH $= (A_{5\,min} - A_{1\,min})/4$. The percentage of apoptotic and necrotic cell death was calculated as follows:

$$\%\,\text{apoptosis} = LDH_A/(LDH_A + LDH_N + LDH_V) \times 100$$

$$\%\,\text{necrosis} = LDH_N/(LDH_A + LDH_N + LDH_V) \times 100$$

### 3.6 DNA fragmentation assay

HeLa cells ($1 \times 10^6$ cells) were seeded and treated with different doses of silymarin with or without p38 inhibitor (SB203580) for 48 h. Both adherent and non-adherent cells were collected by centrifugation at 150*g* for 5 min and washed once with PBS. The cellular pellet was re-suspended in lyses buffer (10 $mmol\,l^{-1}$, Tris–HCl pH 7.4, 10 $mmol\,l^{-1}$ EDTA pH 8.0,

0.5% Triton X-100) on ice for 30 min. The supernatants were incubated with 40 $\mu g\,l^{-1}$ proteinase K and 40 $\mu g\,l^{-1}$ RNase A at 37°C each for 1 h, respectively. Then 5 $mol\,l^{-1}$ NaCl and 50% isopropyl alcohol were added to the mixture at $-20$°C overnight. DNA was separated by 2% agarose gel electrophoresis and stained with 0.1 $mg\,l^{-1}$ ethidium bromide.

### *3.7 Western blot analysis*

Cell extracts were prepared as described by Messmer and Brune [17]. Briefly, cells were suspended in lysis buffer [50 $mmol\,l^{-1}$ Tris, 5 $mmol\,l^{-1}$ edetic acid, 150 $mmol\,l^{-1}$ NaCl, 0.5% NP-40, 10 $\mu g\,ml^{-1}$ leupeptin (Sigma, MO, USA), 10 $\mu g\,ml^{-1}$ aprotinin (Sigma), 1 $mmol\,l^{-1}$ PMSF (Sigma)] on ice for 1 h. Cell lysates were centrifuged at 4000*g*, at 4°C for 15 min, and protein concentration in the supernatant was determined by using Bio-Rad protein assay reagent (Bio-Rad, USA). Total cellular proteins were resolved by 10% SDS-PAGE and blotted onto nitrocellulose membrane. Antibodies against phospho-p38 (rabbit IgG, Oncogene, USA), phospho-p54/p46 SAPK/JNK (mouse IgG, Oncogene), phospho-p44/p42 ERK (mouse IgG, Oncogene), Bcl-2 (rabbit IgG, Oncogene) and Bax (rabbit IgG, Oncogene) special antibodies were applied to detect those proteins. Anti-rabbit IgG conjugated with peroxidase (HRP) and 3,3-diaminobenzidine tetrahydrochloride (Sigma), as the HRP substrate, were used to visualise proteins.

### *3.8 Statistical analysis of the data*

Data were expressed as means $\pm$ standard deviation of $n$ experiments. Statistical significance was evaluated using Student's $t$-test; $p < 0.05$ was considered significant.



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