

Anticancer activity of *Nelumbo nucifera* stamen extract in human colon cancer HCT-116 cells *in vitro*

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Abstract. The aim of the present study was to investigate the anticancer activities of *Nelumbo nucifera* (Ba lotus) stamen ethanol crude extract (BLSEE) in human colon carcinoma HCT-116 cells. MTT assay, flow cytometry analysis and reverse transcription-polymerase chain reaction assay were employed to investigate the anticancer mechanisms of BLSEE (100, 200 and 400 $\mu\text{g/ml}$) in HCT-116 cells. BLSEE reduced HCT-116 cell proliferation in a dose-dependent manner. BLSEE treatment also significantly increased the sub-G1 population in HCT-116 cells ($P=0.0020$ at 400 $\mu\text{g/ml}$), as shown by flow cytometry assay. Following treatment with BLSEE, the mRNA levels of the apoptosis-associated factors Fas, Fas ligand, tumor necrosis factor-related apoptosis-inducing ligand, death receptor 4 (DR4), death receptor 5 (DR5), caspases 3, 8 and 9, and B-cell lymphoma-2 (Bcl-2) associated X protein were increased, and the expression of anti-apoptotic Bcl-2 and Bcl-extra large was decreased in HCT-116 cells. The mRNA levels of matrix metalloproteinase (MMP)-2, MMP-9, TIMP metalloproteinase inhibitor 1 and TIMP metalloproteinase inhibitor 2 were also regulated by BLSEE treatment. In addition, BLSEE was able to modulate

the expression of inflammation-associated nuclear factor- κB , inhibitory $\kappa\text{B}\alpha$, inducible nitric oxide synthase and cyclooxygenase 2 in HCT-116 cells. The present study clearly indicated the cytotoxicity of BLSEE in HCT-116 cells through induced cellular apoptosis. These results also suggested the BLSEE may be a powerful agent against colon cancer cells.

Introduction

Colorectal cancer (CRC) is one of the most frequently diagnosed cancers in the world. In the USA, CRC is the third most common malignant tumor, and there was an estimated 103,170 new cases of CRC throughout the country in 2014 (1). Previously, CRC has become the fourth most common malignant tumor, and the third leading cause of cancer-associated mortality in China (2). CRC also shows a high level of metastasis, and 25% of CRC patients that present with metastatic disease have a 5-year survival of only 10% (3). Surgical resection plus chemotherapy and/or radiation therapy are effective treatments for CRC in the clinic. However, chemotherapy and radiation therapy are commonly associated with serious side effects, including bone marrow suppression, nausea and vomiting, hair loss and loss of appetite (3). Enhancement of cancer cell apoptosis is a good strategy in the clinical treatment of cancer (4).

As classic apoptosis-associated factors, the B-cell lymphoma 2 (Bcl-2) family plays an important role in the regulation of cell apoptosis. The Bcl-2 family is generally divided into anti-apoptotic factors, including Bcl-2, Bcl-extra large (Bcl-xL) and Bcl-W, and proapoptotic factors, including Bcl-2-associated X protein (Bax), Bcl-2 associated agonist of cell death, Bcl-2 interacting mediator of cell death (Bim), Bcl-2 antagonist/killer 1 and p53 upregulated modulator of apoptosis (5,6). In the extrinsic apoptosis pathway, the cell death receptor Fas/Fas ligand (FasL) system plays an important role to induce receptor-ligand mediated cell apoptosis, and also regulates the Fas-associated death domain (FADD) and caspase-8-mediated cell apoptosis (7). In apoptosis, the depolarization of the inner mitochondrial membrane potential causes the release of cytochrome *c* (Cyto *c*) into the

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cytosol (8). The released Cyto *c* also activates caspases by formation of a complex that induces the activation of apoptotic protease-activating factor-1 (Apaf-1) and procaspase-9. Furthermore, activated caspase-8 and -9 cleaved the final executioner caspase-3, and directly induced the chromatin condensation and DNA fragmentation to promoting apoptosis in cells (9,10).

Traditional herbal medications are commonly used to treat colon cancer in the clinic (11-13). Lotus is traditionally used as a Chinese folk medicine to disperse the summer heat, and has shown numerous health benefits and pharmacological activities, including antioxidant (14), anti-diarrheal (15), antiviral (16), anti-obesity (17-20), anti-angiogenic (21), hepatoprotective (22), immunomodulatory (23) and insulin secretagogue activity (24). Therefore, the present study focused on investigating the potential anticancer activity of *Nelumbo nucifera* (Ba lotus) stamen ethanol crude extract (BLSEE), and also to elucidate the mechanisms underlying its anticancer effects in human colon carcinoma HCT-116 cells.

Materials and methods

Chemical reagents. TRIzol reagent, OligodT₁₈ primer, murine Moloney leukemia virus (MMLV) reverse transcriptase, RNase inhibitor, ethidium bromide (EtBr), and agarose were purchased from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). All other reagents were of analytical grade and purchased from Sigma-Aldrich (Merck Millipore, Darmstadt, Germany).

BLSEE preparation. Fresh Ba lotus stamen purchased from Chongqing Enterprise Engineering Research Center of Ba-lotus Breeding and Deep Processing (Chongqing, China) were freeze-dried and then ground into a fine powder. In total, 100 g of powdered Ba lotus stamen was extracted twice with 1,000 ml ethanol (70%, v/v) at 50°C for 1 h. Subsequent to filtering, the sample extraction solution was condensed by vacuum rotary evaporator (Büchi Rotovapor RE 111; Büchi Labortechnik AG, Flawil, Switzerland) at 37°C, freeze-dried and stored at -80°C until further study.

Cell culture. Human colorectal HCT-116 cancer cells were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were routinely maintained in RPMI-1640 medium supplemented with 10% (v/v) FBS, and 1% penicillin-streptomycin in a humidified CO₂ incubator (model 3154; Forma Scientific, Inc., Marietta, OH, USA) with a 5% CO₂ atmosphere at 37°C.

Cell viability assay. Cell viability was measured using an MTT assay. HCT-116 cells were seeded in 96-well plates (Nalge Nunc International, Rochester, NY, USA) at a density of 1x10⁴ cells/well. Following a 24-h incubation, the cells were treated with the various concentrations (100, 200 and 400 µg/ml) of BLSEE for 24 h. Following incubation with BLSEE 0.5 mg/ml of MTT reagent (100 µl) was added to each well and the cells were incubated in a humidified incubator at 37°C to allow the MTT to be metabolized. At total of 4 h later, formazan crystals were dissolved with dimethyl sulfoxide (100 µl in each well). Absorbance of the samples was

measured at a wavelength of 540 nm using a microplate reader (model, 680; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Flow cytometry analysis. The BLSEE-treated HCT-116 cells were first collected following digestion with trypsin, washed twice with cold PBS, and resuspended in 2 ml PBS. The DNA contents of the treated cells were stained with propidium iodide (PI) using a DNA staining kit (CycleTEST™ PLUS DNA reagent kit; Becton Dickinson, Franklin Lakes, NJ, USA), according to the manufacturer's protocol. Fluorescence intensity was determined using a FACScan flow cytometer (EPICS XL-MCL, Beckman Coulter KK, Brea, CA, USA) and analyzed using CellQuest software (Becton Dickinson).

Reverse transcription-polymerase chain reaction (RT-PCR) assay. Total RNA was isolated from BLSEE-treated HCT-116 cells using TRIzol reagent, according to the manufacturer's protocol, and centrifuged at 12,000 x g for 15 min at 25°C following the addition of chloroform (200 µl). Isopropanol was added to the supernatant at a 1:1 ratio and the RNA was pelleted by centrifugation at 12,000 x g for 15 min at 4°C. Subsequent to washing with ethanol, the RNA was solubilized in diethyl pyrocarbonate-treated RNase-free water and quantified by measuring the absorbance at 260 nm using a UV-1750 spectrophotometer (Shimadzu, Kyoto, Japan). Equal amounts of RNA (1 µg) were reverse transcribed in a master mix containing 1X reverse transcriptase buffer, dNTPs (1 mM), oligodT₁₈ primers (500 ng), MMLV reverse transcriptase (140 U), and RNase inhibitor (40 U) for 45 min at 42°C. PCR was then performed in an automatic thermocycler (Bioneer, Daejeon, South Korea) for 40 cycles (94°C for 5 min, 58°C for 30 sec, and 72°C for 90 sec) followed by a 10 min extension at 95°C. The primer sequences as presented at Table I. The PCR products were separated in 2% agarose gels and visualized by EtBr staining. GAPDH was used for normalization of the results. Gene expression was quantified using ImageJ software (version 1.44; National Institutes of Health, Bethesda, MD, USA) and results presented as fold change compared to the control group.

Statistical analysis. Data are presented as the mean ± standard deviation. Differences between the mean values for individual groups were assessed by a one-way analysis of variance with Duncan's multiple range tests. P<0.05 was considered to indicate a statistically significant difference. The SAS v9.1 statistical software package (SAS Institute Inc., Cary, NC, USA) was used for the statistical analysis.

Results

BLSEE reduced HCT-116 cancer cell growth. As shown in Table II, BLSEE significantly inhibited the colon cancer HCT-116 cell growth *in vitro* in a dose-dependent manner. At a high dose of 400 µg/ml, BLSEE showed the highest inhibition activity of HCT-116 cells (86.3%; P=0.0002), compared with doses of 100 (P=0.0006) and 200 µg/ml BLSEE (P=0.0017).

BLSEE induced the apoptosis in HCT-116 cancer cells. Flow cytometry analysis revealed that BLSEE treatment was able to promote apoptosis in HCT-116 cells. As shown in Fig. 1,

Table I. Sequences of reverse transcription-polymerase chain reaction primers.

Gene name	Sequence
Caspase-3	
Forward	5'-CAAAC TTTTTCAGAGGGGATCG-3'
Reverse	5'-GCATACTGTTTCAGCATGGCA-3'
Caspase-8	
Forward	5'-CCCCACCCTCACTTTGCT-3'
Reverse	5'-GGAGGACCAGGCTCACTTA-3'
Caspase-9	
Forward	5'-GGCCCTTCCTCGCTTCATCTC-3'
Reverse	5'-GGTCCTTGGGCCTTCTGGTAT-3'
Bax	
Forward	5'-AAGCTGAGCGAGTGTCTCCGGCG-3'
Reverse	5'-CAGATGCCGGTTCAGGTACTCAGTC-3'
Bcl-2	
Forward	5'-CTCGTCGCTACCGTCGTGACTTGG-3'
Reverse	5'-CAGATGCCGGTTCAGGTACTCAGTC-3'
Bcl-xL	
Forward	5'-CCCAGAAAGGATACAGCTGG-3'
Reverse	5'-GCGATCCGACTACCAATAC-3'
TRAIL	
Forward	5'-GGAACC CAAGGTGGGTAGAT-3'
Reverse	5'-TCTCACCACTGCAACCTC-3'
DR4	
Forward	5'-AAGTCCCTGCACCACGAC-3'
Reverse	5'-CCACAACCTGAGCCGATG-3'
DR5	
Forward	5'-TGAGATAAAGGTGGCTAAA-3'
Reverse	5'-AAAGGTAAACCAGGGAAG-3'
NF-κB	
Forward	5'-CACTTATGGACAACCTATGAGGTCTCTGG-3'
Reverse	5'-CTGTCTTGTGGACAACGCAGTGGAA TTTTAGG-3'
IκBα	
Forward	5'-GCTGAAGAAGGAGCGGCTACT-3'
Reverse	5'-TCGTACTCCTCGTCTTTCATGGA-3'
iNOS	
Forward	5'-AGAGAGATCGGGTTCACA-3'
Reverse	5'-CACAGAACTGAGGGTACA-3'
COX-2	
Forward	5'-TTAAAATGAGATTGTCCGAA-3'
Reverse	5'-AGATCACCTCTGCCTGAGTA-3'
MMP-2	
Forward	5'-CTTCTTCAAGGACCGGTTCA-3'
Reverse	5'-GCTGGCTGAGTACCAGTA-3'
MMP-9	
Forward	5'-TGGGCTACGTGACCTATGAC-3'
Reverse	5'-GCCCAGCCCACCTCCACTCC-3'
TIMP-1	
Forward	5'-GTCAGTGAGAAGCAAGTCGA-3'
Reverse	5'-ATGTTCTTCTGTGACCCA-3'

Table I. Continued.

Gene name	Sequence
TIMP-2	
Forward	5'-TGGGGACACCAGAAGTCAAC-3'
Reverse	5'-TTTTCAGAGCCTTGGAGGAG-3'
Fas	
Forward	5'-GAAATGAAATCCAAAGCT-3'
Reverse	5'-TAATTTAGAGGCCAAAGTGGC-3'
FasL	
Forward	5'-GGATTGGGCCTGGGGATGTTTCA-3'
Reverse	5'-TTGTGGCTCAGGGGCAGGTTGTTG-3'
GAPDH	
Forward	5'-CGGAGTCAACGGATTTGGTC-3'
Reverse	5'-AGCCTTCTCCATGGTCGTGA-3'

Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X protein; Bcl-xL, Bcl-extra large; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; DR4, death receptor 4; DR5, death receptor 5; NF-κB, nuclear factor-κB; IκBα, inhibitory κBα; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase 2; MMP-2, matrix metalloproteinase-2; MMP-9, matrix metalloproteinase-9; TIMP-1, TIMP metalloproteinase inhibitor 1; TIMP-2, TIMP metalloproteinase inhibitor 2; FasL, Fas ligand.

the BLSEE treatment significantly increased the proportion of apoptotic cells to 10.2 (P=0.0043), 19.7 (P=0.0032) and 41.3% (P=0.0020) compared with the non-treated cells (2.3%).

Effect of BLSEE on the expression of Fas, FasL, tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), death receptor 4 (DR4) and death receptor 5 (DR5) in HCT-116 cells. Following treatment with various doses of BLSEE, the mRNA levels of Fas (P=0.0019 at 400 μg/ml) and FasL (P=0.0003 at 400 μg/ml) were upregulated in HCT-116 cells (Fig. 2). In addition, 400 μg/ml of BLSEE significantly upregulated the mRNA levels of TRAIL (24.7-fold; P=0.0015), DR4 (133.2-fold; P=0.0027) and DR5 (7.5-fold; P=0.0064) compared with non-treated HCT-116 cells (Fig. 3).

Effect of BLSEE on the expression of caspases 3, 8 and 9 in HCT-116 cells. The effect of BLSEE on the mRNA caspase family, including caspases 3, 8 and 9 in HCT-116 colon cancer cells was determined by RT-PCR assay. BLSEE treatment significantly increased the mRNA expressions of these caspases in HCT-116 cells in a dose-dependent manner (Fig. 4). The highest dose (400 μg/ml) of BLSEE also increased the mRNA levels of caspase-3 (10.9-fold; P=0.0001), caspase-8 (3.5-fold; P=0.0046) and caspase-9 (11.9-fold; P=0.0014) compared to the non-treated HCT-116 cells.

BLSEE modulated the expressions of Bcl-2, Bcl-xL and Bax in HCT-116 cells. As shown in Fig. 5, BLSEE treatment dose-dependently reduced the expression of Bcl-2 and Bcl-xL in HCT-116 cells. At the highest dose of 400 μg/ml, BLSEE significantly reduced the mRNA levels of Bcl-2 (98.9%; P=0.0009) and Bcl-xL (96.3%; P=0.0006) compared to the

Table II. Growth inhibition of human HCT-116 colon cancer cells by Ba lotus stamen extract as evaluated by MTT assay.

Treatment ($\mu\text{g/ml}$)	OD ₅₄₀	Inhibitory rate, %	P-value
Control	0.488 \pm 0.003		
100 $\mu\text{g/ml}$ BLSEE	0.373 \pm 0.008	23.6 \pm 0.4	0.0017 ^a
200 $\mu\text{g/ml}$ BLSEE	0.238 \pm 0.010	51.2 \pm 0.5	0.0006 ^a
400 $\mu\text{g/ml}$ BLSEE	0.067 \pm 0.002	86.3 \pm 0.4	0.0002 ^a

^aP<0.01 vs. the control group. OD₅₄₀, optical density at 540 nm; BLSEE, Ba lotus stamen extract.

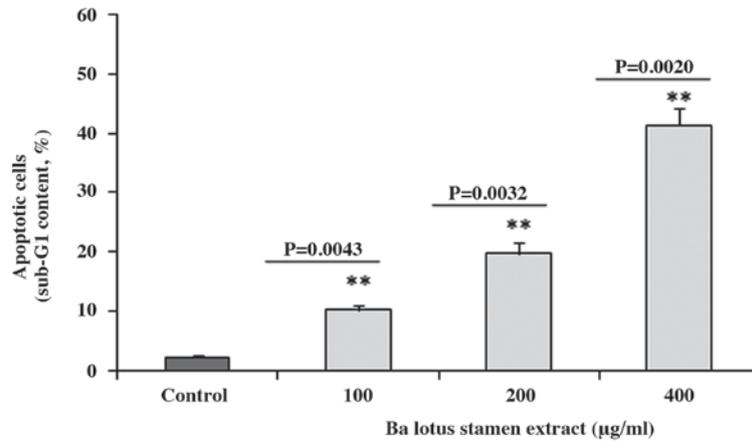


Figure 1. Level of apoptosis (sub-G1 content) induced by Ba lotus stamen extract in HCT-116 human colon cancer cells was evaluated using flow cytometry. ^{**}P<0.01 vs. the control group. Ba lotus, *Nelumbo nucifera*.

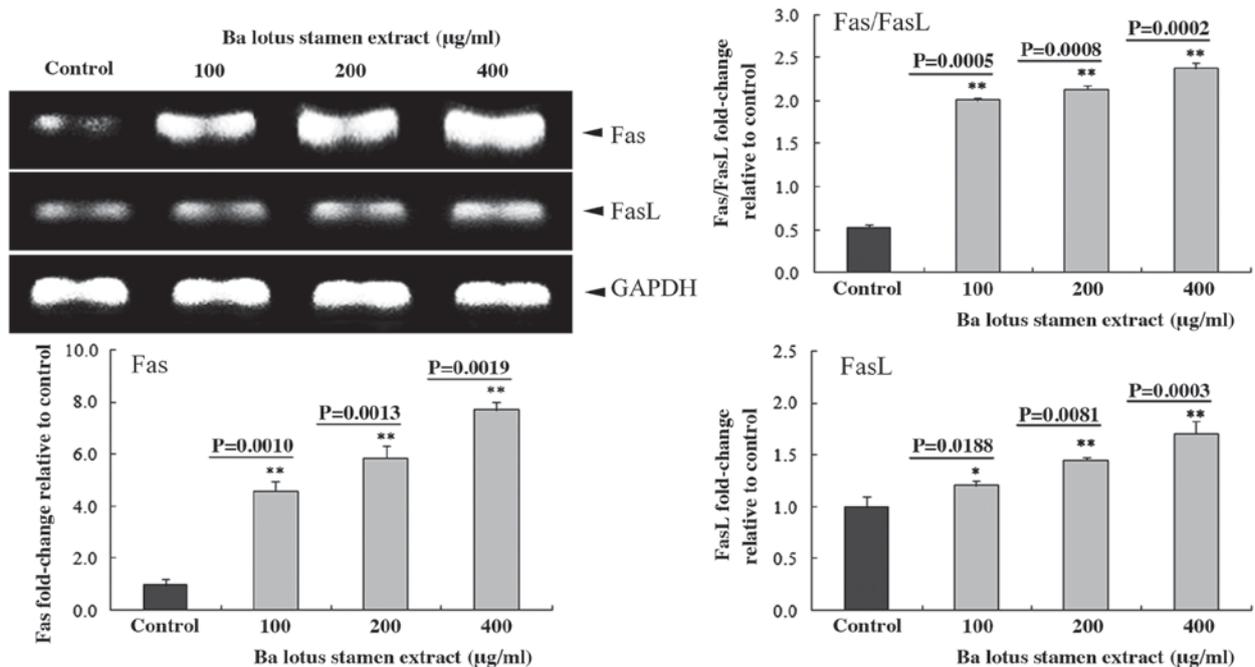


Figure 2. Effect of Ba lotus stamen extract on the mRNA expression of Fas/FasL, Fas and FasL in human colon cancer HCT-116 cells. The fold-ratio was calculated as follows: Gene expression/GAPDH \times control numerical value (control fold ratio, 1). ^{*}P<0.05, ^{**}P<0.01 vs. the control group. FasL, Fas ligand; Ba lotus, *Nelumbo nucifera*.

non-treated HCT-116 cells, respectively. By contrast, 400 $\mu\text{g/ml}$ of BLSEE also enhanced the Bax levels of mRNA (2.9-fold; P=0.0036) compared to non-treated HCT-116 cells.

BLSEE modulated the expression of matrix metalloproteinase (MMP)-2, MMP-9, TIMP metalloproteinase inhibitor 1 (TIMP-1) and TIMP metalloproteinase inhibitor 2 (TIMP-2)

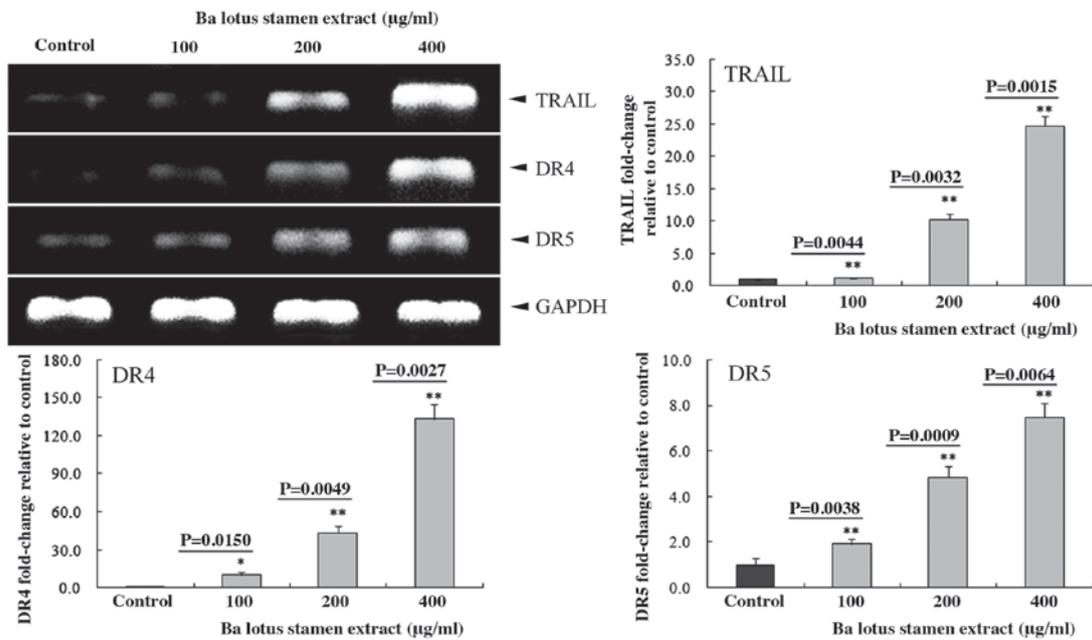


Figure 3. Effect of Ba lotus stamen extract on the mRNA expression of TRAIL, DR4 and DR5 in human colon cancer HCT-116 cells. The fold-ratio was calculated as follows: Gene expression/GAPDH x control numerical value (control fold ratio, 1). *P<0.05, **P<0.01 vs. the control group. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; DR4, death receptor 4; DR5, death receptor 5. Ba lotus, *Nelumbo nucifera*.

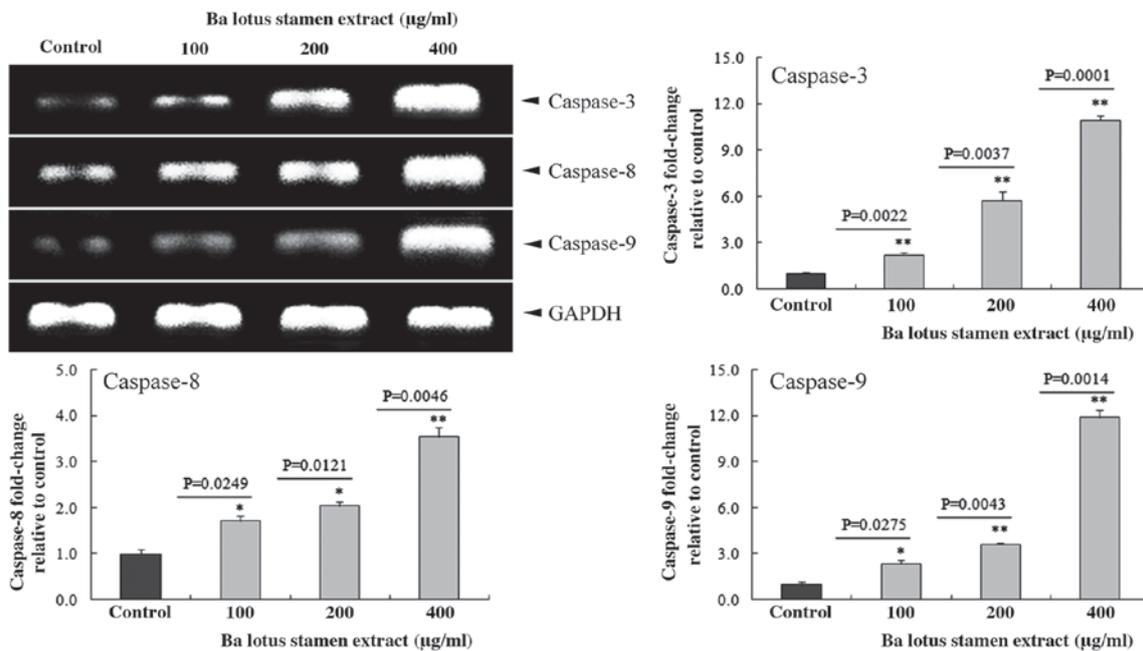


Figure 4. Effect of Ba lotus stamen extract on the mRNA expression of caspase-3, caspase-8 and caspase-9 in human colon cancer HCT-116 cells. The fold-ratio was calculated as follows: Gene expression/GAPDH x control numerical value (control fold ratio, 1). *P<0.05, **P<0.01 vs. the control group. Ba lotus, *Nelumbo nucifera*.

in HCT-116 cells. As shown in Fig. 6, BLSEE treatment dose-dependently reduced the expression of MMP-2 and MMP-9 in HCT-116 cells. At the highest dose of 400 µg/ml, BLSEE significantly reduced the mRNA levels of MMP-2 (95.8%; P=0.0002) and MMP-9 (85.9%; P=0.0002) compared with non-treated HCT-116 cells. By contrast, 400 µg/ml of BLSEE was also able to enhance the mRNA levels of TIMP-1 (17.6-fold; P=0.0004) and TIMP-2 (73.6-fold; P=0.0002) compared with non-treated HCT-116 cells.

BLSEE modulated the expression of inducible nitric oxide synthase (iNOS), cyclooxygenase 2 (COX-2), nuclear factor (NF)-κB and inhibitory κBα (IκBα) in HCT-116 cells. BLSEE treatment significantly decreased the mRNA levels of iNOS, COX-2 and NF-κB, and increased the IκBα levels in HCT-116 cells (Fig. 7). Subsequent to treatment with a dose of 400 µg/ml BLSEE, the mRNA levels of iNOS, COX-2 and NF-κB were decreased by 89.2 (P=0.0008), 88.3 (P=0.0002) and 87.9% (P=0.0007) compared with the non-treated HCT-116 cells.

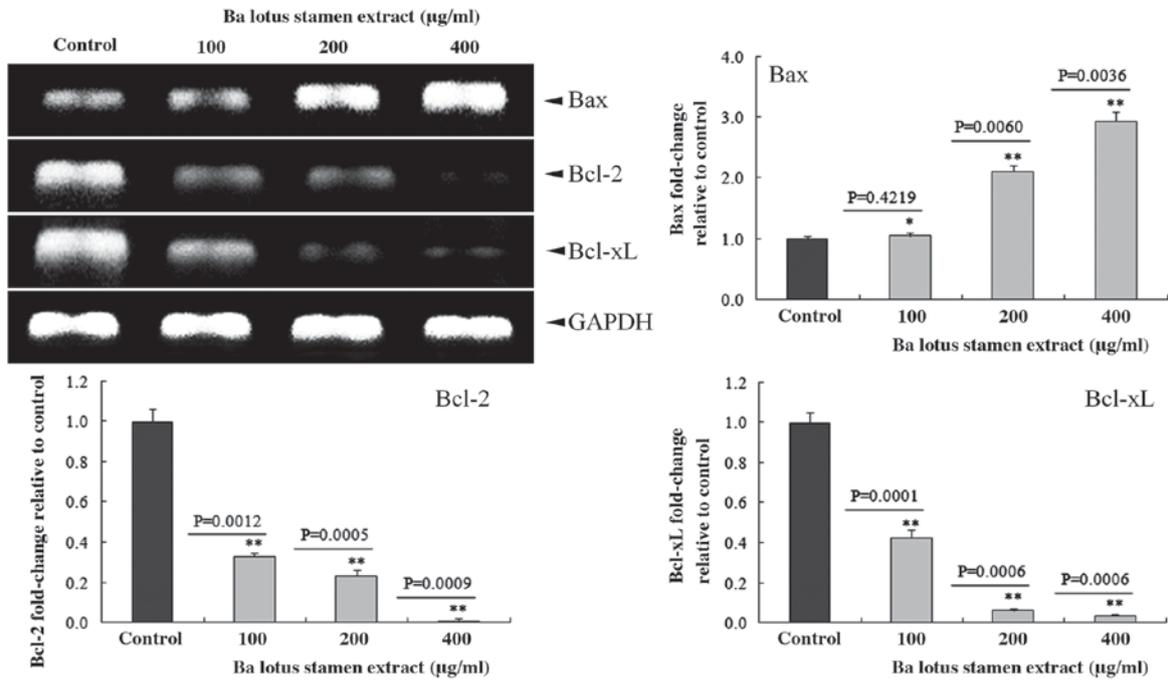


Figure 5. The effects of Ba lotus stamen extract on the mRNA expression of Bax, Bcl-2 and Bcl-xL in human colon cancer HCT-116 cells. The fold-ratio was calculated as follows: Gene expression/GAPDH x control numerical value (control fold ratio, 1). *P<0.05, **P<0.01 vs. the control group. Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X protein; Bcl-xL, Bcl-extra large; Ba lotus, *Nelumbo nucifera*.

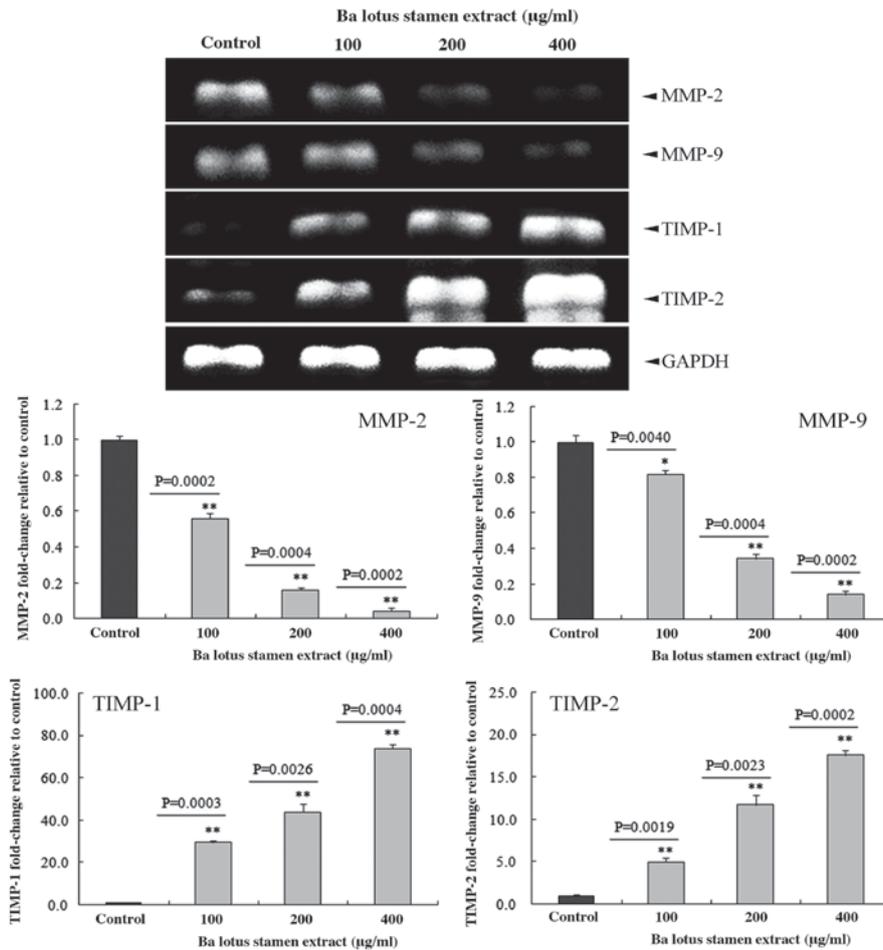


Figure 6. The effects of Ba lotus stamen extract on the mRNA expression of MMP-2, MMP-9, TIMP-1 and TIMP-2 in human colon cancer HCT-116 cells. The fold-ratio was calculated as follows: Gene expression/GAPDH x control numerical value (control fold ratio, 1). *P<0.05, **P<0.01 vs. the control group. MMP-2, matrix metalloproteinase-2; MMP-9, matrix metalloproteinase-9; TIMP-1, TIMP metalloproteinase inhibitor 1; TIMP-2, TIMP metalloproteinase inhibitor 2; Ba lotus, *Nelumbo nucifera*.

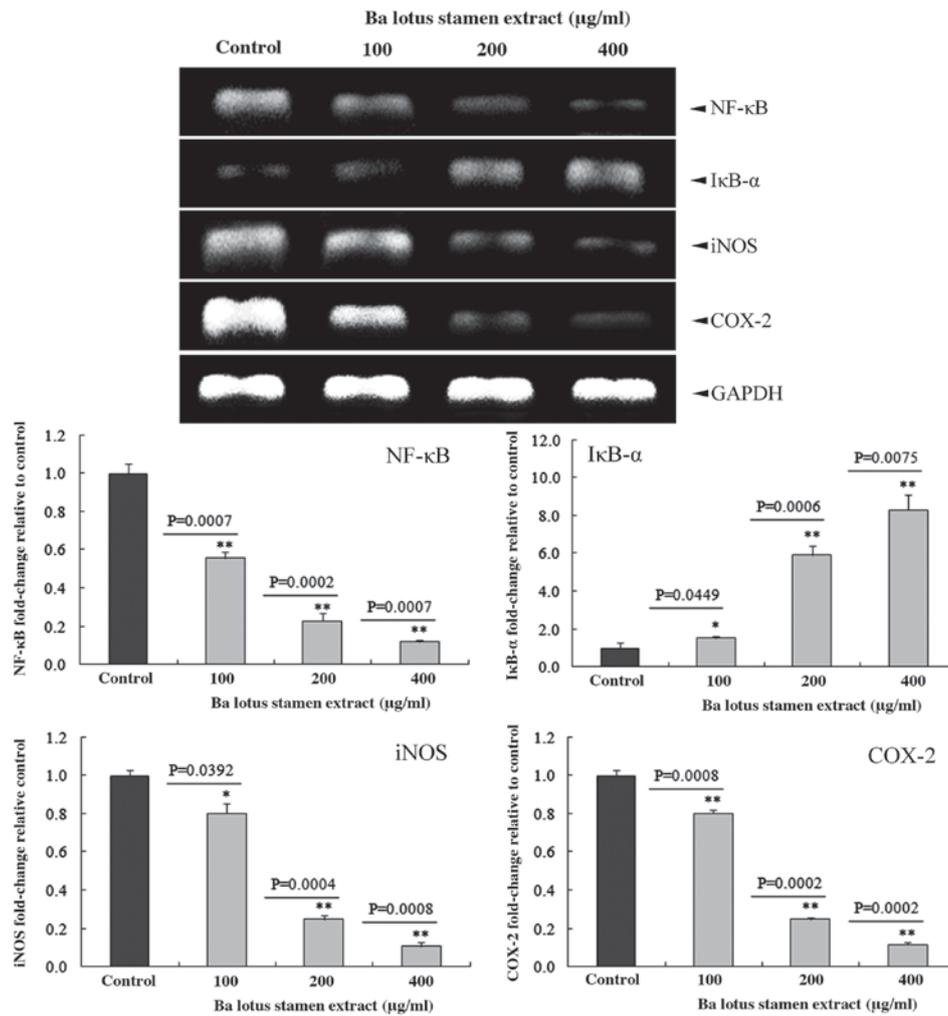


Figure 7. The effects of Ba lotus stamen extract on the mRNA expression of NF-κB, IκBα, iNOS and COX-2 in human colon cancer HCT-116 cells. The fold-ratio was calculated as follows: Gene expression/GAPDH x control numerical value (control fold ratio, 1). *P<0.05, **P<0.01 vs. the control group. NF-κB, nuclear factor-κB; IκBα, inhibitory κBα; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase 2; Ba lotus, *Nelumbo nucifera*.

In addition, BLSEE also increased the mRNA levels of IκBα (8.3-fold; P=0.0075) compared with non-treated HCT-116 cells.

Discussion

In the present study, BLSEE treatment was found to significantly inhibit the cell proliferation, and also increase the proportion of cells in the sub-G1 phase in HCT-116 cancer cells. These results suggested that BLSEE may induce apoptosis in HCT-116 cells.

Based on these observations, the mRNA expression of apoptosis-associated death receptors, including Fas and FasL, was analyzed in HCT-116 cells using an RT-PCR assay. Fas and FasL are apoptosis inducers, and play an important role during death receptor-induced extrinsic apoptosis in cells (25). The present study found that following incubation with 24 h of BLSEE, the mRNA levels of Fas and FasL were each significantly increased in HCT-116 cells. Activation of Fas/FasL was able to recruit FADD and death domain, and subsequently induce the activation of caspases 8, 9 and 10 to promote cellular apoptosis (26). In addition, the present study also observed that mRNA levels of TRAIL, DR4 and DR5 were increased by BLSEE treatment for 24 h.

TRAIL binds to TRAIL receptors, such as DR4 and DR5, to form a trimeric complex, which leads to the recruitment of FADD, an adaptor molecule that recruits and activates caspase-8 (27,28).

The caspase signaling cascade is a key event in extrinsic and intrinsic cell apoptosis, which is characterized by the activation of caspase-8 and caspase-9, respectively (9). Caspase-8 is a main initiator caspase in Fas signaling, and is recruited to the activated Fas receptor, as well as inducing death receptor-induced cell apoptosis (29). In addition, caspase-9 is an apoptosis effector molecule in the mitochondrial channel and starts programmed cell death subsequent to activation (10). Activated caspase-8 and caspase-9 are each able to activate caspase-3, which is an executor of apoptosis that subsequently induces apoptosis in cells (8). In the present study, it was found that the mRNA levels of caspases 3, 8 and 9 were significantly increased in BLSEE-treated HCT-116 cells.

It was also found that BLSEE treatment was able to modulate the mRNA expression of apoptosis-associated Bcl-2 family members in HCT-116 cells. BLSEE treatment significantly reduced the mRNA levels of anti-apoptotic Bcl-2 and Bcl-xL in HCT-116 cells. Bcl-2 and Bcl-xL are generally known as factors that prevent the release of Cyto c

from the mitochondria into the cytosol, inhibiting apoptosis and promoting the survival of cells (5). Bcl-xL can also block the formation of the apoptosome, which converges with Cyto *c*, Apaf-1 and procaspase-9, to reduce the activation of caspase-3 and inhibit apoptosis (30,31). The balance between anti-apoptosis and pro-apoptosis factors may affect the occurrence of apoptosis in cells, and is also associated with the success or failure of chemotherapy in cancer patients (32). By contrast, BLSEE was able to increase the mRNA levels of Bax, which is a pro-apoptotic protein, to promote apoptosis in cells (5,7). The activated Bax may be directly engaged by Bim to promote apoptosis in cells (33). In addition, Bax also induces the activation of caspase-8 and the release of Cyto *c* from the mitochondria, resulting in the cleavage of caspase-9, and contributes to the activation of executor caspase-3 (5,34). The present results suggest that the BLSEE may modulate the balance between anti-apoptosis and pro-apoptosis factors, in particular enhancing the expression levels of pro-apoptotic Bax to promote apoptosis in HCT-116 cells.

MMPs are an important family of proteolytic enzymes that not only contribute to organ development and tissue regeneration, but also show an extremely important role in the regulation of tumor invasion, angiogenesis and metastasis (35,36). In particular, MMP-2 exhibits a key role in the progression of colon cancer and the promotion of metastasis (37). Additionally, overexpression of MMP-9 is closely associated with poor prognosis in patients with colon cancer (38). Numerous studies have reported that herbs extracts were able to inhibit the migration or invasion of colorectal cancer cells by downregulating MMP-2 and MMP-9 *in vivo* (39-41). In the present study, it was found that BLSEE significantly reduced the expression of MMP-2 and MMP-9, and also increased the activation of TIMP-1 and TIMP-2 in HCT-116 colon cancer cells. As natural MMP inhibitors, TIMP-1 and TIMP-2 are able to inhibit the activation of MMP-2 and MMP-9 to reduce the invasion and metastasis in patients with colon cancer (42,43). Subsequent to treatment with BLSEE, the expression levels of TIMP-1 and TIMP-2 were significantly increased. Furthermore, maintaining an increased level of TIMP-1 and TIMP-2 may improve the poor survival of CRC patients (37,44).

Over activation of NF- κ B has been found in numerous human cancers (45,46). NF- κ B promotes cell proliferation, negatively regulates cell apoptosis (47), reduces cell death in tumorigenesis (48) and induces the inflammatory reaction in human disease (46,49). The activation of NF- κ B is able to reduce TNF- α -induced cell apoptosis (50). TNF- α directly activates the apoptosis inhibitor Bcl-xL (51), and suppresses anti-apoptosis factors, including inhibitor of apoptosis, caspase 8-FADD-like IL-1 β -converting enzyme inhibitory protein, TNF receptor associated factor (TRAF) 1 and TRAF2 to regulate the pro- or anti-apoptotic pathways (52). In the present study, BLSEE treatment significantly reduced the mRNA levels of NF- κ B, and also increased the mRNA levels of I κ B α in HCT-116 cells. Enhancement of the I κ B α activation is a potential strategy to reduce cancer cell growth in clinical chemotherapy (53,54).

In conclusion, the present study has clearly indicated that BLSEE suppresses the proliferation of human HCT-116 colon carcinoma cells *in vitro*. BLSEE may effectively induce the apoptosis through reducing the ratio of anti-apoptotic Bcl-2

to pro-apoptotic Bax, and increasing the activation of the death receptor system, as well as promoting the cleavage of caspases-3, 8 and 9 in HCT-116 cells. These results suggest that the BLSEE is able to induce HCT-116 cell apoptosis through activating death receptor and mitochondrial apoptotic pathways.

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