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Original Article

Alhagi-honey and its compound abnormal savda munziq (ASMq) inhibit proliferation and enhance apoptosis of human hepatoma BEL-7402 cells

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Abstract: Objective: To investigate the effects of alhagi-honey and abnormal savda munziq (ASMq) on the proliferation and apoptosis of human hepatoma BEL-7402 cells and the related molecular mechanisms. Method: Human hepatoma BEL-7402 cells were treated with alhagi-honey, ASMq, or 5-FU (positive control). Cell proliferation was assessed with the CCK-8 assay, and the cell cycle and cellular apoptotic process were detected with flow cytometry. The mRNA and protein expression levels of apoptosis-related proteins were determined with quantitative real-time PCR and Western blot analysis, respectively. Results: Both the treatments of alhagi-honey and ASMq significantly increased the inhibitory rates of cell proliferation in human hepatoma BEL-7402 cells, in a dose-dependent manner. Moreover, the treatments of alhagi-honey and ASMq induce the G0/G1 phase arrest of human hepatoma BEL-7402 cells. Furthermore, both the alhagi-honey and ASMq treatments significantly elevated the apoptosis rates of human hepatoma BEL-7402 cells. In addition, the treatments of alhagi-honey and ASMq significantly decreased the expression levels of anti-apoptotic proteins Bcl-2 and EGFR, while increased the expression level of pro-apoptotic protein Bax in human hepatoma BEL-7402 cells. Conclusion: Alhagi-honey and its compound ASMq could significantly inhibit the proliferation and enhance the apoptosis of human hepatoma BEL-7402 cells, which would be associated with the down-regulated Bcl-2 and EGFR expression levels and the up-regulated Bax expression level in these cells. These findings might provide evidence for the application of alhagi-honey and its compound ASMq in the treatment of hepatocellular carcinoma (HCC) in clinic.

Keywords: Alhagi-honey, abnormal savda munziq (ASMq), human hepatoma BEL-7402 cells, proliferation and apoptosis

Introduction

Hepatocellular carcinoma (HCC) is one of most common cancers, andthe third leading cause of cancer-induced death,throughout the world [1]. There are approximately one million newly-reported HCC cases each year, and 600 thousand subjects die of HCC in the world, making it a serious threat to human health [2]. Therefore, it is of great importance to discover and develop efficient therapeutic strategies for HCC in clinic.

Abnormal savda munziq (ASMq) is a commonly used clinical compound in Uyghur medicine. Recent studies have shown that, ASMq could effectively inhibit the tumor cells in liver cancer, lymphoma, and breast cancer [3]. As one of the single medicines in ASMq, saccharum alhagi accounts for a large proportion (about 42.3%) in the prescription. Alhagi-honey is the main component in saccharum alhagi. In recent years, alhagi-honey has also been shown to be able to exert potent anti-tumor effects, and has been widely used in the clinical treatment of malignant tumors. Alhagi-honey could stimulate the maturation, differentiation, and proliferation of immunocompetent cells, regulating the body immune system, which could phagocyte and clear the tumor cells [4].

In this study, the effects of alhagi-honey and its compound ASMq on the proliferation and apoptosis of human hepatoma BEL-7402 cells and the related molecular mechanisms were investigated. Human hepatoma BEL-7402 cells were

treated with alhagi-honey or ASMq, and their cell proliferation-inhibiting and apoptosis-enhancing effects were analyzed and compared.

Materials and methods

Cell line and cell culture

Human hepatoma BEL-7402 cell line was provided by the Cell Resource Center, Shanghai Institutes for Biological Sciences, CAS, Shanghai, China. These cells were cultured with the RPMI-1640 complete medium (Life Technologies, Carlsbad, CA, USA) containing 10% fetal bovine serum and 1 mL penicillin and streptomycin, in a 5% CO_2, 37°C incubator.

Cell counting kit-8 (CCK-8) assay

Cell proliferation was assessed with the CCK-8 assay (Beyotime, Haimen, Jiangsu, China). Human hepatoma BEL-7402 cells in the logarithmic growth phase were collected and planted onto a 96-well plate at the density of 5×10^4 cells/mL. After 48 h, the medium was discarded, and the cells were treated with alhagi-honey (Xinjiang Maidisen Uyghur Pharmaceutical Co., Ltd., Hetian, Xinjiang, China; which had been qualified by Prof. Palida Abuliziat Uyghur Medical College, Xinjiang Medical University) or ASMq (Xinjiang Ciconhabo Uyghur Pharmaceutical Co., Ltd., Urumqi, Xinjiang, China) at 0, 6, 8, 10, 12, 14, 16, and 20 mg/mL, respectively, for 48 h. The 5-FU was used as positive control, and the treatment concentrations were set at 0, 0.1, 0.5, 1, 5, 20, 40, and 80 μg/mL, respectively. After discarding the medium, the cells were treated with 10% CCK-8 at 37°C for 1.5 h. Then $OD_{450\ nm}$ was read with a microplate reader (Bio-Rad, Hercules, CA, USA). The IC_{50} values were obtained, and the inhibitory rates were calculated according to the following formulation: inhibitory rate = $(OD_{450\ nm\ control} - OD_{450\ nm\ treatment})/OD_{450\ nm\ control} \times 100\%$.

Flow cytometry

Cell cycle and cellular apoptotic process were detected with flow cytometry. Human hepatoma BEL-7402 cells were collected and planted onto the 25-cm^2 culture bottle at the density of 1×10^6 cells/bottle. After 24 h, the cells were treated with 8 mg/mL alhagi-honey, 8 mg/mL ASMq, and 0.6 μg/mL 5-FU, respectively, for 48 h. After washing with PBS, these cells were digested with trypsin and at 37°C. Then the cells were collected into a centrifuge tube, and subjected to centrifugation at 1000 rpm for 5 min. The cells were incubated with pre-chilled 75% ethanol at 4°C overnight. After centrifugation at 2500 rpm for 5 min, single cell suspension was prepared with 200-mesh grit. For the cell cycle detection, the cells were treated with 0.2 mg RNA enzyme and 10 μL propidium iodide (PI) at 4°C in dark for 30 min. The fluorescence was detected with a flow cytometer. For the apoptosis detection, the cell suspension was treated with 400 μL AnnexinV binding solution and 5 μL AnnexinV-FITC in dark for 15 min, and then 10 μL PI at 4°C in dark for 30 min, followed by flow cytometry.

Quantitative real-time PCR

The mRNA expression levels of Bcl-2, Bax, and EGFR in human hepatoma BEL-7402 cells were detected with quantitative real-time PCR. After drug administration for 48 h, the cells were collected and total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA, USA). The cDNA template was obtained with the reverse transcription kit (Taingen, Beijing, China). Quantitative real-time PCR was performed with the SYBR Select Master Mix (ABI, Grand Island, NY, USA) on the MyCycler thermal cycler (Bio-Rad, Hercules, CA, USA). The primer sequences were as follows: Bcl-2, forward 5'-GATAACGGAGGCTGGGATGC-3' and reverse 5'-TCACTTGTGGCCCAGATAGG-3'; Bax, forward 5'-CCCCCGAGAGGTCTTTTTCC-3' and reverse 5'-CTGATCAGTTCCGGCACCTT-3'; EGFR, forward 5'-TTGCCGCAAAGTGTGTAACG-3' and reverse 5'-AGTCACCCCTAAATGCCACC-3'; and β-actin, forward 5'-ATGATGATATCGCCGCGCTC-3' and reverse 5'-TCGATGGGGTACTTCAGGG-3'. The 20-μL PCR system consisted of 0.4 μL primer each, 10 μL SYBRR Select Master Mix (2 ×), 1 μL template, and 8.2 μL ddH_2O. The reaction condition was set as 50°C for 2 min, 95°C for 2 min, 95°C for 15 s, 60°C for 1 min, for totally 40 cycles.

Western blot analysis

The protein expression levels of Bcl-2, Bax, and EGFR were detected with Western blot analysis. After drug administration for 48 h, the cells were lysed with RIPA buffer. Protein concentration was determined with the BCA method (Tiangen). 20 μg protein sample was subjected

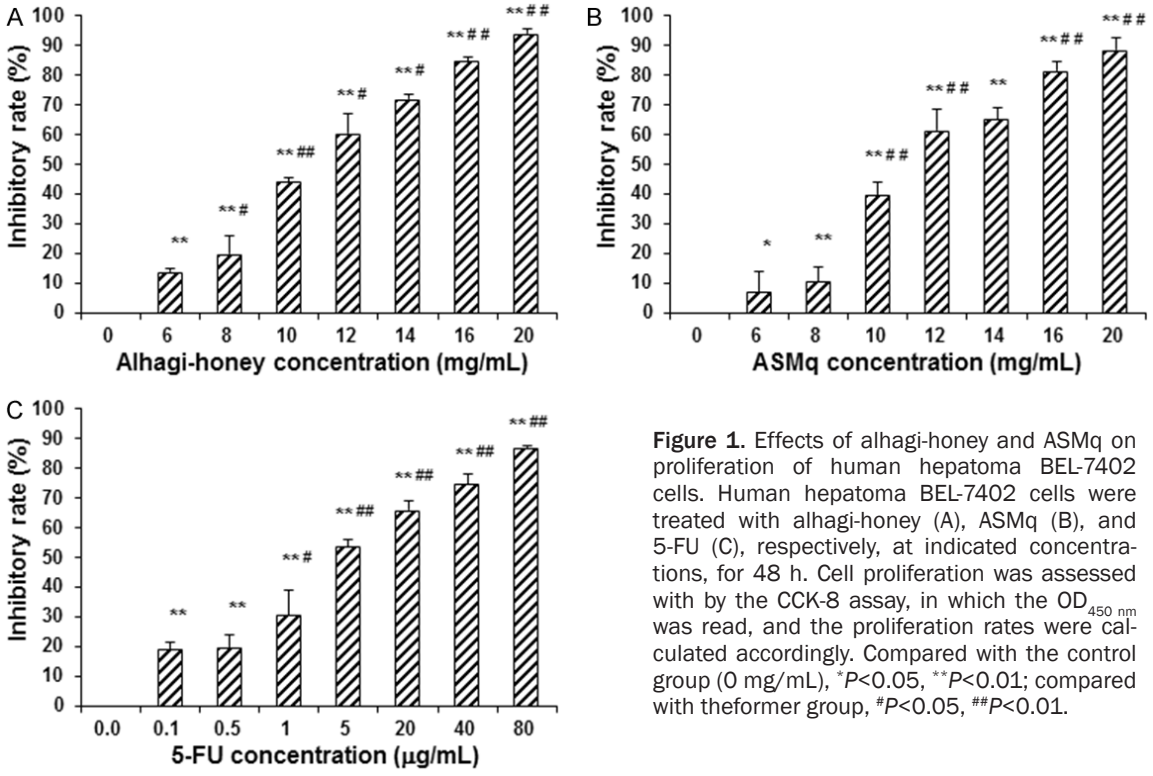

Figure 1. Effects of alhagi-honey and ASMq on proliferation of human hepatoma BEL-7402 cells. Human hepatoma BEL-7402 cells were treated with alhagi-honey (A), ASMq (B), and 5-FU (C), respectively, at indicated concentrations, for 48 h. Cell proliferation was assessed with by the CCK-8 assay, in which the $OD_{450\ nm}$ was read, and the proliferation rates were calculated accordingly. Compared with the control group (0 mg/mL), $^{*}P<0.05$, $^{**}P<0.01$; compared with theformer group, $^{\#}P<0.05$, $^{\#\#}P<0.01$.

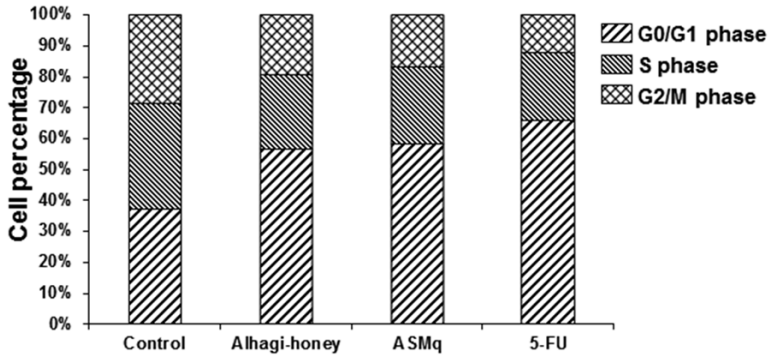

Figure 2. Effects of alhagi-honey and ASMq on cell cycle of human hepatoma BEL-7402 cells. Human hepatoma BEL-7402 cells were treated with 8 mg/mL alhagi-honey, 8 mg/mL ASMq, and 6 mg/mL 5-FU (positive control), respectively, for 48 h. Then cell cycle was detected with flow cytometry.

to SDS-PAGE, and then electronically transferred onto a PVDF membrane. After blocking with 5% non-fat milk for 1 h, the membrane was incubated with anti-Bcl-2 primary antibody (1:200 dilution; Abcam, Cambridge, MA, USA), anti-Bax primary antibody (1:500 dilution; Abcam), anti-EGFR primary antibody (1:500 dilution; Abcam), or anti-β-actin primary antibody (1:2000 dilution; Abcam), at 4°C overnight. Then the membrane was treated with goat anti-mouse IgG (Pierce, Rockford, IL, USA) at room temperature for 1 h. After incubated with colorization solution A and B, the chemiluminescence was detected with the ChemiScope mini-imaging system.

Statistical analysis

Data were expressed as mean ± SD. SPSS 19.0 software was used for statistical analysis. ANOVA was performed for multiple comparisons, with LSD and Dunnett's T tests. $P<0.05$ was considered as statistically significant.

Results

Effects of alhagi-honey and ASMq on proliferation of human hepatoma BEL-7402 cells

To investigate the effects of alhagi-honey and its compound ASMq on the proliferation of human hepatoma BEL-7402 cells, these cells were first treated with alhagi-honey (0, 6, 8, 10, 12, 14, 16, and 20 mg/mL), ASMq (0, 6, 8, 10, 12, 14, 16, and 20 mg/mL), and 5-FU (0, 0.1,

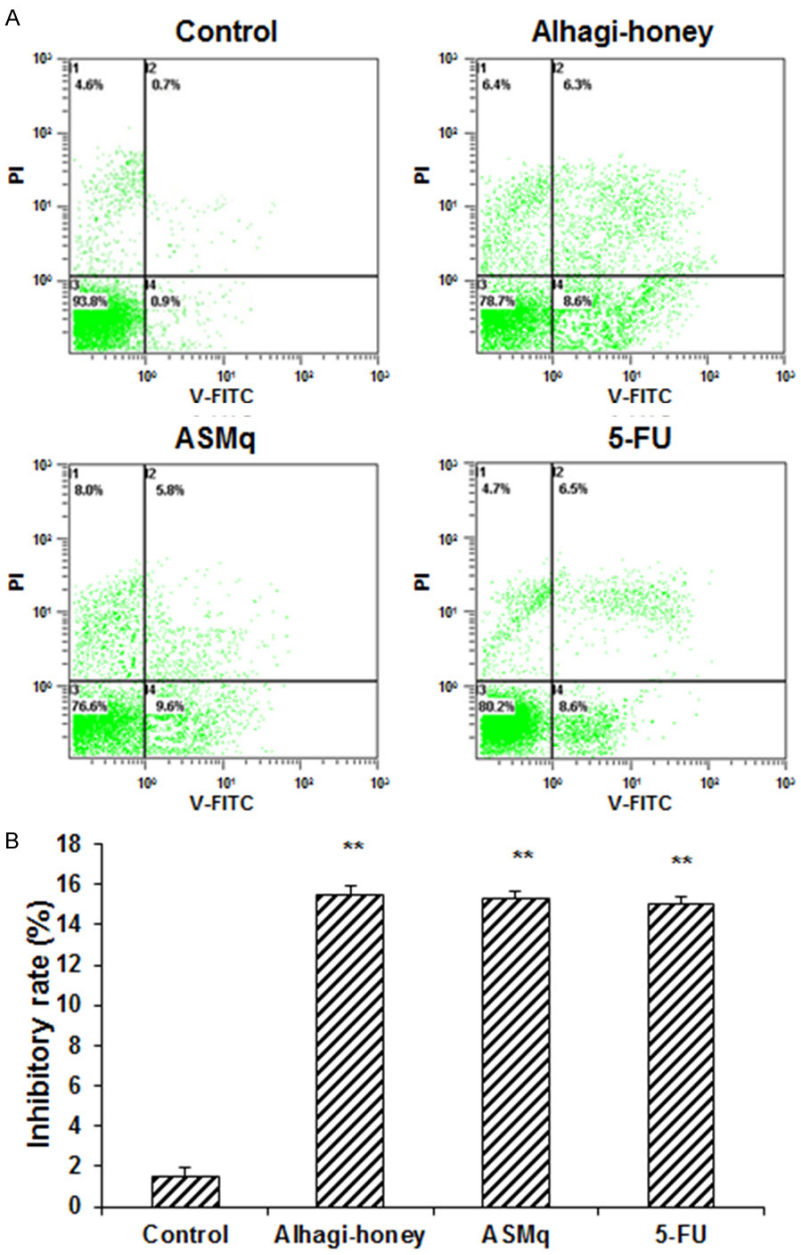

Figure 3. Effects of alhagi-honey and ASMq on apoptosis of human hepatoma BEL-7402 cells. Human hepatoma BEL-7402 cells were treated with 8 mg/mL alhagi-honey, 8 mg/mL ASMq, and 6 mg/mL 5-FU (positive control), respectively, for 48 h. A. Cellular apoptotic process was detected using AnnexinV-FITC/PI staining by flow cytometry. B. Statistical analysis of apoptosis rates of human hepatoma BEL-7402 cells. Compared with the control group, $^{*}P<0.05$, $^{**}P<0.01$.

0.5, 1, 5, 20, 40, and 80 μg/mL), respectively, for 48 h, and the cell proliferation was assessed with the CCK-8 assay. Our results showed that, the IC_{50} values for alhagi-honey, ASMq, and 5-Fu treatments were 10.75 mg/mL, 11.30 mg/mL and 4.55 μg/mL respectively. For the treatments of alhagi-honey and ASMq, the inhibitory rates of cell proliferation of human hepatoma BEL-7402 cells were significantly increased with the increasing treatment concentrations ($P<0.01$) (**Figure 1**). These results suggest that, the treatments of alhagi-honey and ASMq could significantly inhibit the proliferation of human hepatoma BEL-7402 cells, in a dose-dependent manner.

Effects of alhagi-honey and ASMq on cell cycle of human hepatoma BEL-7402 cells

To investigate the effects of alhagi-honey and ASMq on the cell cycle of human hepatoma BEL-7402 cells, flow cytometry was performed. As shown in **Figure 2**, after treated with 8 mg/mL alhagi-honey for 48 h, the G0/G1 cell proportion was significantly elevated ($P<0.05$), while the proportions of S and G2/M cells were significantly declined (both $P<0.05$). Similar results were obtained for the ASMq treatment, which significantly increased the G0/G1 cell proportion and decreased the S and G2/M cell proportions in human hepatoma BEL-7402 cells ($P<0.05$). These results suggest that, the treatments of alhagi-honey and ASMq could induce the G0/G1 phase arrest in human hepatoma BEL-7402 cells.

Effects of alhagi-honey and ASMq on apoptosis of human hepatoma BEL-7402 cells

To investigate the effects of alhagi-honey and ASMq on the apoptotic process of human hepatoma BEL-7402 cells, the AnnexinV-FITC/PI staining was performed, and the fluorescence was detected with flow cytometry. As shown in

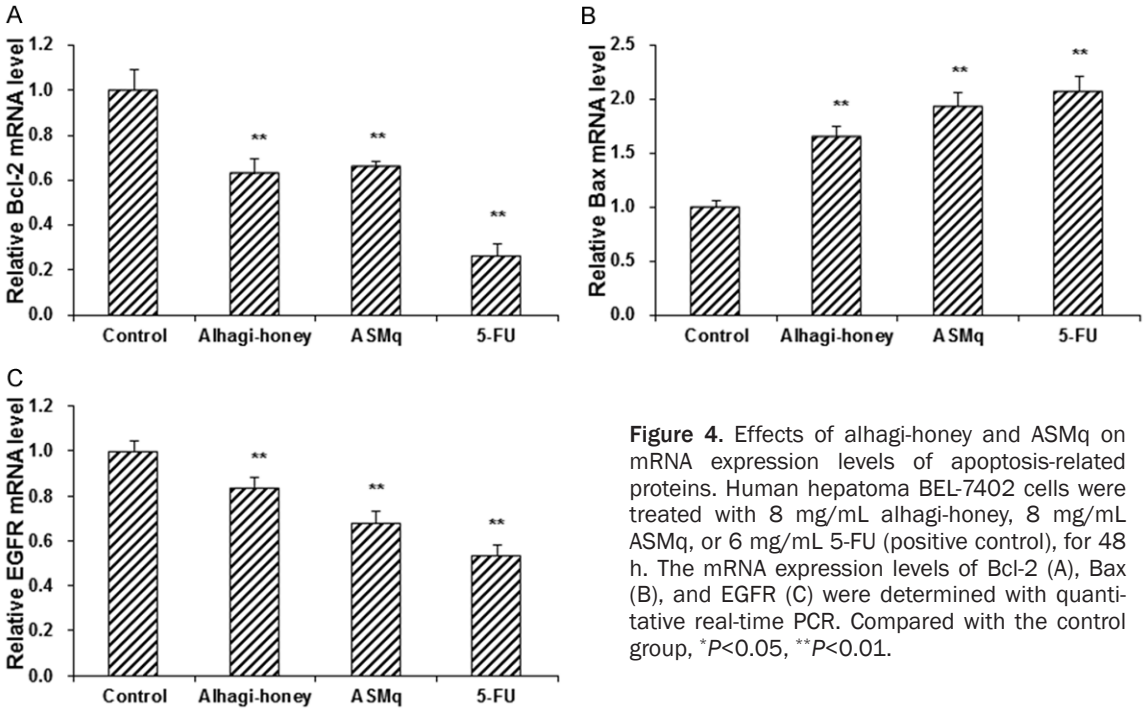

Figure 4. Effects of alhagi-honey and ASMq on mRNA expression levels of apoptosis-related proteins. Human hepatoma BEL-7402 cells were treated with 8 mg/mL alhagi-honey, 8 mg/mL ASMq, or 6 mg/mL 5-FU (positive control), for 48 h. The mRNA expression levels of Bcl-2 (A), Bax (B), and EGFR (C) were determined with quantitative real-time PCR. Compared with the control group, $^{*}P<0.05$, $^{**}P<0.01$.

Figure 3, the apoptosis rate for the control group was 1.60±1.00%, which was significantly elevated by the treatments of alhagi-honey and ASMq, to 14.97±0.15% and 15.07±0.29%, respectively (both $P<0.05$). These results suggest that, the treatments of alhagi-honey and ASMq could significantly enhance the apoptosis of human hepatoma BEL-7402 cells.

Effects of alhagi-honey and ASMq on expression of apoptosis-related proteins

The effects of alhagi-honey and ASMq on expression of apoptosis-related proteins in human hepatoma BEL-7402 cells were next investigated. These cells were first treated with 8 mg/mL alhagi-honey or ASMq for 48 h, and the mRNA and protein expression levels of Bcl-2, Bax, and EGFR were determined with quantitative real-time PCR and Western blot analysis, respectively. Our results from the quantitative real-time PCR showed that, compared with the control group, the mRNA expression levels of Bcl-2 and EGFR were significantly declined, while the mRNA expression level of Bax was significantly elevated, by the treatments of alhagi-honey and ASMq, in human hepatoma BEL-7402 cells ($P<0.05$) (**Figure 4**). Similar results were observed for the Western blot analysis. Compared with the control group, the treatments of alhagi-honey and ASMq significantly decreased the expression levels of anti-apoptotic proteins Bcl-2 and EGFR, while increased the expression level of pro-apoptotic protein Bax in human hepatoma BEL-7402 cells ($P<0.05$) (**Figure 5**). These results suggest that, the treatments of alhagi-honey and ASMq could significantly change the expression levels of apoptosis-related proteins in human hepatoma BEL-7402 cells, which was in line with its apoptosis-inducing effects.

Discussion

Modern researches have confirmed that ASMq could exert diuretic, anti-inflammatory, and detoxication functions in clinic, with negligible-side effects. At present, the investigation and application of ASMq are no longer limited to the treatment of diabetes and cardiovascular diseases. The anti-tumor activity of ASMq has been attracting more and more attention, especially concerning its apoptosis-inducing effects in tumor cells. Wang *et al.* [5] have found that, ASMq could down-regulate the expression of p53 in cervical cancer cells, thus promoting the cellular apoptotic process. Moreover, Yusup *et al.* [6] have shown that, the apoptosis-promoting effects of ASMq in human hepatoma cells might be associated with the down-regulated

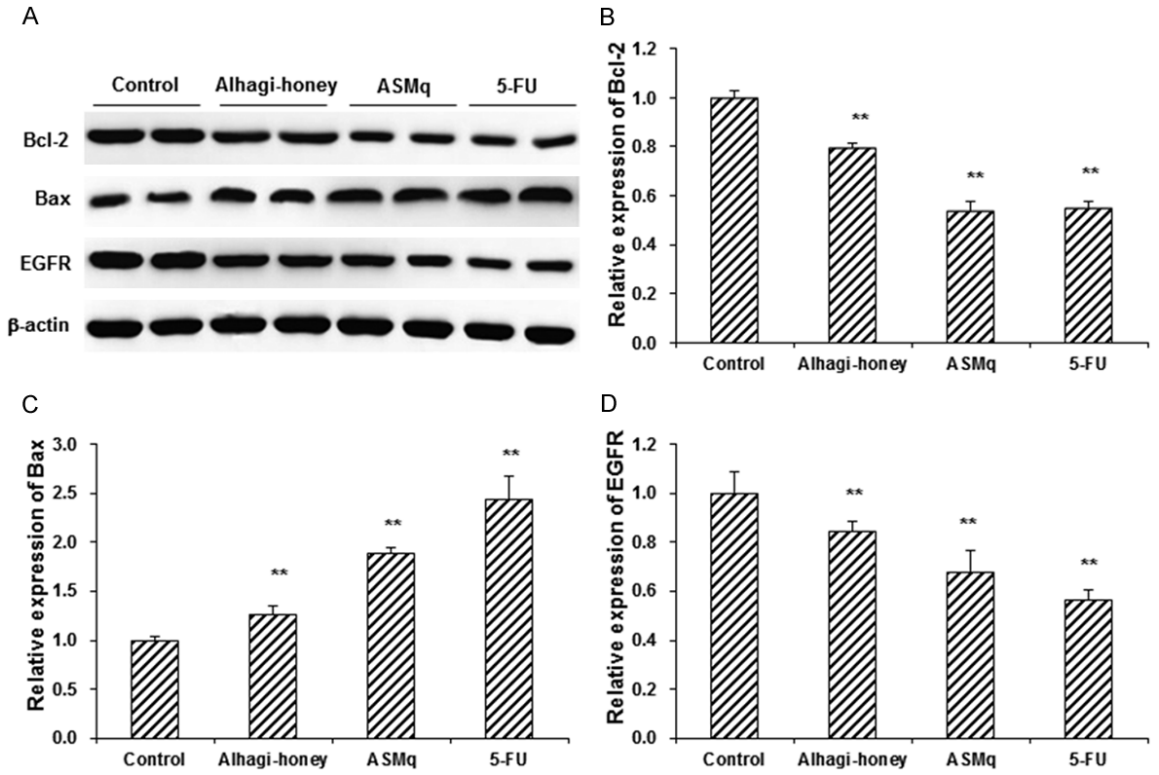

Figure 5. Effects of alhagi-honey and ASMq on apoptosis-related protein expression. Human hepatoma BEL-7402 cells were treated with 8 mg/mL alhagi-honey, 8 mg/mL ASMq, or 6 mg/mL 5-FU (positive control), for 48 h. (A) The protein expression levels of Bcl-2, Bax, and EGFR were detected with Western blot analysis. (B-D) Statistical analysis of protein expression levels of Bcl-2 (B), Bax (C), and EGFR (D). Compared with the control group, $^{*}P<0.05$, $^{**}P<0.01$.

Bcl-2 mRNA expression level and the up-regulated mRNA expression levels of p53, p21, and Bax. As one of the single medicines in ASMq, saccharum alhagi accounts for about 42.3% in the prescription, and alhagi-honey is the main component in saccharum alhagi [7]. Numerous studies have demonstrated that polysaccharides could exert potent anti-tumor effects. Polysaccharides do not directly kill the tumor cells, but they work as highly efficientimmunomodulators in the body. They stimulate the maturation, differentiation, and proliferation of immunocompetent cells, and recover the balance of immune system, which could phagocyte and clear the tumor cells [8-15]. Therefore, polysaccharides have been widely used in the clinical treatment of malignant tumors nowadays [16-19].

Due to the complex structure of polysaccharides, the investigation of their molecular mechanism and structure-activity relationship has become a research hotspot in recent years. In this study, the anti-tumor effects of both alhagi-honey and its compound ASMq have been analyzed and compared. The effects of alhagi-honey and ASMq on the proliferation and apoptosis of human hepatoma BEL-7402 cells were investigated with the CCK-8 assay, flow cytometry, quantitative real-time PCR, and Western blot analysis, respectively.

In this study, our results from the CCK-8 assay showed that, the treatments of alhagi-honey and ASMq could significantly inhibit the proliferation of human hepatoma BEL-7402 cells, in a dose-dependent manner. Moreover, when the cell cycle was detected with flow cytometry, our results showed that, the alhagi-honey and ASMq treatments could significantly elevate the cell proportion in the G0/G1 phase, and decline the cell proportion in the S and G2/M phases. These results suggest that the treatments of alhagi-honey and ASMq could induce G0/G1 phase arrest in human hepatoma BEL-7402 cells, indicating significantly inhibited cell proliferation.

Induction of apoptosis in human hepatoma BEL-7402 cells is an important therapeutic method for the disease in clinic. In this study, the cellular apoptotic process was detected using the AnnexinV-FITC/PI staining by flow cytometry. Our results showed that, when these cells were treated with alhagi-honey and ASMq for 48 h, the apoptosis rate would be significantly elevated. The expression levels of Bcl-2 and Bax could reflect the cellular apoptotic process [20, 21]. Bcl-2 family is well known for the anti-apoptotic properties, which could prevent and block cell shrinking, DNA cleavage, and chromatin shrinkage, thereby inhibiting apoptosis. However, Bax in the cytoplasm would bind to the mitochondrial membrane and increasethe membrane permeability, thus inducingapoptosis. Studies have shown that, the interaction between Bcl-2 and Bax could promote the apoptotic process. In this study, our results from the quantitative real-time PCR and Western blot analysis showed that, the treatments of alhagi-honey and ASMq could significantly decline the Bcl-2 expression and elevate the Bax expression in human hepatoma BEL-7402 cells. On the other hand, EGFR is a common receptor involved in the cellular proliferation and signal transduction, whose mutation and/or over-expression might lead to tumorigenesis. In this study, our results showed that, the treatments of alhagi-honey and ASMq could significantly decrease the expression level of EGFR in human hepatoma BEL-7402 cells, which might contribute to the apoptosis-enhancing effects of alhagi-honey and ASMq.

In conclusion, our results showed that, the treatments of alhagi-honey and ASMq could significantly inhibit the proliferation and enhance the apoptosis of human hepatoma BEL-7402 cells. Moreover, the treatments of alhagi-honey and ASMq could induce G0/G1 phase arrest in these cells. Furthermore, the treatments of alhagi-honey and ASMq significantly down-regulated Bcl-2 and EGFR expression levels and up-regulated Bax expression level in human hepatoma BEL-7402 cells. These findings might provide evidence for the application of alhagi-honey and its compound ASMq in the treatment of HCC in clinic.

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Disclosure of conflict of interest

None.

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