RESEARCH ARTICLE

Alkaloids from Beach Spider Lily (*Hymenocallis littoralis*) Induce Apoptosis of HepG-2 Cells by the Fas-signaling Pathway

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Abstract

Alkaloids are the most extensively featured compounds of natural anti-tumor herbs, which have attracted much attention in pharmaceutical research. In our previous studies, a mixture of major three alkaloid components (5, 6-dihydrobicolorine, 7-deoxy-trans-dihydronarciclasine, littoraline) from *Hymenocallis littoralis* were extracted, analyzed and designated as AHL. In this paper, AHL extracts were added to human liver hepatocellular cells HepG-2, human gastric cancer cell SGC-7901, human breast adenocarcinoma cell MCF-7 and human umbilical vein endothelial cell EVC-304, to screen one or more AHL-sensitive tumor cell. Among these cells, HepG-2 was the most sensitive to AHL treatment, a very low dose (0.8μg/ml) significantly inhibiting proliferation . The non-tumor cell EVC-304, however, was not apparently affected. Effect of AHL on HepG-2 cells was then explored. We found that the AHL could cause HepG-2 cycle arrest at G2/M checkpoint, induce apoptosis, and interrupt polymerization of microtubules. In addition, expression of two cell cycle-regulated proteins, CyclinB1 and CDK1, was up-regulated upon AHL treatment. Up-regulation of the Fas, Fas ligand, Caspase-8 and Caspase-3 was observed as well, which might imply roles for the Fas/FsaL signaling pathway in the AHL-induced apoptosis of HepG-2 cells.

Keywords: Beach spider lily - AHL - HepG-2 - apoptosis - cell arrest - cell cycle-regulated protein - Fas signaling

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Introduction

Natural herbs products (mainly herbal extract formulations) with anti-tumor effects have attracted much attention in pharmaceutical research. There are a number of herbs that have shown to have the abilities to induce apoptosis of tumor cells and play important roles in cancer prevention. Genistein, daidzein and isoflavonoids in soybean are thought to play an important role in breast cancer prevention (Wang et al., 2002). CKBM and Genistein were found to induce G2/M cell cycle arrest in MCF-7 cells (Chen et al., 2003; Luk et al., 2005). Schisandra chinensis has the ability to reduce prostate cancer cell growth and induce apoptosis by inhibiting androgen receptor expression (Hsieh et al., 2002). Shin et al. found that Panax ginseng and Ziziphus jujube could stimulate activation of macrophages and induce cytokine release (Shin et al., 2002). Moreover, Lee et al. found that soluble β-glucans exacted from *Saccharomyces cerevisiae* could activate macrophage activity and stimulate high-level TNF-α secretion (Lee et al., 2002), which may implies its pro-apoptotic effect on human cancer cells (Gillio et al., 1996).

To date, the most extensively characterized active compounds of these herds are alkaloids. Alkaloids are a highly diverse group of compounds that contain a ring structure and a nitrogen atom, in most case, the nitrogen atom is located inside the heterocyclic ring structure. Certain alkaloids from varieties of natural herbs have demonstrated potent *in vitro* cytotoxicity against cancer cells and potent *in vivo* antitumor activity (Kekre et al., 2005; Mclachlan et al., 2005; Griffin et al., 2010; Lu et al., 2012). Two alkaloids, camptothecin (CPT) and vinblastine have already been successfully developed into anticancer drugs for their clinical applications against cancer and HIV-I (El-Galley et al., 2003).

More than 19 alkaloids have been extracted and identified in *Hymenocallis Littoralis* until now, some of which showed anti-tumor activity. They inhibit cancer cell lines by variety of approaches, such as, promoting cell apoptosis, inhibiting tumor cell invasion and metastasis, reducing the proliferative activity of drug resistant tumor cell lines, etc. Some recent insights regarding the mode of action of pancratistatin (PST) include inhibition of the cell cycle from G0/G1 to S phase and powerful antiparasite activity (Kekre et al., 2005; Mclachlan et al.,

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2005; Griffin et al., 2010). Narciclasine, lycoricidine and PST, especially PST from Spider Lily, display selective potent toxicity against human tumor cells. Another distinct feature of PST as an antitumor monomer is its ability to induce apoptosis in tumor cells via mitochondrial pathways, while leaving the normal cells unaffected (McLachlan et al., 2005; Ingrassia et al., 2008). In our previous studies, a mixture of alkaloids were extracted by n-butanol extraction method and identified by LS-MS, and designated as AHL. AHL comprises of three major components [5, 6-dihydrobicolorine (41.37%), 7-deoxy-trans-dihydronarciclasine (27.65%), littoraline (18.52%)]. In this paper, the anti-tumor effect and its preliminary mechanism of this alkaloids mixture AHL were studied. Fully understanding of the effect and mechanism of this mixture on tumor cells will help to explore the possibilities of developing these alkaloids with high efficiency and low toxicity features as antitumor drugs.

Materials and Methods

Alkaloids AHL preparation and cell culture

AHL extract was prepared by n-butanol extraction method, Briefly, bulbs of Beach Spider Lily (*Hymenocallis Littoralis*) (1kg, wet weight) were soaked in 3L methanol/dichloromethane (1:1, v/v) mixture for overnight, followed by ultrasonic extraction. The extract was filtrated into separatory funnel; the upper phase was then concentrated in vacuum and extracted by n-butanol. Then the concentrates were dissolved in methanol and acetone, alkaloids from *H. littoralis* (AHL) was obtained when the final solution was evaporated just to dryness; Hydroxycamptothecin (HCPT) was a commercial product from Harbin Medisan Pharmaceutical Co., Ltd; Human liver hepatocellular cells HepG2, human gastric cancer cell line SGC-7901, human breast adenocarcinoma cell line MCF-7 and human umbilical vein endothelial cell line EVC-304 were maintained in Dulbecco's minimum essential medium (DMEM; Invitrogen, Life Technologies, MA, USA) or RPMI1640 medium (Invitrogen, Life Technologies, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Life Technologies, MA, USA), 2mM L-glutamine, 100units/ml penicillin and 100μg/ml streptomycin at 5% CO_2, 37°C.

Cell viability assay

Viability of cells was determined by the MTT assay (Sun et al., 2014), briefly, HepG2, SGC-7901, MCF-7 and EVC-304 cells were seeded in a 96-well flat bottom plate (Corning, NY, USA) at a density of 1×10^4 cells/well respectively. 12-18h later, 20μl AHL was added in each well to reach a final concentration of 0.8μg/ml, 3.2μg/ml , 12.5μg/ml, 25.0μg/ml, and 50.0μg/ml respectively and incubated for another 48 h. HCPT were added as positive control and growth medium as negative control respectively. Cells were then treated with 0.5 mg/ml freshly-made 3-[4, 5-dimethyl-2-thiazolyl]-2, 5 diphenyltetrazolium bromide (MTT; Sigma-Aldrich, MA, USA) for 4h, resuspended in 150μl Dimethyl sulfoxide (DMSO; Sigma-Aldrich, MA, USA) followed by agitation, read optical density at 560nm and subtract background at 670nm. Inhibitory concentration (IC) of AHL (IC_{50}) was calculated according to following formula: percent of inhibitory concentration= (OD value of control group-OD value of AHL treated group)/ OD value of control group×100%.

Cell proliferation assay (SRB)

Four types of cells (HepG2, SGC-7901, MCF-7 and EVC-304) were seeded in 96-well plates (Corning, NY, USA) at approx. 60% confluence and allowed to attach for 24 h. Different concentrations of AHL were added in each well incubated for 24h, 48h and 72h at 37° C. After incubation, cells were treated for 30 min with 0.4% (w/v) sulforhodamine blue (SRB; Sigma-Aldrich, MA, USA) dissolved in 1% acetic acid. Protein-bound dye was extracted with unbuffered 10mM Tris base. Absorbance was measured at 490 nm using a 96-well microtiter plate reader. Growth inhibition (GI50) was calculated accordingly.

Annexin V-FITC/Propidium Iodide and Hoechst 33258 Staining

HepG2 cells in logarithmic growth phase were seed in 6-well plates and allowed to attach overnight. AHL was added to the wells in a volume of 2.0 ml per well to a final concentration of 2.0μg/ml, 4.0μg/ml and 8.0μg/ml . HCPT was also added to the wells to rich a final concentration of 10.0μg/ml as positive control. 24 h later, cells were transferred to a staining tube and washed with 4 ml of 4°C PBS containing 1% (v/v) FBS (Gibco, Life Technologies, MA, USA), followed by centrifugation for 10 minutes at 1 000× g at 4°C. 100μl of 2μg/ml Annexin V-FITC (Beyotime, Jiangsu, China) in Annexin V-binding buffer was added to the cells and the staining tubes were incubated for 10 minutes on ice in the dark. Propidium Iodide (PI) single color control cells were treated with 100ul of Annexin V-binding buffer alone. After adjusting the total volume of each tube to 0.5ml with annexin V-binding buffer, 1μg per tube of PI (Sigma) was added. The cells were analyzed within 20 minutes by fluorescence microscopy.

We also detected apoptotic cells by microscopy with cell permeable Hoechst 33258 dye (Sigma-Aldrich, MA, USA). Briefly, cell were AHL treated or mock treated for 24h, cells were then washes twice with PBS, fixed in PBS, 4% paraformaldehyde (Sigma-Aldrich, MA, USA), 0.5% Triton X-100 for 20 min at room temperature and stained for 10 min with 200ng/ml Hoescht in PBS before mounting. Representative images of typical apoptotic cells were captured on an inverted fluorescence microscope (Leica, Mannheim, Germany).

Flow cytometry

For cell cycle distribution analysis, HepG-2 and EVC-304 cells were first treated with 0.8μg/ml, 2.0μg/ml, 4.0μg/ml and 8.0μg/ml AHL respectively, 48h later, cells were fixed with 70% ethanol followed by permeabilizing with 0.5% TritonX-100. Fluorescence-activated cell sorting (FACS) was performed using 20μg/ml propidiun iodide (PI) staining and 10μg/ml RNase (Sigma-Aldrich, MA, USA) treatment to reduce background staining. The cells

were then gently vortex and incubated for 15 min at RT in the dark. Cell cycle distribution was then analyzed with flow cytometry (Beckman Coulter, USA) within 1 h. Cells treated with 10.0mg/L HCPT were considered as parallel controls.

Western blotting

Cells were treated with AHL at final concentration 2.0μg/ml, 4.0μg/ml and 8.0μg/ml. At different time points (6h, 24h, 48h and 72h), cells were washed twice with PBS then treated with cell lysis buffer (50mM Tris-HCl pH 8.0, 150mM NaCl, 1% Triton X-100, 100μg/ml phenylmethanesulfonyl fluoride) for 20 min. After centrifugation at 4°C, cell lysates were mixed with 5×SDS sample buffer (250mM Tris-HCl pH 6.8, 10% SDS, 5% β-mercaptoethanol, 50% glycerol, 0.05% bromophenol blue), boiled for 5 min and then analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Fractionated proteins were electrotransferred onto a nitrocellulose membrane (Millipore, MA, USA), after which the membrane was incubated in blocking solution (0.1% Tween-20 and 5% nonfat dry milk in PBS) at 4°C overnight, followed by reaction for 1 h at room temperature with mouse anti cyclin B1 monoclonal antibodies (Santa Cruz Biotechnology, CA, USA) and anti-β actin monoclonal antibodies (Sigma-Aldrich, MA, USA) in appropriate dilution (1:200 for anti cyclin B1 and 1:5, 000 for anti-β actin), washed extensively in PBST (0.1% Tween-20 in PBS) followed by reaction for 2 h with 1:5000 diluted horseradish peroxidase-conjugated immunoglobulin (Sigma-Aldrich, MA, USA). After several washes in PBST, the bands were visualized by incubating the membrane in freshly prepared DAB solution (2.5 mg of 3, 3-diaminobenzidine tetrahydrochloride in 10 ml of Tris-HCl buffer, pH 7.5) containing 0.03% H_2O_2.

Immunofluorescence

HepG-2 and EVC-304 cells were grown on Fluorodishes (WPI, FL, USA.) and were treated with AHL at final concentration of 0.825μg/ml, 1.65μg/ml and 3.3μg/ml. 48h later, cells were fixed with 4% paraformaldehyde (Sigma-Aldrich, MA, USA) for 20 min at room temperature, followed by permeabilizing with 0.5% Trixon X-100 for 10 min. After brief washing, cells were blocked in 10% horse serum to avoid high background levels, and then incubated in 1:400 diluted anti-alpha-tubulin monoclonal antibodies (Invitrogen, Life Technologies, MA, USA) overnight at 4°C as recommended by the manufacturer. Cells were rinsed extensively, and reacted with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin (Sigma-Aldrich, MA, USA) diluted 1:80. Laser scanning confocal microscopy was performed with a Leica TCS SP5 microsystem (Leica, Mannheim, Germany).

Statistical Analysis

Data are expressed as the mean±SD from triplicate experiments performed in a parallel manner. Statistical analysis of group differences was performed using Student's t-test. A value of $p<0.05$ (*) or $p<0.01$ (**) was considered statistically significant.

Results

AHL affects HepG-2 cell proliferation

In order to screen one or more AHL-sensitive tumor cell lines, cytotoxicity by MTT assay and cell proliferation by SRB assay were employed to determine antitumor effect of AHL on human liver hepatocellular cells HepG-2, human gastric cancer cell line SGC-7901, human breast adenocarcinoma cell line MCF-7 and human umbilical vein endothelial cell line EVC-304. As is shown in Figure 1A, proliferation of all tested cell lines were inhibited by treating with different concentrations of AHL, and this inhibitory effect, in all cases, showed in AHL-dosage and treating-time dependent manner. Especially, the cytotoxicity of AHL on HepG-2 was more fascinating: significant inhibitory effect, IR of 35.38%, was observed even in a very low dosage of AHL treatment (0.8μg/ml), other test cell line showed similar cytotoxicity assay, however, not as potent as in HepG-2 cells ($p<0.05$ over the dose above 12.5μg/ml), whereas the human endothelial cells were less affected as a non-tumor cell control.

AHL induces HepG-2 cell cycle arrest at G2/M checkpoint

The flow cytometry (FCM) results showed that HepG-2 cell cycles arrested in G2/M phase over the treatment of AHL at the final concentrations of 1.0μg/ml, 2.0μg/ml and 4.0μg/ml after 48 hours, and the arrest intensity gradually increased with the dose of AHL added, indicating a potent cell arrest by AHL with a dose-dependent manner (Figure 1B and 1C). On the contrary, no significant cell cycle arrest was observed on the EVC-304 cells treated with different concentrations of AHL. The results thus indicated an important mechanism of AHL employed on human hepatoma HepG-2 cell cycle, while leaves normal cell unaffected with respect to cell cycle arrest.

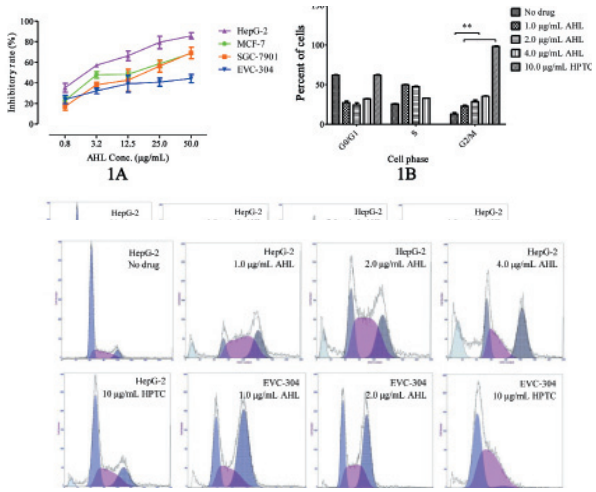

Figure 1. Effect of AHL on proliferation of tumor cells and cell-division cycle. 1A) Inhibitory effect of AHL on proliferation of HepG-2, SGC-7901, MCF-7 and EVC-304 cell lines. **1B and 1C**) Cell cycle analysis of HepG-2 and EVC treated with AHL. HepG-2 cells were treated with different concentration of AHL followed by FCM analysis. EVC-304 was set as nontumor cell control and 10μg/ml HPTC treatment of HepG-2 and EVC-304 were positive controls. The analysis of HepG-2 FCM results were shown in 1B, while typical raw data were shown in 1C. ** represents $P<0.01$ between untreated and AHL or HPTC treated samples

AHL interrupts polymerization of microtubules in HepG-2

Microtubules depolymerize and repolymerize continually in animal cells, this dynamics of polymerization and depolymerization of microtubules during M phase is of vital importance for the spindle microtubules to form and completion of mitosis (Hammond et al., 2008). To further investigate the effect of AHL on the polymerization of microtubules of HepG-2, we observed the morphology of microtubules of the HepG-2 cells using immunofluorescence assay. As is shown in Figure 2, HepG-2 cells in mock treated group showed clear bundle-like, morphologically normal microtubules patterns, whereas, microtubules of AHL treated HepG-2 cell showed disorder distribution patterns, typical bundle fiber become diffusing and disappeared gradually, in addition, the morphology of the cells became abnormal compared to the untreated cells. The microtubules of EVC-304 cells, whether treated with AHL, did not change significantly with tubulin surrounding radiated along the axial direction and the distribution in cells was clear. The abnormality in microtubules dynamics is in correspond to the G2/M arrest of HepG-2 cells treated with AHL, which may lead to the G2/M phase cell arrest.

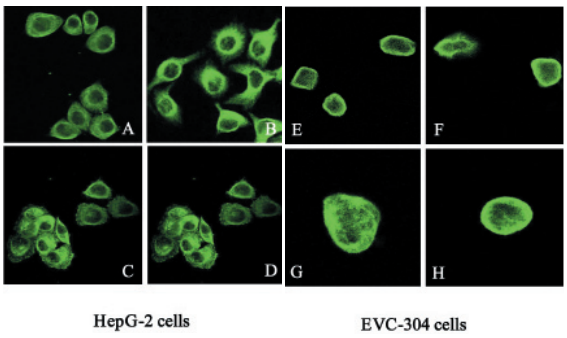

Figure 2. Confocal analysis of the effect of AHL on polymerization of microtubules HepG-2 and EVC-304 cells. HepG-2 and EVC-304 cells were treatment with 4.0μg/ml **C and G groups)** and 8.0μg/ml **D and H groups)** AHL respectively. 10μg/ml HPTC treatment of both cells were positive controls **B and F group)**, while **A and E)** represent no drug treatment groups

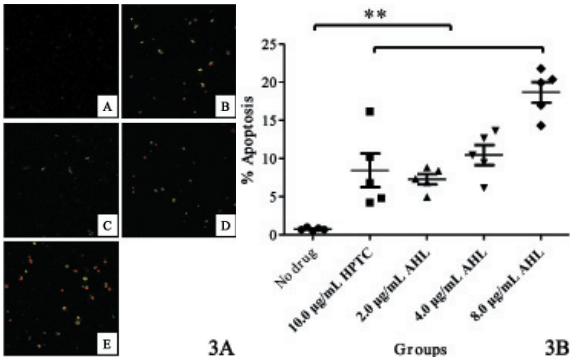

Figure 3. Detection of apoptosis in HepG-2 cells treated for 24h with AHL or HPTC. Cells were treated with different concentration (2.0μg/ml, 4.0μg/ml and 8.0μg/ml respectively) of AHL. 24h later, Annexin V-FITC/PI staining was performed to detect apoptosis; results were shown in microscopic images **3A)** group C, D and E. No drug and 10.0 μg/ml HPTC control groups were shown in group A and B respectively. Percent of apoptosis cells were shown in **3B)** 5 fields with 100 cells per field were counted and statistical analysis was performed (unpaired t-test, two-tailed *p* value); ** represents $P<0.01$ between untreated and AHL or HPTC treated samples

AHL induces apoptosis in cultured HepG-2 cells

Morphological change is the main feature of cells undergo apoptosis (Krysko et al., 2008), in order to visualize apoptotic body formation and nuclear morphological changes, which are the characteristic of cell apoptosis, HepG-2 cells were treated with AHL of different concentration (2.0μg/ml, 4.0μg/ml and 8.0μg/ml respectively) followed by Hoechst 33258 staining. This bisbenzimidazole dye could specifically penetrate plasma membrane and stain DNA in cells without permeabilization. In this assay, apoptotic cells have a stronger blue fluorescence compared with nonapoptotic cells. The untreated HepG-2 cells demonstrated clear nuclear boundaries, uniform nuclei with diffused and homogeneous fluorescence, on the contrary, AHL-treated cells exhibit highly condensed chromatin that was uniformly stained by Hoechst, Apoptotic bodies increased gradually with the dose of AHL added on cells (data not shown). In Annexin V-FITC/PI staining assay, cells in control group were not dyed by Annexin V-FITC or PI, only showed limited nonspecific fluorescence (Figure 3). After treatment with AHL for 24h, cells in the low concentration group (2.0μg/ml) were dyed by Annexin V-FITC and showed green fluorescence, which indicates the early phase of apoptosis (3C). As AHL concentration increased to 4.0μg/ml and 8.0μg/ml respectively, cells were dyed by Annexin V-FITC and PI and showed red fluorescence inside the cell, and green fluorescence outside, this corresponds to the late phase of apoptosis

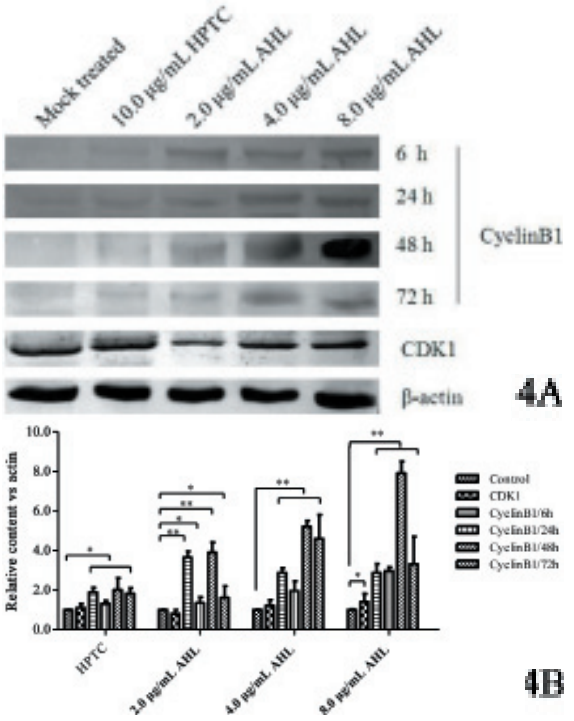

Figure 4. Effect of AHL on expression levels of CyclinB1 and CDK1 in HepG-2. Expression level of CyclinB1 and CDK1 in HepG-2 AHL-treated cells were visualized using Tannon GIS-2019 gel imaging system **4A)** and plotted as relative levels **4B)**. * represents $P<0.05$ *vs*. control; ** represents $P<0.01$ *vs*. control

(Figure 3D and 3E). Based on these morphologic findings, AHL appears to cause apoptosis in HepG-2 cells, which is in a dose dependent manner.

AHL up-regulates CyclinB1and CDK1 expression in HepG-2

To characterize the differentially expression of CyclinB1 and CDK1, these two proteins were analyzed by Western blot technique, followed by Tannon GIS-2019 gel imaging system analysis as indicated by Figure 4. The results showed that the AHL significantly affected the expression of CyclinB1 and CDK1 proteins in human hepatoma HepG-2 cell, CyclinB1 proteins expression level in HepG-2 cells was unregulated upon the treatment of three concentrations of AHL (2.0μg/ml, 4.0μg/ml and 8.0μg/ml respectively) (P<0.01), Whereas, the CDK1 showed unregulated expression upon the treatment of moderate dose (4.0μg/ml) and high dose (8.0μg/ml) of AHL (P<0.05). Lower dose of AHL (2.0μg/ml) did not significant affect the expression level of CDK1 (P>0.05).

Fas-signaling pathway involves in AHL induced apoptosis

Different concentrations of AHL were added to the medium of the culture cells, expression levels of Fas and its Fas ligand, Fas-L were measured by Western blot (Figure 5). The results demonstrated that Fas and FasL were unregulated upon the treatment of AHL. Dose-dependent effect was observed according to the data. Similar results were observed in the expression level of Caspase-8 and Caspase-3 as well. Both protein were unregulated which showed in a dose dependent manner. Collectively, these results may lead to the suggestion that AHL unregulated expression of Fas and FasL, the two proteins formed a dimer and activated Caspase-8, consequently, Caspase-3 was activated and induced apoptosis of the HepG-2 cells. In this notion, the Fas/FasL pathway plays an important role in the AHL-induced apoptosis of HepG-2 cells. However, silencing of key effectors in this pathway by interfering or knockdown techniques is needed and carefully interpreted.

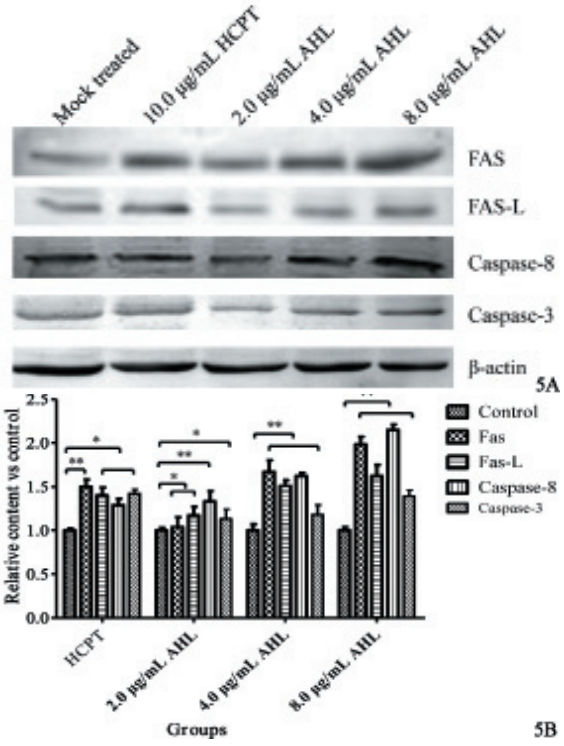

Figure 5. Expression of Fas pathways proteins in HepG-2 cells upon treatment of AHL. Up-regulation of Fas pathway proteins expression in HepG-2 cells treated with AHL **5A)** and plotted as relative levels **5B)**. * represents P<0.05 *vs*. control; ** represents P<0.01 vs. control

Discussion

In this study, we conducted experiments to screen AHL-sensitive tumor cells, and found that HepG-2 was the most sensitive to the treatment of AHL, we also showed that this mixture of major three alkaloid components (5, 6-dihydrobicolorine, 7-deoxy-trans-dihydronarciclasine, littoraline) extracted by n-butanol from *Hymenocallis Littoralis* could cause HepG-2 cell cycle arrest at G2/M checkpoint, interrupt polymerization of microtubules and induce apoptosis. In addition, expressions of two cell cycle-regulated proteins, CyclinB1 and CDK1, were up-regulated upon AHL treatment. Up-regulations of the Fas, Fas ligand, Caspase-8 and Caspase-3 were observed as well. In conclusion, AHL cause HepG-2 cell cycle arrest at G2/M checkpoint and induce apoptosis. Fas signaling pathway may be involved in this process.

Alkaloids are the main active component of the anti-tumor in natural medicines *Hymenocallis Littoralis*. In recent years, experiential studies indicated that alkaloids of *H. littoralis* had significant antitumor activity. Natural products inhibit many kinds of cancer cell lines by various approaches, such as promoting cell apoptosis, inhibition of tumor cell invasion and metastasis, reducing the proliferative activity of the drug resistant tumor cell lines. However, one certain alkaloid sometimes does not meet the need of effectively inhabiting proliferation of tumor cells. In tradition Chinese medicine, different herbs or different component of one herb are assumed to mix together, in such way, different component work coordinately, some shortcomes of one active component are made up by another one (s). This mutual promotion and restraint between the elements theory are commonly used in Chinese medicine (Fang et al., 2012). Thus, we explore the anti-tumor effect of mixture alkaloids by n-butanol extraction, the mechanism of underlying this are studied as well. The results showed that the Alkaloids of *H. littoralis* could inhabit proliferation of tumor cell HepG-2, induce apoptosis of the cells, which has an important significance in developing anti-cancer drug with high efficiency and low toxicity features.

Cell-cycle checkpoints help ensures the accuracy of DNA replication and division. Cells that have a defective G2-M checkpoint enter mitosis before repairing their DNA, leading to apoptosis and death after cell division (Cuddihy et al., 2003). No significant cell cycle arrest was observed on the EVC-304 cells treated with different concentrations of AHL. The results indicated an important mechanism of AHL employed on human hepatoma HepG-2 cell cycle, while left normal cell unaffected with respect to cell cycle arrest.

Cyclin-dependent kinase 1 (CDK1) is a catalytic

subunit of the highly conserved protein kinase complex known as M-phase promoting factor (MPF), which is essential for G1/S and G2/M phase transitions of eukaryotic cell cycle, and cyclin B1 is critical to the completion of G2/M transition. Activation of the CDK1-cyclin B brings the onset of mitosis and is tightly regulated. During G2 phase, CDK1-cyclin B complex is held in an inactive state by phosphorylation of CDK1 at the two negative regulatory sites (Lindqvist et al., 2007; Gavet et al., 2010; Wu et al., 2013). AHL significantly affected the expression of CyclinB1 and CDK1 proteins in HepG-2 cell, CyclinB1 proteins expression level in HepG-2 cells was unregulated upon the treatment of three concentrations of AHL (2.0μg/ml, 4.0μg/ml and 8.0μg/ml respectively) (p<0.01), Whereas, the CDK1 showed unregulated expression upon the treatment of moderate dose (4.0μg/ml) and high dose (8.0μg/ml) of AHL (p<0.05). Lower dose of AHL (2.0μg/ml) did not significantly affect the expression level of CDK1 (p>0.05). Thus, we conclude that AHL, in relatively higher dose, up-regulates CyclinB1and CDK1 expression in HepG-2. An interesting fact is that these effects seem to be dose-dependent rather other time-dependent. Flow cytometry showed that the cells treated with AHL have effectively arrested in G2/M phase. Abnormality in cell cycle regulation implies the following apoptosis event, which leads to the proliferation inhibition of HepG-2 cells (Xue et al., 2014).

Apoptosis is a distinct event that triggers characteristic morphological and biological changes in the cellular life cycle. One of the early events that happened in the apoptotic pathway is the loss of plasma membrane asymmetry. In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. Annexin V when labeled with FITC can be used with microscopy or flow cytometry to measure this event. Annexin V- FITC staining can identify apoptosis at an earlier stage of the apoptosis. Staining with Annexin V-FITC is typically used in conjunction with a live/dead dye such as propidium iodide (PI) or 7-Amino-Actinomycin (7-AAD) to allow the investigator to identify early apoptotic cells (PI negative, Annexin V-FITC positive) from dead cells (PI positive, AnnexinV-FITC positive). Viable cells with intact membranes exclude PI, whereas the membranes of dead and damaged cells are permeable to PI. For this reason, Annexin V staining has to be performed on live cells. Cells that are viable are both Annexin V-FITC and PI negative. While cells that are in early apoptosis are Annexin V-FITC positive and PI negative and cells that are in late apoptosis or already dead are both FITC Annexin V and PI positive. In this study, HepG-2 cells treated with AHL of different concentration (2.0μg/ml, 4.0μg/ml and 8.0μg/ml respectively) showed different Annexin V-FITC/PI staining. Cells treated with low dose of AHL showed small amount of green Annexin V-FITC positive percent, this indicated cells underwent early apoptosis stage, while in higher dose of AHL treated groups, cells appeared both FITC Annexin V and PI positive. In addition, percentage of apoptotic cells became higher over the higher concentration of treated AHL, which implied the dose-dependent of HepG-2 apoptosis induced by AHL.

Fas (also called Apo1 or CD95) is a death domain-containing member of the TNFR (Tumor Necrosis Factor Receptor) superfamily, and Fas ligand (FasL or CD95L) is a type-II transmembrane protein that belongs to the tumor necrosis factor (TNF) family 1. Fas ligand/receptor interactions play an important role in the regulation of the immune system and the progression of cancer. upon binding to the FasL, the Fas trimerizes and induces apoptosis through the cytoplasmic domain DD (Death Domain) that interacts with signaling adaptors like FADD (Fas-Associated Death Domain). FADD carries a DED (Death Effector Domain) and it recruits the DED containing Procaspase8 protein which is in inactive state. Procaspase8 is proteolytically activated to Caspase8. FADD also helps in the activation of Caspase10. Upon activation, Caspase8 and Caspase10 cleave and activate downstream effector Caspases, including Caspase3, 6 and 7. In most case, Caspase3 plays central roles in this signal pathway (Xu et al., 2014). Therefore, Fas, FasL, and two key downstream effector Caspases, Caspase8 and Caspase3 were measure to reveal whether Fas/FasL signaling pathway involved in inducing apoptosis of HepG-2 cells upon treatment of AHL. According to figure 5, up-regulation of Fas, FasL, Caspase8 and Caspase3 were observed, which might indicate that AHL induced HepG-2 cells apoptosis may involve in Fas/FasL interactions, enhanced expression of caspase-8, caspase-3. These results suggest that the Fas/FasL pathway may play an important role in the AHL-induced apoptosis of HepG-2 cells, or this event involves in a pathway that crosstalk with the Fas pathway. However, protein expression did not mean the involvement of such signaling for apoptosis. Therefore, the blockade of caspase activation and Fas/FasL activation pharmacologically and genetically are needed to test its essential role in apoptosis. To elucidate the mechanism of the apoptosis induced by AHL, silencing of key effectors in this pathway by RNA interfering or knockdown techniques is needed and carefully interpreted.

Acknowledgements

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