

Research Article

Actinidia arguta Polysaccharide Induces Apoptosis in Hep G2 Cells

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Abstract: In our previous study, a potential Antitumor Polysaccharide (AAP-3b) was extracted from the fruit of *Actinidia arguta*. Several earlier studies have indicated that AAP-3b exhibits antioxidant activity. This study was designed to determine the effect of AAP-3b on cytotoxicity in human hepatoma cell line, Hep G2 cells. Hep G2 cells were cultured in the presence of AAP-3b at various concentrations (0.005-1 mg/mL) from 24 to 96 h and the percentage of cell viability was evaluated by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl Tetrazolium bromide (MTT) assay. The results showed that AAP-3b inhibited the cells viability in concentration and time dependent characteristics at concentrations (0.05-0.5 mg/mL) from 24 to 72 h. The IC₅₀ (50% cell growth inhibitory concentration) of tumor cell proliferation inhibition from 24 to 72 h were 0.193 (95% confidence interval 0.159 to 0.234), 0.114 (95% confidence interval 0.073 to 0.170), 0.076 (95% confidence interval 0.034 to 0.137) mg/mL respectively. We found that anti-proliferative effect of AAP-3b was associated with cell cycle blocked and apoptosis on Hep G2 cells by determinations of morphological changes, cell cycle and apoptosis. These results provide new insight into the health functions of *Actinidia arguta* polysaccharide.

Keywords: AAP-3b, *Actinidia arguta*, antitumor, Hep G2 cells, polysaccharide

INTRODUCTION

Nowadays, hepatoma locate one of the first place in all malignant tumor. Although most chemotherapeutic agents in clinical used currently are effective in combating cancer cells, also induce adverse effects (Kawaguchi *et al.*, 2009), to extract and look for active component of anti-tumor from natural products become new method and wish to treating cancer.

Actinidia arguta Sieb. et Zucc belongs to Actinidiaceae, is one of the most widely distributed wild fruit trees in China. It contains multifunctional compounds with various pharmacological activities (Krupa *et al.*, 2011). Polysaccharide is one of the main components of *Actinidia arguta*, we have purified polysaccharide from *Actinidia arguta* named AAP-3b, the monosaccharide composition of AAP-3b was determined by PMP pre-column derivative HPLC, the results showed that AAP-3b was composed of mannose, rhamnose, galacturonic acid, glucose, galactose and arabinose. Infrared spectrum scanning results showed that AAP-3b was pectic polysaccharide, in which the content of β -glycosidic bond was predominant (Xuan *et al.*, 2013).

In recent years, polysaccharides from food plants have emerged as an important class of bioactive natural products that are being widely studied (Sun *et al.*, 2011; Yi *et al.*, 2012), more and more polysaccharides have

been reported to exhibit a variety of biological activities, including anti-aging, anti-tumor, anti-hiv and adjust immunity. Medicinal plant extracts are generally considered to be relatively no-toxic at low doses and are not thought to cause major side effects compared to those observed with drugs (Wang *et al.*, 2011). We reported for the first time that Polysaccharide (AAP-3b) from the fruit of *Actinidia arguta* exhibited a potent anti-proliferation effect on Hep G2 cells in vitro. *Actinidia arguta* polysaccharide provide an alternative to currently employed hepatoma therapies and maybe used as a natural health food with antitumor actions.

MATERIALS AND METHODS

Materials: Dulbecco's Modified Eagle's Medium (DMEM), Fetal Bovine Serum (FBS), Hank's Balance Salt Solution (HBSS), trypsin, Phosphate Buffered Saline (PBS), RNase A and Propidium Iodide (PI) were purchased from GIBCO (Burlington, Ontario, Canada). Dimethyl Sulfoxide (DMSO) was from Amresco (99.9% purity) (USA). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenylterazolium bromide (MTT) was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Olympus IX-71 inversion microscope and Olympus CKX31 inversion microscope were made in Japan. FACSCanto™ II flow cytometer and data acquisition/analysis software Modfit LI 3.2 were from

Becton Dickinson Company (USA). Synergy HT ELIASA was from Gene Co.

Actinidia arguta Polysaccharide (AAP-3b) was supplied by Dr. Xuan Li at the *Actinidia arguta* lab of Shenyang Agriculture University and dissolved in culture medium to give the concentrations that needed in the experiments.

Hep G2 cells were obtained from Shenyang Pharmaceutical University (China).

Cell culture: Hep G2 Cells were cultured in DMEM medium supplemented with 10% (v/v) heat-inactivated FBS, 1% (v/v) penicillin and streptomycin in an incubator at 37°C and in a humid atmosphere with 5% CO₂. The cells with medium changed every other day were sub cultured at 1:3 every 3 days and were then plated at an appropriate density according to each experimental scale. Stock of cells were routinely frozen and stored in liquid N₂.

Cell proliferation assay: The effect of AAP-3b on hepatomas was assessed by the colorimetric MTT assay (Mosmann, 1983). Hep G2 cells were seeded into four 96-well culturing plates at a density of 5×10^3 cells/well in 100 µL of culture medium. After 24 h of incubation, cells were synchronized and attached to plates, after which the medium in the plates was discarded and replaced with 100 µL fresh medium that contains different concentrations of AAP-3b. The cells were divided into two groups as followings: The DMEM control group; AAP-3b group supplemented at final AAP-3b concentrations of 0.005, 0.05, 0.1, 0.2, 0.5 and 1 mg/mL, respectively. Each sample had six replicates. Cells were cultured for 24, 48, 72 and 96 h separately under 5% CO₂ at 37°C. At the end of the treatment, the medium containing various tested samples were discarded and the cells were washed twice with HBSS. MTT (20 µL) at the final concentration was added to each well. The cells were then incubated for another 4 h and then 150 µL DMSO was added to each well. Absorbance at 560 nm was read and cell viability (%) was calculated as $(A_{560} \text{ of drug-treated sample} / A_{560} \text{ of control}) \times 100\%$ (Shen *et al.*, 1995).

Morphological observation: Hep G2 cells were seeded into 6-well culturing plates at a density of 1×10^5 cells/well in 3 mL of culture medium. After 24 h of incubation under 5% CO₂ at 37°C, cells were synchronized and attached to plates, after which the medium in the plates was discarded and replaced with 3 mL fresh medium that containing different concentrations of AAP-3b. The cells were divided into two groups as followings: The DMEM control group; AAP-3b group supplemented at final AAP-3b concentrations of 0.005, 0.05, 0.1, 0.2 and 0.5 mg/mL, respectively. Each sample had three replicates. Cells were cultured for 72 h. Cellular morphology was

observed under inverted microscope and recorded by the image system at 200× magnification in 24, 48 and 72 h, respectively.

Cell cycle analysis: Hep G2 cells (5×10^5) were seeded into 6-well plates and incubated with AAP-3b (0.05, 0.1 and 0.2 mg/mL) in a humidified incubator at 37°C under 5% CO₂ for specific periods of time (24, 48 and 72 h, respectively). The DMEM control group was also prepared. Each sample had six replicates. After the treatment, the cells were collected by trypsinization, centrifuged at 2000 r for 10 min, washed two times with PBS, the cells were suspended in 70% ethanol at 4°C overnight. Then the cells were centrifuged to remove ethanol and washed twice in PBS, stained with 500 µL PI stain solution (50 µg/mL PI, 20 µg/mL DNase free RNase A in PBS) in the dark for 30 min at room temperature (Di Leonardo *et al.*, 1994; Tsai *et al.*, 2010). Cell cycles (G₀/G₁, S and G₂/M) were analyzed by DNA histograms determined by flow cytometry (Becton Dickinson, FACSCanto™ II) using Modfit LI 3.2 software.

Apoptosis assay: Hep G2 cells were seeded into 6-well culturing plates at a density of 1×10^5 cells/well for 24 h, after which the medium in the plates was discarded and replaced with fresh medium that containing different concentrations of AAP-3b (0.05, 0.1 and 0.2 mg/mL, respectively) and the DMEM control group was prepared at the same time. Each sample had six replicates and incubated for 48 and 72 h in a humidified incubator at 37°C under 5% CO₂. Then the cells were digested with trypsin and washed with PBS for twice, discarded the supernatant, fixed with ice-cold 70% ethanol overnight, washed with PBS, stained with 500 µL PI stain solution (50 µg/mL PI, 20 µg/mL DNase free RNase A dissolved in PBS) in the dark for 30 min at room temperature before DNA quantification by flow cytometry. The apoptosis rate was analyzed by software FACS Diva v 6.1.3.

Statistical analysis: Data was expressed as means±Standard Deviation (S.D.) of at least three independent experiments performed in triplicate. Differences between the groups were evaluated by the t-test and inter-group differences were evaluated by a one-way ANOVA. $p < 0.01$ was considered statistically significant. Cytotoxicity against tumor cells (value of IC₅₀) were done using the SPSS 21.0 software package.

RESULTS AND DISCUSSION

Results:

Effect of AAP-3b on cell proliferation inhibition: The effect of the exposure to AAP-3b on the cell viability of Hep G2 cells, as assessed by the MTT test,

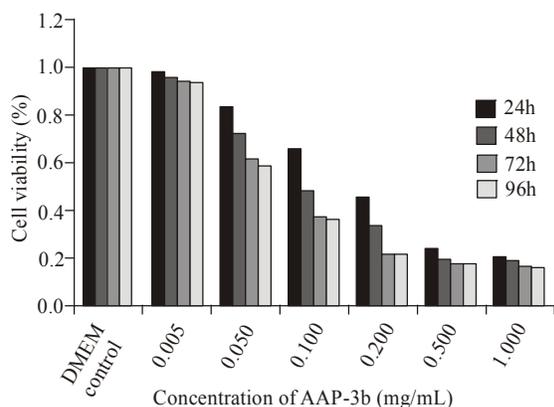


Fig. 1: Effect of AAP-3b in the cell viability in Hep G2 cells. At the concentrations from 0.005 to 1 mg/mL, time from 24 to 96 h, AAP-3b significantly inhibited the Hep-G2 cells viability and the effects were in dose and time dependent manner from 0.05 to 0.5 mg/mL, from 24 to 72 h

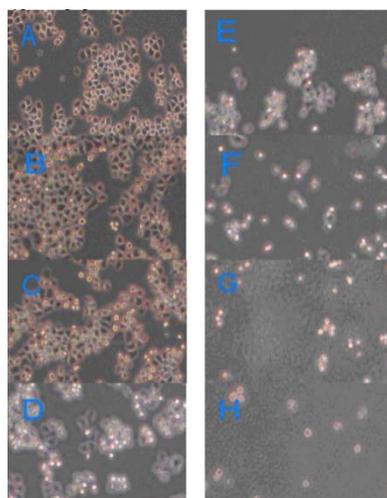


Fig. 2: Morphological changes of Hep G2 cells under the action of AAP-3b. (A) control-24 h; (B) control-72 h, (C) 0.005 mg/mL-72 h, (D) 0.05 mg/mL-48 h, (E) 0.05 mg/mL-72 h, (F) 0.1 mg/mL-48 h, (G) 0.2 mg/mL-24 h, (H) 0.5 mg/mL-24 h

is shown in Fig. 1, Hep G2 cells were not obvious affected when exposed to low concentrations of AAP-3b (0.005 mg/mL) at 24 and 48 h and the cells exposed to high concentrations of AAP-3b (0.05-0.5 mg/mL)

revealed that AAP-3b caused a time dependent and partially dose dependent reduction in cell viability for up to 72 h.

The inhibition of proliferation by AAP-3b in the human hepatoma cell Hep G2 cell line is shown in Table 1 and 2, the inhibition was not obvious at 0.005 mg/mL, but as concentration increased, proliferation of Hep G2 cells was markedly inhibited by AAP-3b in dose and time dependent manners at the concentration of 0.05-0.5 mg/mL for 24, 48 and 72 h, respectively in vitro ($p < 0.01$). The inhibition rates did not increase at 1 mg/mL and for 96 h. The IC_{50} of tumor cell proliferation inhibition from 24 to 72 h were 0.193 (95% confidence interval 0.159 to 0.234), 0.114 (95% confidence interval 0.073 to 0.170), 0.076 (95% confidence interval 0.034 to 0.137) mg/mL, respectively.

Morphological changes: Morphological changes induced by AAP-3b were observed at the concentrations of 0.005, 0.05, 0.1, 0.2 and 0.5 mg/mL under inversion microscopy (Fig. 2). The control group cells arranged closely and to grow with time. After exposure to AAP-3b at the concentration of 0.005 mg/mL for 72 h (Fig. 2C), the number of cells reduced, but morphological changes were not obvious, at the concentration of 0.05 mg/mL for 48 h (Fig. 2D), it presented a significant decline in cell density and the morphological changes happened, Hep G2 cells round in shape with shrinkage and retracted from their neighbors, accompanied with floating apoptotic cells in the culture medium. The changes above were more obvious with time and dose increasing (Fig. 2E and F), especially after exposed to AAP-3b at the concentration of 0.2 and 0.5 mg/mL for 24 h (Fig. 2G and H), cells morphology changed obviously, their form extremely irregular, cells fragmentation and typical apoptosis bodies were seen.

Effect of AAP-3b on the cell cycle distribution of Hep G2 cells: Controlling cell-cycle progression for cancer cells might prevent or delay tumor progression. Many anticancer agents arrest cancer cell cycles in G_1 , S and G_2/M phase (Mork *et al.*, 2005). To investigate the mechanisms leading to decrease of cell viability by AAP-3b, the percentages of G_0/G_1 , S and G_2/M cells

Table 1: Absorbance value changes of each group cell after the action of different concentration of *Actinidia arguta* polysaccharide

Group	Concentration (mg/mL)	n	24 h	48 h	72 h	96 h
Control test	0	6	0.61±0.040	0.98±0.049	1.42±0.037	2.23±0.034
	0.005	6	0.60±0.085	0.94±0.061	1.33±0.083☆	2.08±0.090★
	0.050	6	0.51±0.043★	0.71±0.059★	0.88±0.022★	1.32±0.066★
	0.100	6	0.40±0.039★	0.48±0.068★	0.53±0.033★	0.82±0.049★
	0.200	6	0.27±0.015★	0.31±0.049★	0.31±0.053★	0.47±0.042★
	0.500	6	0.14±0.018★	0.18±0.031★	0.25±0.047★	0.39±0.032★
	1.000	6	0.13±0.013★	0.18±0.024★	0.24±0.040★	0.36±0.027★

★: $p < 0.01$ compared with the control group; ☆: $p < 0.05$ compared with the control group

Table 2: Hep G2 cells growth inhibition rate after the action of different concentration of *Actinidia arguta* polysaccharide

Group	Concentration (mg/mL)	n	24 h	48 h	72 h	96 h
Control test	0	6	-	-	-	-
	0.005	6	1.62	4.57	5.94	6.92
	0.050	6	16.68	27.36	37.98	40.97
	0.100	6	34.72	50.65	62.28	63.38
	0.200	6	55.09	68.12	78.16	78.92
	0.500	6	76.80	81.20	82.08	82.58
	1.000	6	79.11	82.13	82.91	83.83

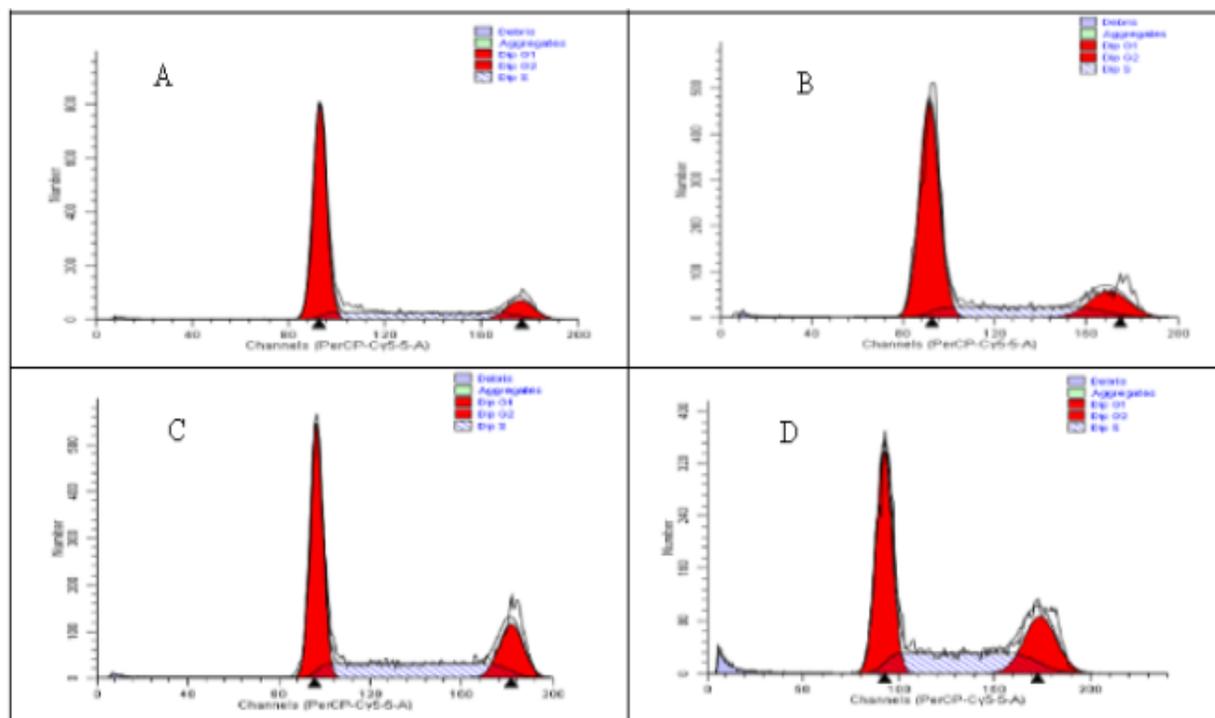


Fig. 3: Effect of AAP-3b on the cell cycle distribution of Hep G2 cells. (A) control-24 h, (B) 0.05 mg/mL-24 h, (C) 0.1 mg/mL-24 h, (D) 0.2 mg/mL-24 h

were evaluated by PI staining and flow cytometric analysis. Cell cycle redistributions at various time points (24, 48 and 72 h, respectively) and concentrations (0.05, 0.1 and 0.2 mg/mL, respectively) were evaluated. As shown in Fig. 3, when Hep G2 cells were treated with AAP-3b for 24 h, the percentage of cells arrested at the G₂/M and G₀/G₁ phase of the cell cycle were 11.48 and 65.46% for control cells (Fig. 3A), 15.51 and 62.96% for 0.05 mg/mL (Fig. 3B), 17.48 and 55.69% for 0.1 mg/mL (Fig. 3C), 25.01 and 48.31% for 0.2 mg/mL (Fig. 3D), respectively. The dose dependency of the cell cycle redistribution on Hep G2 cells was displayed. For the experiments, after 48 h of incubation, apoptosis was found.

Effect of AAP-3b on the cell apoptosis of Hep G2 cells: Apoptotic cells could be separated from normal ones by their lower DNA contents. Since nuclear fragmentation was a hallmark of apoptosis. To quantify apoptosis in Hep G2 cells triggered by AAP-3b, Hep

G2 cells were treated with different concentration (0.05, 0.1 and 0.2 mg/mL, respectively) of AAP-3b for 48 and 72 h, the intensity of nuclear DNA was detected by flow cytometric. It was found that AAP-3b induced cellular apoptosis effectively. As shown in Figure 4, when cells were exposed to 0.05, 0.1 and 0.2 mg/mL for 48 h (Fig. 4C to G), the apoptotic rates were 10.4, 19.6 and 51.5%, respectively. The apoptotic rates induced by 0.05, 0.1 and 0.2 mg/mL AAP-3b for 72 h (Fig. 4D to H) were 28.1, 35.1 and 63.4%, respectively. In contrast, the apoptotic rate for control cells at 48 and 72 h (Fig. 4A and B) were 1.4 and 5.0%, respectively.

Discussion: Hepatoma remains a major public health threat and one of the most common cause of death from cancer. Usually, chemotherapy and radiotherapy are the most frequently used palliative treatment for liver and other cancers. However, some normal cells are destroyed as well by this method. Therefore to find novel natural compounds with low toxicity and high

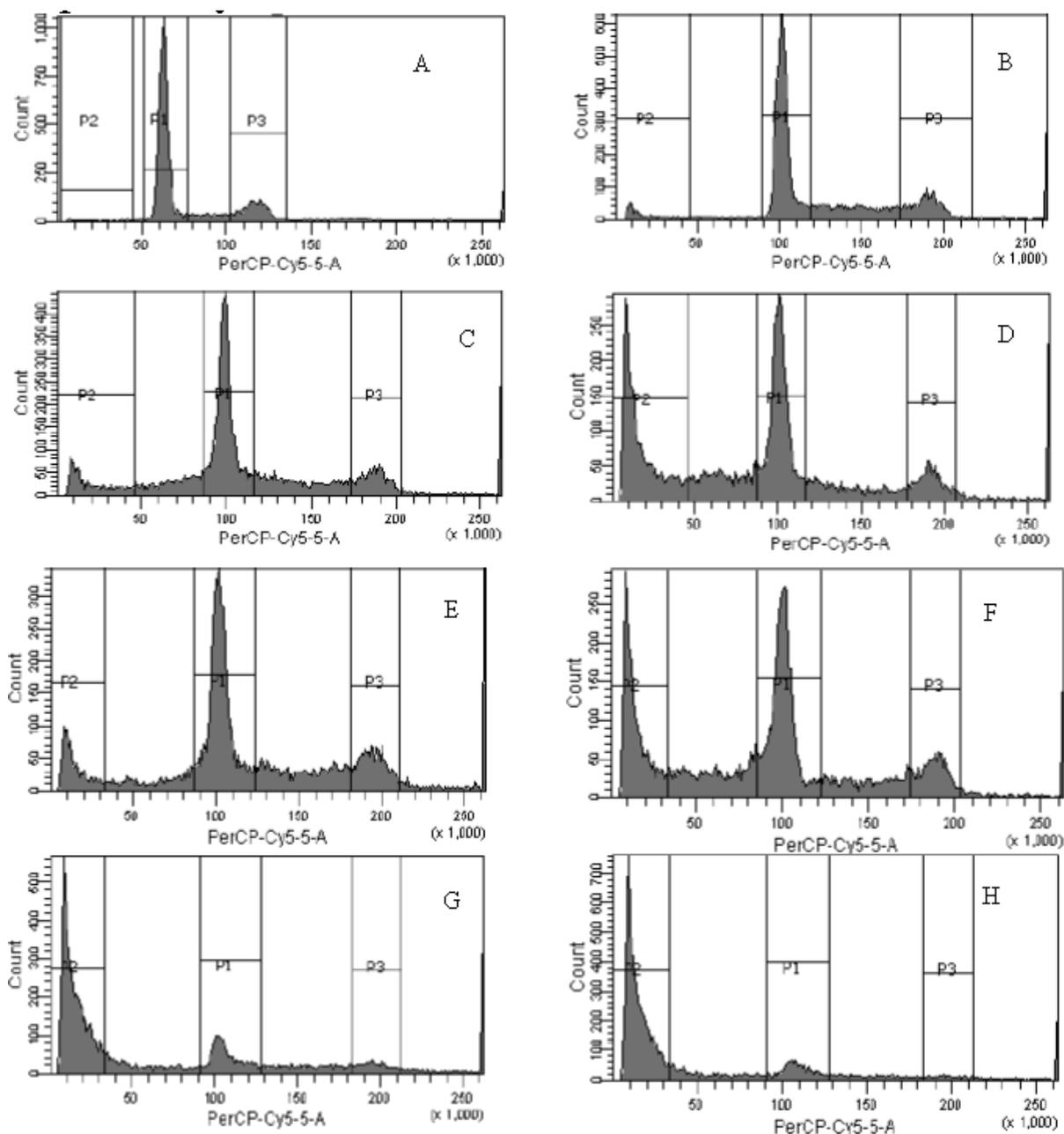


Fig. 4: Effect of AAP-3b on apoptosis of Hep G2 cells, (A) control-48 h, (B) control-72 h, (C) 0.05 mg/mL-48 h, (D) 0.05 mg/mL-72 h, (E) 0.1 mg/mL-48 h, (F) 0.1 mg/mL-72 h, (G) 0.2 mg/mL-48 h, (H) 0.2 mg/mL-72 h

selectivity of killing cancer cells is an important area in cancer research. To date, some anticancer drugs have been developed and applied by clinical doctors due to the wide range of biological activities and low toxicity in animal models. Recently, resistance to anticancer drugs was discovered (Doroshov, 2013). Therefore, research and development of new and safe drugs by the pharmaceutical industry have become necessary.

The northeast wild *Actinidia arguta* is an important wild fruit resource. *Actinidia* is perennial and deciduous liana, its root, stem and fruit are all useful for us in their

medical value and healthy function. Current biological activity research of *Actinidia arguta* focuses on the root and stem, the biological activity research of fruit is limited to fruit juice or fruit extract, unable to determine the biological activity of a specific substance. During this study water-soluble polysaccharide AAP-3b from the fruit of *Actinidia arguta* was studied to evaluate the cytotoxicity in human hepatoma cell line and we demonstrated that AAP-3b had significant effects on inhibiting hepatoma cell growth and inducing hepatoma cell apoptosis.

Morphological changes can reflect the strength of the effect of drugs to living cells. In this study, we used inverted microscope to observe the morphological changes of Hep G2 cells after treated with several concentrations of AAP-3b for different time, the result suggested that when the concentration of AAP-3b was less than or equal to 0.005 mg/mL, the morphological changes were not obvious, however, when the concentration of AAP-3b was greater than or equal to 0.05 mg/mL, the morphological changes happened (Hep G2 cells round in shape with shrinkage and retracted from their neighbors, accompanied with floating apoptotic cells in the culture medium), more and more obvious with the concentration increasing and time extended. This indicated that AAP-3b has obviously inhibition effect on Hep G2 cells in a dose and time dependent manner *in vitro*.

MTT assay was first reported by Mosmain in 1983, recently, it was widely used in the antitumor drug testing and research in vitro screening (Chang *et al.*, 2010). Our study of MTT assay clearly demonstrated that AAP-3b has evident cell proliferation inhibition effect on Hep G2 cells in a dose and time dependent from 0.05 to 0.5 mg/mL, from 24 to 72 h.

The cell cycles and tumor have close relationship (Pendergast *et al.*, 2010). Flow Cytometry (FCM) can be applied to the cell DNA content detection, then analysis the rate of cells in every cell cycle phase. It is widely used in the research of tumor cell proliferation and apoptosis (Darzynkiewicz *et al.*, 2001; Darzynkiewicz *et al.*, 2001). The result of FCM in this study displayed that Hep G2 cells cycle were blocked in a dose and time dependent manner. Hep G2 cells in G₂/M phase were increased after 24 h of treatment with AAP-3b compared with the control and cells in G₀/G₁ phase were decreased.

Apoptotic defects are a common event in oncogenesis and contribute to drug resistance. Tumor growth is determined not only by the rate of cell proliferation, but also by the rate of cell death. In our study, AAP-3b significantly induced cell death of human hepatoma cells from 0.05 to 0.2 mg/mL, from 48 to 72 h. We can assume that AAP-3b acts as an inhibitor of tumor cell growth.

CONCLUSION

In conclusion, AAP-3b inhibited the proliferation of Hep G2 cells by blocking the cell cycle and inducing apoptosis and further research on the molecular mechanisms of AAP-3b induced apoptosis in Hep G2 cells may be helpful for AAP-3b toxicity activity in cellular and molecular level. In addition, it will be interestingly to investigate whether other cell types are also susceptible to AAP-3b. Furthermore, the

toxicological analysis such as the effect of AAP-3b to normal human cells need to be evaluated. The findings of the study demonstrate that *Actinidia arguta* polysaccharide maybe a potential source of natural antitumor and maybe used as a natural health food with antitumor actions.

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