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Effects of the Extract of *Ammopiptanthus mongolicus* cheng f. (JA1) on Induction of Apoptosis of HepG2 *in vitro* and Its Molecular Mechanisms[1]

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Objective To study the effects and the mechanisms of extract from a leguminous plant (*Ammopiptanthus mongolicus* cheng f.) (JA1) in northwest China on inducing apoptosis and inhibiting proliferation of HepG2 hepatocarcinoma cell *in vitro*. **Methods** The HepG2 cell line was used as target cells. The effect of JA1 on HepG2 cell growth was detected by microculture tetrazolium assay (MTT), flow cytometry assay, DNA agarose gel electrophoresis and transmission electronic microscopy. The expressive effect of the wt-p53 in HepG2 cells was analyzed with p53 protein test-reagent. **Results** JA1 not only had significant anti-proliferative effects depending upon time and dosage, but also induced apoptosis of HepG2 cells. Apoptotic typical morphological changes were observed in JA1-treated HepG2 cells under transmission electronic microscope, "Sub-G1" phase peak occurred in flow cytometry and DNA "ladder" was found in DNA agarose gel electrophoresis. The expression of the wt-p53 increased *in vitro*, and JA1-treated HepG2 and the positive cell percentage of the wt-p53 protein also increased. **Conclusions** JA1 could obviously induce apoptosis and inhibit proliferation of HepG2 cells *in vitro*, and these effects are closely related with the increase of wt-p53 expression. JA1 can be used as a good source of medicinal plant for the treatment of hepatocarcinoma.

Key words: Extract from *Ammopiptanthus mongolicus* cheng f. (JA1); HepG2; Apoptosis; Induction; wt-p53

INTRODUCTION

Researches in recent years indicate that disorder in the apoptosis mechanisms of cells has a close relationship with the occurrence and development of malignant tumors[1-3]. Therefore, inducing apoptosis of tumor cells presents a new method for tumor treatment. It has been reported that natural medicines can induce apoptosis of tumor cells through a number of approaches, such as direct destruction of tumor cells, improvement of body immunity by inducing tumor necrosis factor-α (TNF-α) and cell differentiation, as well as by increasing hormone level, *etc*.[2-3, 11].

JA1 is an extract from a leguminous plant (*Ammopiptanthus mongolicus* cheng f.) in northwest China. Professer Hui-Xian JIA first reported in 1991 that the extract (JA1) could inhibit tumor cell growth in mice[4-5]. Unfortunately, so far no other reports on its inhibiting functionare available. In this study, we examined the effects of JA1 on inhibition and induction of apoptosis of human HepG2 *in vitro* and investigated the possible mechanisms therein as the basis of more thorough research in this field and the development of such an important resource.

MATERIALS AND METHODS

Culture of Hepatocarcinoma Cell Strain

HepG2 was purchased from the Animal Laboratory Centre of the Fourth Military Medical University, Xi'an, China. Common culture of tumor cells was conducted in 100 mL culture flasks containing RPMI-1640 culture solution with 100 mL/L calf serum, 8×10^5 U/L penicillin, and 8×10^4 U/L gentamicin in an incubator under 5% CO_2 at 37℃. When the anchorage-dependent cells grew to united

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condition, 0.25% trypsin was used for digestion before subculture. All cells used in the experiments were at the logarithmic growth phase.

JA1 Reagent

JA1 was extracted from the seed oil of *Ammopiptanthus mongolicus*. This species is the only evergreen plant in the north desolate region of China and is the remnant plant of the ancient subtropical zone in Tertiary Period. Because it grows over a long period under stress conditions such as drought, salinity and low temperature, it may contain activated materials. The local inhabitants often use its leaves to treat frostbite. Its anticancer effective composition is water-extraction. The effective ingredients are found to be series compounds of Lupanine through thin-layer chromatography and mass spectroscopy (the result will be reported in a separated article). In this experiment, the oil-like extract was diluted into 10% solution of the original concentration by double-distilled water. The diluent was then blended and homogenized. After 1 h of repose, the solution was filtrated with a glass funnel filter, sealed off and preserved at 4℃. Before the experiment, limpid and light yellow solution were then filtrated twice with a sterile syringe filter under aseptic condition.

MTT Assay

Tumor cells of HepG2 at the logarithmic growth phase were diluted at 1×10^5 mL^{-1} concentration as previously described[6]. HepG2 in suspension was inoculated in 96-well plates with 0.2 mL in each well. Meanwhile, the preserved JA1 filtrate was added to four parallel wells for each concentration of 20 mL/L, 30 mL/L, 40 mL/L, 50 mL/L, 60 mL/L, and 70 mL/L respectively. Culture solution of equal volume was added for the control wells. The culture incubators were placed in a CO_2 incubator under 5% (volume percentage) CO_2 and 37℃. After 48h, the culture medium was gently aspirated from each well and replaced with 50 μL of 1 mg/mL MTT solution, cultured under the same condition for 4 h. Then the MTT was again removed while 150 μL DMSO was put in each well and vibrated. After the purple blue precipitate was fully dissolved, the A value at 570 nm was measured on a microplate reader (DG-3020). The inhibitory rate was calculated with the following equation:

Rate of tumor cell inhibition (%)=(1− the treated group A / the control group A)×100%

Detection of Cell Cycle Changes by Flow Cytometry

Tumor cells of HepG2 including the adherent and suspending cells in the JA1-treated groups of each concentration and the control groups were collected respectively at different time points. The cells were prepared into mono-cell suspension, washed twice with PBS, fixed in precooled ethanol (70%-75%), and stored at 4℃ for at least 24 h. Before analysis, the cells were washed and suspended again in PBS at a final concentration of 1×10^6 cells/mL. Fifty μL suspension was put in the test tube, dyed with 1000 μL propidium iodide (PI) and cultured in dark for 30 min. Sample analysis of apoptosis of cells and cell counting at different phases of the cell cycle (10 000 cells counted) were performed with a flow cytometer (Coulter EPICS XL, USA). The histogram results were analyzed using the Muticycle AV software.

DNA Agarose Gel Electrophoresis and Ladder Detection Assay

HepG2 cells (4×10^6) treated with JA1 at each concentration in the experiment were collected at different time points and centrifuged to remove the culture medium. One mL splitting solution [10 mmol/L Tris-HCI, ph 8.0, 0.1 μmol/L EDTA-Na2, SDS 5 g/L] was added in each sample tube, cultured at 37℃ overnight after protease K was added to make the final concentration into 100 μg/mL. The cells in the samples were first extracted in phenol-chloroform-isopentanol (25:24:1), and then in chloroform-isopentanol. Three mol/L sodium acetic acid (1/10 of the DNA) and 100% ethanol (Double DNA) were put in before stored at -20℃ for over 1 h. After centrifugation at 12 000×g for 15 min at room temperature, the precipitate was dissolved into 70% (v:v) ethanol and then centrifuged at 12 000×g for 10min. The precipitate was dissolved in TE buffer, RNase A (at the final concentration of 0.1 μL/mL) was added and cultured for 1h at 37℃, and extracted again. Three mol/L sodium acetic acid (1/10 of the DNA) and 100% ethanol (Double DNA) were put in, stored at -20℃ for over 1 h. After centrifugation at 12 000×g for 15 min at room temperature, the precipitate was washed with 70% (v:v) ethanol, centrifuged and dissolved in TE buffer. Electrophoresis was performed on a 2% agarose gel containing ethidium bromide. Ladder formation of oligonucleosomal DNA was detected under ultraviolet light.

Morphological Change of HepG2 Cells

Tumor cells of HepG2 in JA1 treated group and control groups were collected separately, centrifuged to remove culture medium, suspended in 5 mL of 0.01 mol/L PBS, transferred into centrifuge tubes with empty trough of agar, centrifuged at 2000×g for 20 min for cellconglobation. Culture medium was

then removed, the conglobated cells were fixed in 2.5% glutaraldehyde solution. The agar remaining in the centrifuge tube was collected and the agar pieces containing conglobated cells were taken and put in 2.5% glutaraldehyde fixed solution and stored at 4℃. The stored samples were then washed with 0.1 mol/L PBS buffer, fixed in 1% osmic acid PBS for 60 min, dehydrated in acetone:ethanol series and embedded in Epon812. Slices were cut with an ultra-thin microtome (LKB8800), stained with acetic uranium and lead nitrate, and observed under an electron microscope (JEM-100CX, Japan).

Changes in Expression of p53

HepG2 cells at the logarithmic growth phase were diluted to 3×10^5 mL^{-1} and inoculated in 100 mL culture flasks. The filtrated JA1 solution was then inoculated into the experiment flasks at the final concentrations of 20 mL/L, 40 mL/L, and 60 mL/L respectively, while equal volume of distilled water was inoculated into control flasks. The cells were collected after cultured for 24 h and 48 h respectively, and prepared into mono-cell suspension, washed twice in PBS. One hundred μL cell suspension was inoculated in analytical tubes and negative control tubes respectively, then 100 μL Reagent A from Fix/Perm (Germany, Onocogene Company) to each tube, mixed and stored at room temperature for 15 min. The samples were washed twice in PBS and centrifuged to remove culture medium. One hundred μL Reagent B from Fix/Perm reagent box was put into all tubes and mixed. Then 5 μL IgG2a-FITC was added to each negative control tube and 25 μL p53-FITC was put in each analytical tube. The samples were then stored in the dark for 20 min at room temperature, washed twice in PBS, centrifuged to remove the culture medium. The cells were suspended in 1 mL PBS again, and detected immediately with a flow cytometer (American, COULTER EPICS XL) adjusted with forward scatter (FSC) and side scatter (SSC) using a negative control sample. Gates were installed for different cells to detect the positive cell percentage of wt-p53 (10 000 cells counted).

Statistical Analysis

All data shown were the means from at least 3 experiments and expressed as $\bar{x} + s$. The data analysis was performed with SPSS software (version 11.0) and Student's *t*-test was used for comparison.

RESULTS

Results of Cell Proliferation Inhibition

JA1 showed significant anti-proliferation effects on HepG2 in a time-time and dosage–dependent manner (Table 1).

TABLE 1

Analysis of Effects of JA1 on Inhibition of Proliferation of HepG2 *in vitro* (n=6, $\bar{x} + s$)

Group	Final Concentration	A Value			Inhibition of Proliferation (%)		
		24 h	48 h	72 h	24 h	48 h	72 h
JA1	70 mL/L	$0.307\pm0.015^{**}$	$0.173\pm0.014^{**}$	$0.152\pm0.028^{**}$	75.3	87.7	85.9
JA1	60 mL/L	$0.399\pm0.037^{**}$	$0.265\pm0.041^{**}$	$0.141\pm0.013^{**}$	67.9	81.2	86.9
JA1	50 mL/L	$0.627\pm0.029^{**}$	$0.317\pm0.014^{**}$	$0.198\pm0.027^{**}$	49.6	77.5	81.6
JA1	40 mL/L	$0.994\pm0.026^{**}$	$0.810\pm0.037^{**}$	$0.435\pm0.033^{**}$	20.1	42.4	59.5
JA1	30 Ml/L	$1.103\pm0.039^{*}$	$1.121\pm0.029^{**}$	$1.121\pm0.029^{**}$	11.3	20.3	40.6
JA1	20 mL/L	1.174 ± 0.046	$1.230\pm0.034^{*}$	$0.874\pm0.054^{*}$	5.6	12.6	18.7
Control		1.244 ± 0.004	1.407 ± 0.024	1.075 ± 0.012	0	0	0

Note. $^{*}P<0.05$, $^{**}P<0.01$, vs control.

Flow Cytometry Results

The obvious sub-G1 peak on DNA histogram was detected in JA1-treated HepG2 samples. The apoptotic exponents of HepG2 samples cultured *in vitro* for 48 h with JA1 at the final concentrations of 20 mL/L, 30 mL/L, 40 mL/L, 50 mL/L, and 60 mL/L were 6.8%, 11.6%, 22.0%, 34.1%, and 40.3% respectively (Table 2, Fig.1), indicating that the JA1 treatment could significantly induce HepG2 apoptosis *in vitro* and the inducing effect was obviously related to JA1 dosage. Analysis of the cell cycle showed that the phase distribution of the cell cycle also changed after 48 h culture with JA1 at all concentrations. As the JA1 concentration increased, the G1 phase cell percentage increased while the S phase cell percentage decreased compared to the control (Table 2, Fig. 1). The apoptotic exponents of the HepG2 samples cultured with 50 mL/L JA1 *in vitro* for 12 h, 24 h and 48 h were 5.5%, 29.9%, and 34.1% respectively (Table 3), suggesting that the inducing effect of JA1 on HepG2 apoptosis *in vitro* was significantly dependent upon treating time.

TABLE 2

Apoptotic Rate and Changes in Cell Cycle of HepG2 Cultured With JA1 *in vitro* for 48 h (n=3, $\bar{x} + s$)

Group	JA1 Concentration (mL/L)	G0/G1 (%)	S (%)	G2/M (%)	Apoptotic Rate (%)
Control		52.6±3.2	42.5±1.2	4.9±1.1	0.5±0.0
20 mL/L JA1	20	59.0±2.6*	31.0±2.0*	10.0±0.9	6.8±1.1*
30 mL/L JA1	30	65.7±4.2*	25.1±1.3**	9.2±1.8	11.6±0.8**
40 mL/L JA1	40	70.9±2.7**	27.3±1.9**	1.8±0.7	32.0±2.1**
50 mL/L JA1	50	78.2±3.4**	4.1±2.3**	17.9±3.5	34.1±0.9**
60 mL/L JA1	60	83.6±3.8**	13.9±1.4**	2.5±1.7	40.3±4.4**

Note. *$P<0.05$, **$P<0.01$, vs control.

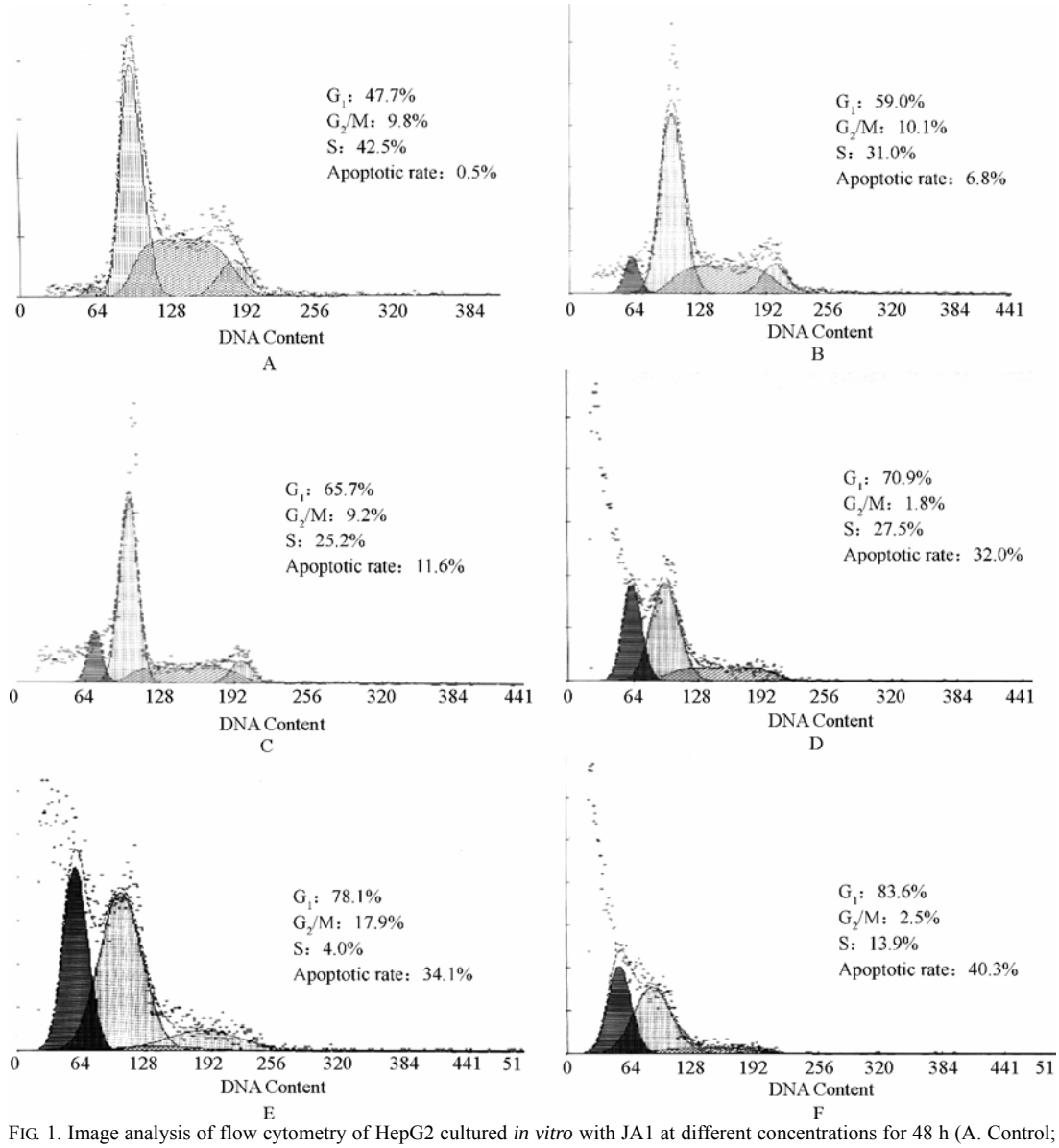

FIG. 1. Image analysis of flow cytometry of HepG2 cultured *in vitro* with JA1 at different concentrations for 48 h (A. Control; B. 20 ml/L JA1; C. 30 ml/L JA1; D. 40 mL/L JA1; E. 50 ml/L JA1; F. 60 ml/L JA1.)

TABLE 3

Apoptotic Rates and Cell Cycle Changes of HepG2 Cultured *in vitro* With 50 mL/L JA1 for Different Periods of Time (n=3, $\bar{x} + s$)

Culture Time	Group	Final Concentration (ml/L)	G0/G1	S	G2/M	Apoptotic Rate (%)
12 h	Control	50	73.6±1.8	24.7±1.1	1.7±0.6	0.0±0.0
	Experiment		61.1±2.2	29.6±0.9*	9.3±2.4*	5.5±0.6**
24 h	Control	50	64.4±1.5	23.3±3.1	12.3±4.3	0.3±0.0
	Experiment		80.1±4.1*	0.5±0.1**	19.1±1.7*	29.9±2.4**
48 h	Control	50	52.6±2.3	42.5±5.3	4.9±0.6	0.5±0.1
	Experiment		78.1±4.9*	4.0±1.1**	17.9±2.5**	34.1±4.7**

Note. *$P<0.05$, **$P<0.01$, vs control.

DNA Ladder

Agarose gel electrophoresis showed that the DNA of HepG2 treated with JA1 at different concentrations for 48 h was broken into 200 bp fragments, exhibiting apoptotic typical DNA ladder, while the DNA of control cells did not undergo such breaking process (Fig. 2), suggesting that JA1 could induce apoptosis of HepG2.

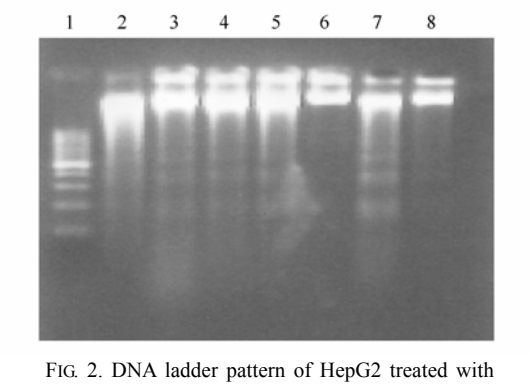

FIG. 2. DNA ladder pattern of HepG2 treated with JA1 at different concentrations for 48 h (1. Marker; 2. 30 mL/L JA1; 3. 40 mL/L JA1; 4. 50 mL/L JA1; 5. 60 mL/L JA1; 6. control; 7. 70 mL/L JA1; 8. 20 mL/L JA1).

Morphological Changes Under Electronic Microscope (EM)

Apoptotic typical morphologic changes of HepG2 were clearly observed under EM after treated with JA1. Marginal gathering of nuclear chromatin occurred in the early phase and crescent-shaped pattern was formed on the edges of the nuclear envelope (NE). Furthermore, the chromosome pycnosis was seenwith electronic density increased, while irregular nuclear deformation and accidental nuclear envelope surface and even Karyorrhexis occurred. Meanwhile, nuclear fragments with increased electronic density were formed in the cell serosa. Then swelling and expansion in mitochondrion and endoplasmic reticulum were observed, empty vesicles in the cell serosa increased, the blebbing phenomenon and formation of apoptotic bodies might take place. However, the cell membrane remained intact (Fig. 3).

Expression of wt-p53 Protein

The wt-p53 expression and the percentage of the wt-p53 protein positive cells increased in HepG2 cultured with JA1 *in vitro*, suggesting that JA1 significantly increased the expression of wt-p53, in a time- and dosage-dependent manner (Table 4).

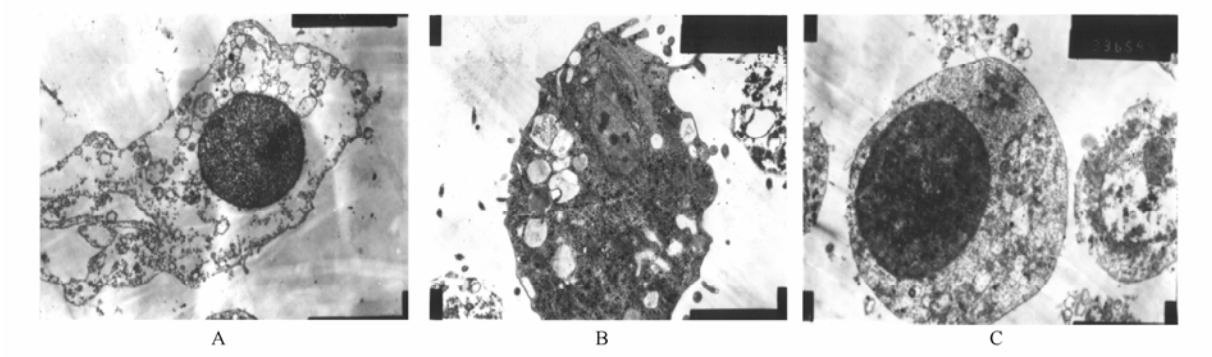

FIG. 3. Electron microscope images of apoptosis in HepG2 treated with 50 mL/L JA1 for 48 h (A: The nucleus underwent pycnosis, endoplasmic reticulum expanded and surface protuberance of the cell increased; B: Cell pycnosis and surface protuberances occurred, apoptotic bodies formed, while the cell membrane remained intact; C: The nucleus underwent pycnosis, but cell membrane remained intact.).

TABLE 4

wt-p53 Protein Positive Cell Percentage in HepG2 Cultured With JA1 *in vitro* (n=4, $\bar{x} + s$)

Group	JA1 Concentration	wt-P53 Protein Positive Cells (%)	
		24 h	48 h
	20 mL/L	9.14±0.26**	15.28±0.46**
	40 mL/L	11.82±0.36**	21.84±1.22**
	60 mL/L	27.06±1.48**	65.38±2.56**
Control		0.88±0.04	0.78±0.06

Note. ** P< 0.01, vs Control.

DISCUSSION

At present, the clinical effect of chemical treatment on hepatocarcinoma is extremely limited. The catabatic rate of hepatocarcinoma is less than 20% for many chemotherapeutic medicines. On the other hand, these medicines have strong toxic and side effects and violent reactions which are insufferable for many patients. Therefore, high attention has been paid to selecting new and better Chinese medicines with higher efficacy, lower toxicity and cost, for combined treatment with Western medicines in clinical practice.

The study reported for the first time, that HepG2 cells turned out obviously apoptotic after treated with JA1 at different concentrations. In our experiments, the HepG2 cells treated with JA1 showed obvious "Sub-G1" phase peak in DNA histogram of flow cytometry and DNA "ladder" was detected in agarose gel electrophoresis (Fig. 1). HepG2 showed typical morphologic changes of apoptotic cells under transmission electronic microscope, indicating that JA1 can induce apoptosis of HepG2 cultured in a time- and dosage-dependent manner.

Previous studies showed that the stimulating signal can affect both the apoptosis of cells and the fission and proliferation of cells[1-3,7,11]. Our experiments showed that the phase distribution of HepG2 cell cycle changed after treated with JA1 for 48 h, and the number of cells in G1 phase increased progressively and decreased gradually in S phase with the increase of JA1 concentration, indicating that JA1 can block HepG2 at G1 phase, delay its transformation in S phase and induce apoptosis of HepG2.

The gene of the wild-type p53 (wt-p53), participating in the regulation and control of the growth, differentiation and death of tumor cells, is one of the anti-cancer genes closely related to the growth of tumor. The p53 protein, normally encoded by wt-p53 gene, is a nuclear binding protein and exists in the nucleus[9-10]. Since it serves as a checkpoint from G1 phase to S phase in the cell cycle and can prolong the G1 phase, thus inducing apoptosis of tumor cells. P53 protein is called "the molecular police"[10]. Our study showed that JA1 was able to increase the expression and positive cell percentage of wt-p53 protein. It may be an important molecular mechanism by which JA1 induces apoptosis and inhibits the proliferation of HepG2.

In conclusion, the JA1 from *Ammopiptanthus mongolicus* has obvious effect on inducing apoptosis of hepatocarcinoma cells, with an inhibitory rate of 87.7% for HepG2. *Ammopiptanthus mongolicus* is distributed over the whole Northwest China, the effective component can be extracted from its seeds and the natural plant resources should not be destroyed by the development of JA1 derived medicine. Therefore, JA1 can be a new and good medicinal plant for the treatment of hepatocarcinoma.

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