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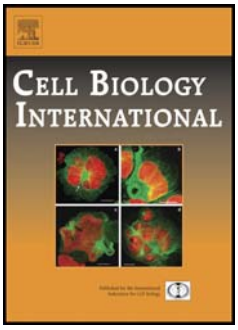

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The apoptotic effect of Sarsasapogenin from *Anemarrhena asphodeloides* on HepG2 human hepatoma cells

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Abstract

Sarsasapogenin, a kind of mainly effective components of *Anemarrhena asphodeloides* Bunge (*Liliaceae*), has the effects of being anti-diabetes and improving memory. However, there are few reports focusing on its anti-tumor effects. In this study, the sarsasapogenin was extracted from rhizomes of *Anemarrhena asphodeloides* Bunge and applied to inhibit HepG2 human hepatoma cells. MTT assay showed that sarsasapogenin induced a distinct dose- and time-dependent diminution of cell viability with IC_{50} of 42.4 ± 1.0 μg/ml for 48 h. Furthermore, sarsasapogenin-induced apoptosis of HepG2 cells was verified by Hoechst 33258 staining, electron microscopy, DNA fragmentation and PI staining. Flow cytometry analysis showed that sarsasapogenin-induced cell apoptosis was through arrest of cell cycle in G_2 /M phase. We hence proposed that sarsasapogenin could be used as an anti-liver cancer drug for future studies.

Keywords: Anemarrhena asphodeloides; sarsasapogenin; HepG2 human hepatoma cell; apoptosis

1. Introduction

Hepatocellular carcinoma (HCC) affects more than 500,000 people globally annually, and five year mortality exceeds 95%. More than half of these people are in China, and the incidence in sub-Saharan Africa is also high (Andraw and Christopher, 2003). Despite extensive research on treatment modalities towards liver cancer, it has been being a world problem. In recent years, the development of new anticancer drugs is a key issue for cancer chemotherapy, because of the reality that cancer cells, which are resistant to current chemotherapy, will eventually dominate the cell population and cause mortality (Kang *et al*., 2005). Furthermore, traditional or folk herbal medicine as alternative cancer therapy has attracted a great deal of recent attention due to its low toxicity and costs.

Anemarrhena asphodeloides Bunge (*Liliaceae*) is a perennial herb and widely grows in most parts of China. Anemarrhenae Rhizoma (rhizomes of *Anemarrhena asphodeloides* Bunge) has been demonstrated not only to have anti-diabetic activity, platelet aggregation inhibitory activity, antipyretic activity, sedative activity, diuretic activity, molluscicidal activity, anti-fungal activity and anti-yeast activity, but also to have inhibiting effects on

cAMP phosphodiesterase (Hoa *et al*., 2004; Iida *et al*., 1999; Miura *et al*., 2001; Tsukamoto *et al*., 2005; Zhang *et al*., 1999). In traditional Chinese medicine, Anemarrhenae Rhizoma is used for the treatment of lung disease, fever, diabetes and constipation (Hua *et al*., 2006). Analysis of chemical compositions of Anemarrhenae Rhizoma found that steroidal saponins, xanthone C-glyco-sides, polysaccharides and norlignans were the major active compounds. The content of steroidal saponins is more than 5% and sarsasapogenin is the main group (about 76.59%) of steroidal saponins (Aritomi and Kawasaki, 1969; Ichiki *et al*., 1998; Jeong *et al*., 2003; Meng *et al*., 1999). Sarsasapogenin (Fig. 1) has the effect of being anti-diabetes and improving memory (Attele *et al*., 2002; Hu *et al*., 2005). Therefore, sarsasapogenin has an extensive application possibility in the clinical medicine. However, there are few reports focusing on its anti-tumor effects.

In this study, we extracted sarsasapogenin from Anemarrhenae Rhizoma, and then the viability of HepG2 human hepatoma cells was detected by MTT assay. Hoechst 33258 staining, electron microscopy, DNA fragmentation and fluorescent microscope were used to verify the apoptotic effects of sarsasapogenin on HepG2 cells. Our experiments demonstrated that sarsasapogenin could dose- and time-dependently inhibit HepG2 cell proliferation, as well as induced HepG2 cell apoptosis with the characteristic of a DNA ladder, a significant accumulation of cells in G_2/M phase, chromatin condensation, cell shrinkage, nuclear fragmentation with formation of apoptotic bodies.

2. Materials and methods

2.1. Materials

Anemarrhena asphodeloides (dry) was purchased from Kangyi Biotechnology Development Limited Company (Hangzhou, China) and identified by Prof Hanchen Zheng, Department of Pharmacognosy, School of Pharmacy, Second Military Medical University, China. A reference specimen (voucher No 20040903) was deposited in the Herbarium of the Second Military Medical University, China. Standard samples of sarsasapogenin were purchased from Institution of Drug Test, China. HepG2 human hepatoma cells were purchased from Cancer Research Department of Zhejiang University, China**.** Fetal bovine serum (FBS) was purchased from Sijiqing, Hangzhou, China. Dulbecco's modified Eagle medium (DMEM)

was purchased from GibcoBRL. Methylthiazolyl tetrazoliun (MTT), penicillin, /BRL fromrtmentdepartment of zhejiang university, streptomycin and propidium iodide (PI) were purchased from Sigma. Hoechst 33258 cell apoptosis stained kit was purchased from Beyotime, Jiangsu, China. All other chemicals were of the highest purity commercially available.

2.2. Isolation, purification and identification of sarsasapogenin

Anemarrhenae Rhizoma (1 kg, dry weight) was ground into powder. The powder was added to a container and extracted by percolation once with a 20 L volume of 95% aqueous ethanol at 70 °C for 4 h. The ethanolic extracts were filtered and concentrated under reduced pressure at 50 °C to a volume of 1 L.

The soluble extracts were chromatographed on macroporous resin (D101, Tianxing, Bengbu, China), eluted with 10%, 30%, 50% and 90% ethanol consecutively. The elutes of 90% ethanol were concentrated under reduced pressure at 50 °C. Equal volume of 10% HCl was added to the concentrated fluid and incubated for 2 h at 50 °C. After concentrating, the residues were dissolved with absolute ethyl alcohol, decolorized with activated carbon for 30 min, filtrated, saturated the residues with absolute ethyl alcohol at 60 °C and formed crystals at RT. White acicular crystals (4.6 g) were obtained after another twice recrystallizations using before-mentioned methods. The identification of sarsasapogenin was accomplished by spectroscopic methods (IR, MS, ^{1}H-NMR, ^{13}C-NMR), and comparison with those of published data (Agrawal *et al*., 1998; Hong *et al*., 1999; Ji *et al*., 2001; Kobayashi *et al*., 1993; Wawer *et al*., 2001) and reference compounds (Fig. 1). Its purity was more than 98% based on HPLC analysis comparisons with standard sample. The crystal was dissolved in methanol and 20 μl solution was injected into the HPLC system (Waters 515-PU, 2410-UV). HPLC conditions: column; Hypersil ODS (150 × 4.6 mm, 5 μm) at 30 °C, mobile phase; methanol:water in the ratio of 98:2, flow rate; 0.9 ml/min, detection; UV 254nm.

2.3. Cell culture and drug treatment

HepG2 cells were maintained in DMEM supplemented with 15% FBS, 100 units/ml of penicillin and 100 µg/ml of streptomycin in a water-saturated atmosphere of 5% $C0_2$ at 37^{o}C. Sarsasapogenin was dissolved in ethanol and the final ethanol concentration in all cultures was 0.5%.

2.4. Analysis of cell viability

After exposure to sarsasapogenin, Cells in 96-well plates were incubated with MTT. The formazan precipitate was dissolved in 200 µl dimethyl sulphoxide and absorbance at 550 nm was measured with a Benchmark microplate reader (Bio-Rad, CA, USA) (Liu *et al*., 2004a).

2.5. Hoechst 33258 staining

Cells were treated with sarsasapogenin, harvested, cytospun onto glass slides and fixed with a 50% solution of fixative (3:1 methanol/acetic acid). The preparations were stained with Hoechst 33258 for 5 min at RT, rinsed, dried and examined using fluorescence microscope (Nikon, Japan) (Li *et al*., 2001).

2.6. Electron microscope

Cells were treated with sarsasapogenin, harvested, fixed with 2.5% glutaraldehyde and as a pellet for 2 h. The pellets were postfixed with 1% osmium tetroxide, stained *en bloc* with aqueous uranyl acetate, dehydrated in a graded series of acetone, and embedded in Epon812 resin. Ultra-thin sections were cut, equipped, counterstained with lead citrate and examined under a HITACHI-600 electron microscope (Hitachi, Tokyo, Japan) (Polla *et al*., 1996).

2.7. Analysis of DNA fragmentation

Cells were treated with sarsasapogenin and DNA was extracted according to Wang *et al*. (2001). Then the DNA fragments were separated on 1.5% agarose gel electrophoresis.

2.8. Fluorescent microscope

Cells were treated with sarsasapogenin, harvested, fixed with ice-cold 70^{o}C ethanol and re-suspended in 40 µg/ml PI, 0.1 mg/ml RNase and 0.1% triton X-100 at 37 °C for 30 min,

followed by flow cytometric analysis at 488 nm (Beckman-Coulter Inc., Fullerton, CA, USA) (Kim *et al*., 2004).

2.9. Statistic analysis

Data are presented as the mean ± S.E.M. for the indicated number of experiments and evaluated by *t*-test. *P*-values below 0.05 were regarded as statistically significant.

3. Results

3.1. Effect of sarsasapogenin on the growth of HepG2 cells

To illustrate whether sarsasapogenin decreases the survival of HepG2 cells, they were treated with four different concentrations of sarsasapogenin i.e., 12.5 μg/ml, 25 μg/ml, 50 μg/ml or 75 μg/ml for 6 h, 12 h, 24 h, 48 h and 72 h respectively. As shown in Fig. 2, sarsasapogenin induced a distinct dose- and time-dependent diminution of cell viability with IC_{50} of 42.4 μg/ml after 48 h treatment or 25 μg/ml after 72 h treatment.

3.2. Hoechst 33258 staining

Hoechst 33258 staining showed that there were significant morphological changes in the nuclear chromatin (Fig. 3). In the untreated group, the nuclei were stained a less bright blue and the color was homogeneous. After treating with 50 μg/ml sarsasapogenin for 72 h, the blue emission light in apoptotic cells was much bright than the control cells. Condensed chromatin could also be found in many treated cells and some of them formed the structure of apoptotic bodies, which is one of the classic characteristics of apoptotic cells.

3.3. Electron mircroscope

As displayed by electron micrographs (Fig. 4), control cells showed integrated nuclear membrane, relatively homogeneous chromatin and extensive membrane interdigitations and microvilli. After treated with 50 μg/ml sarsasapogenin for 72 h, HepG2 cells were characterized by condensation into dense granule or blocks, migration of nuclear chromatin, formation apoptosis bodies and many vacuoles in cytoplasm, at the same time, membrane microvilli were also disappeared. All the changes indicated the apoptosis of HepG2 cells.

3.4. DNA fragmentation

DNA fragmentation is the typical biochemical index of cell apoptosis. As shown in the result (Fig. 5), cell genomic DNA showed the typical formation of DNA fragments as ladders in a time-dependent manner, and the interval of the ladders is about 200bp. The DNA ladder was observed for 24 h after treatment with 50 μg/ml sarsasapogenin. With the time went on, the ladder showed more obviously. This phenomenon indicated that the apoptosis of HepG2 cells was induced by sarsasapogenin.

3.5. Flow cytometry

Apoptotic cells can be recognized by flow cytometry through their diminished stainability with the DNA-specific fluorochrome PI, in which the hypodiploid population can be quantified by DNA content frequency histograms. As shown in Fig. 6, the mean apoptotic population of HepG2 cells was 1.79% under control conditions, while it was increased to 28.71% after treatment with 50 μg/ml sarsasapogenin for 72 h. At the same time, the proportion of G_2/M period cells increased. Therefore, the anti-cancer functions of sarsasapogenin and cell apoptosis induction are related to the accumulation of the G_2/M period cells.

4. Discussion

HCC is one of the most common malignant tumors in some areas of the world. In order to reduce the morbidity and mortality of HCC, early diagnosis and the development of novel systemic therapies for advanced disease, including drugs, gene and immune therapies as well as primary HCC prevention are of paramount importance (Hubert, 2005). Therefore, searching for new anti-tumor and other medical substances and studying their medical value has become a matter of great significance. In this work, we found that sarsasapogenin from *Anemarrhena asphodeloides*, a herbal medicine in China, has significant toxicity against HepG2 human hepatoma cells and induced HepG2 cells apoptosis with the characteristic of a DNA ladder, a significant increase of G_2/M period cells, chromatin condensation, nuclear indentations, disappearance of membrane microvilli, cytoplasmic mass decrease, formation

apoptosis bodies and overall cell shrinkage.

Trouillas *et al.* (2005) reported that sarsasapogenin could inhibit the proliferation of human 1547 osteosarcoma cells and arrested cell cycle in G_2/M. Simillarly, in our study, the DNA content appeared the accumulation of cells in G_2/M phase, according to the results of flow cytometric analysis. Sarsasapogenin and diosgenin, belonging to steroid saponins, are structurally close natural molecules and the only differences are at C-25 and 5, 6-bond. At present, most anti-tumor mechanisms are focusing on diosgenin (Liagre *et al*., 2005; Liu *et al*., 2004b; Liu *et al*., 2005; Trouillas *et al*., 2005). According to their studies, the cell G_2/M arrest was usually involved in diosgenin-induce tumor cell apoptosis. It implied our sarsasapogenin would induce cell apoptosis through the same pathway with disruption of Ca^{2+} homeostasis and mitochondrial dysfunction (Liu *et al*., 2005).

In conclusion, for the first time, we found that sarsasapogenin could induce HepG2 cell apoptosis, leading inhibition of tumor cell growth, through cell cycle arrest on G_2/M. Further studies are being performed to uncover the underlying apoptosis mechanism. Moreover it will be interesting to study. From our studies, sarsasapogenin is of potential as a candidate for cancer therapy, bearing the multiple functions and high safety.

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FIGURE LEGENDS

Fig. 1. Structure of sarsasapogenin

Fig. 2. Time- and concentration-dependent effect of sarsasapogenin on HepG2 cells. HepG2 cells were treated with 12.5 μg/ml, 25μg/ml, 50 μg/ml, 75 μg/ml sarsasapogenin for 6 h, 12 h, 24 h, 48 h, 72 h respectively. Cells were incubated with MTT, and then detected the absorbance at 550 nm. Results are expressed as the mean ± S.E.M. of data obtained in three independent experiments (*p*-value relative to control group, $p<0.05$).

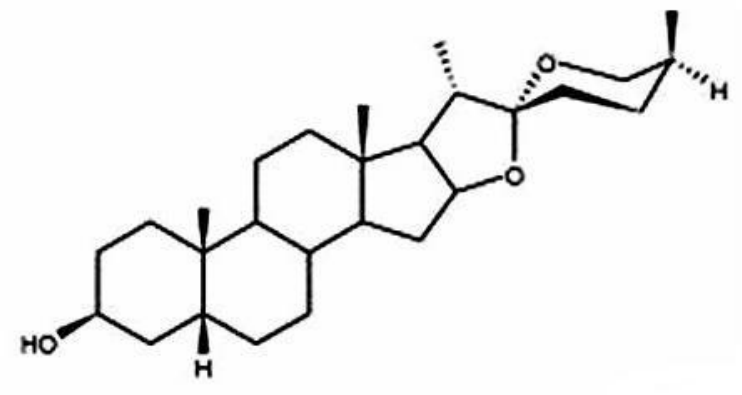
Fig. 3. Fluorescence photomicrograph of HepG2 cells stained with Hoechst 33258. HepG2 cells were exposed to 50 μg/ml sarsasapogenin for 72 h, harvested, and cytospun onto glass slides for fixing. The preparations were stained with Hoechst 33258 and examined under fluorescence microscope. (A) Control (100×): the nuclei were stained homogeneous and less bright. (B) Treated cells (100×): chromatin condensed and apoptotic bodies formed. Pictures in the bottom right-hand corner were amplified to show obviously.

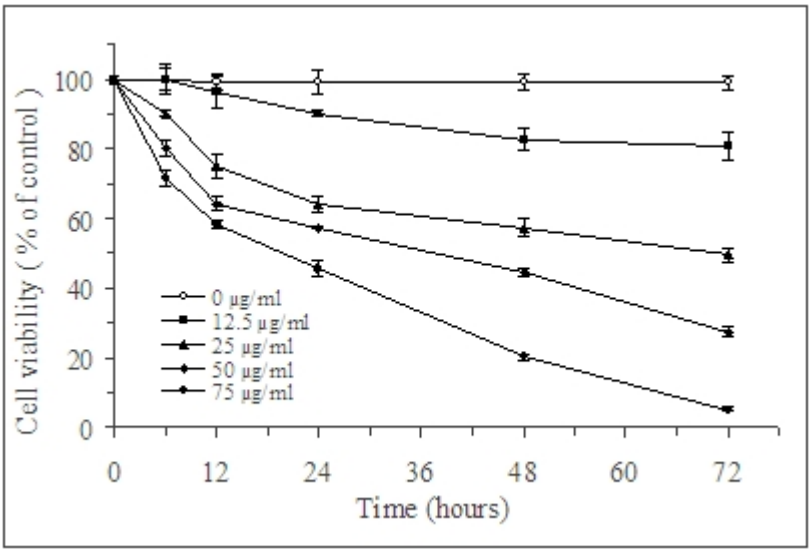
Fig. 4. Electron micrographs of HepG2 cells stained with uranyl acetate and lead citrate. HepG2 cells were exposed to 50 μg/ml sarsasapogenin for 72 h, harvested and fixed with glutaraldehyde. Ultra-thin sections were cut, equipped, counterstained with lead citrate and then examined under electron microscope. (A) Control: showing integrated nuclear membrane, relatively homogeneous chromatin and extensive membrane interdigitations and microvill. (B) Treated cells: showing chromatin condensation into dense granule. (C) Treated cells: showing margination of nuclear chromatin and cytoplasm vacuolization. (D) Treated cells: showing

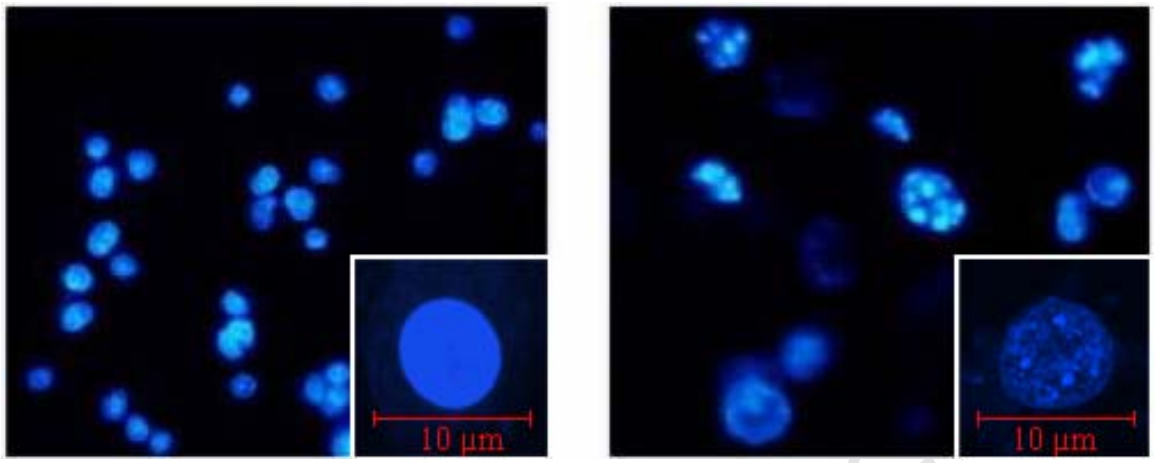
formation apoptosis bodies, disappearance of membrane microvill and many vacuoles in cytoplasm.

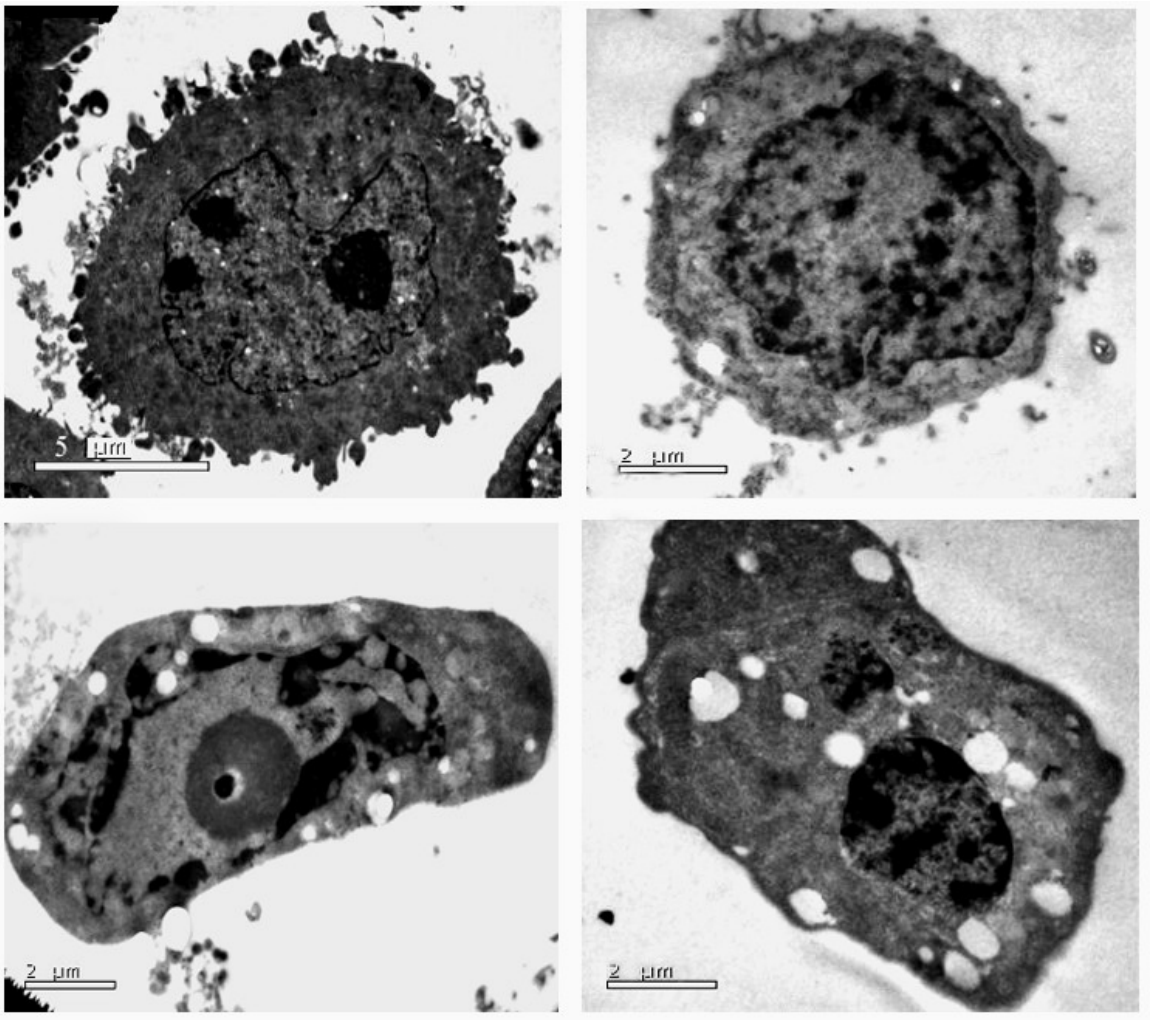
Fig. 5. Gel electrophoresis of DNA extracted from HepG2 cells. HepG2 cells were exposed to 50 µg/ml sarsasapogenin for different times (0 h, 24 h, 48 h, 72 h). Cells were harvested by centrifugation and DNA was extracted. The DNA fragments was separated on 1.5% agarose gel electrophoresis and visualized under ultraviolet light after staining with ethidium bromide.

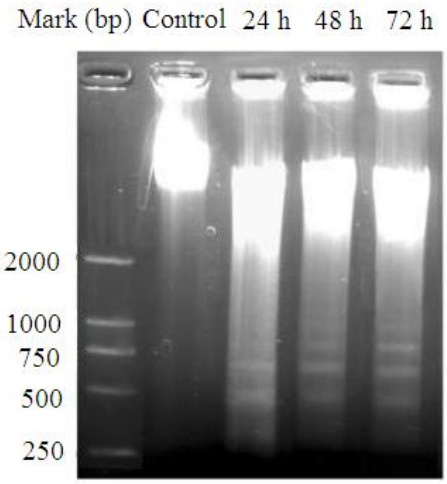
Fig. 6. Flow cytometric analysis of DNA fragmentation for HepG2 cells. HepG2 cells were exposed to 50 µg/ml sarsasapogenin for 72 h. The harvest cells were fixed in 70% ethanol and stained with PI, followed by flow cytometric analysis. (A) Control; (B) Treated cells.

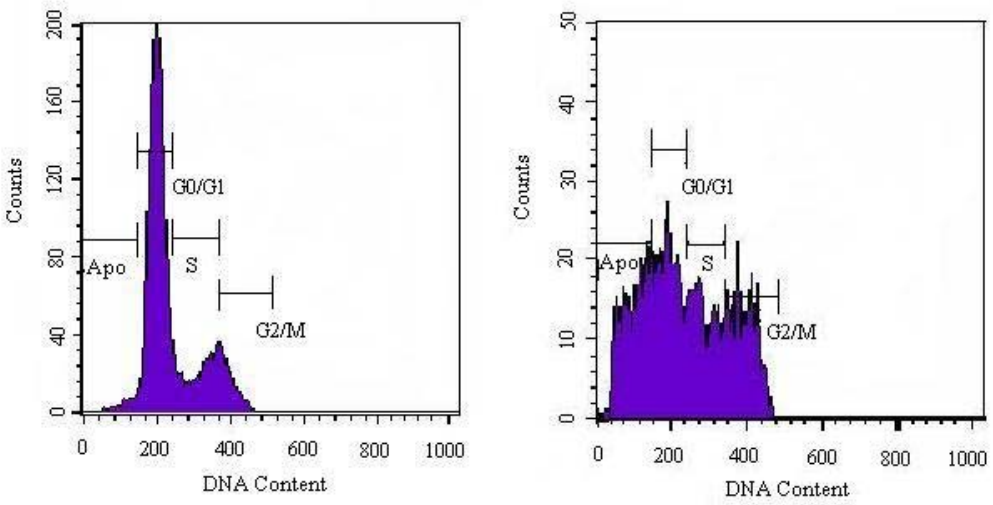








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