

*Full Length Research Paper*

## **Inhibitory and apoptosis-inducing effects of *Lophophora williamsii* extracts on HeLa cells**

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The *in vitro* inhibitory and apoptosis-inducing effects of three different *Lophophora williamsii* extracts were observed in HeLa cells. Methanol, hydrochloric acid, and sodium hydroxide were used to extract active compounds from the caudices of *L. williamsii*. The inhibitory effects of the extracts on HeLa cells were detected by Methyl Thiazolyl Tetrazolium (MTT) assay and the apoptosis-inducing effects were assessed by analyzing caspase 3 and PARP expression using Western blotting. The methanolic, HCl, and NaOH extracts at 150 µg/ml, exhibited 91.98, 96.14, and 25.64% inhibition rates, respectively. The median effective concentrations EC<sub>50</sub> of the methanolic and HCl extracts were 4.2 and 7.8 µg/ml, respectively. The HeLa cells showed a DNA ladder pattern in the Western blot, as well as upregulation of caspase 3 and PARP after treatment with the methanolic extract. Therefore, both the methanolic and HCl extracts inhibit the growth of HeLa cells, with the former capable of inducing apoptosis.

**Key words:** *Lophophora williamsii*, HeLa cells, extracts inhibitive, apoptosis.

### **INTRODUCTION**

The *Lophophora williamsii* cactus, or peyote, is a succulent plant which originates primarily from the deserts of Central Mexico. It is bluish green, button-like, low growing, and tufted with thick and fleshy perennial roots, which feature high storage capacities for water (Borchers et al., 2000). As a hallucinogen, the roots of peyote can significantly affect the physique, vision, and perception of those who consume it (Hermle et al., 1992). It is commonly used as a ceremonial sacrament and a traditional medicine by the Arapaho, Blackfoot, Comanche, Huichol, Kickapoo, Kiowa, Lakota, Navajo, Omaha, and Winnebago Indian Tribes (McLaughlin, 1973; Borchers et al., 2000) to treat infections, arthritis, asthma, influenza, intestinal disorders, and diabetes, as well as snake and scorpion bites (Franco-Molina et al., 2003).

Modern research has discovered that peyote root contains a substance called mescaline, which possesses hallucinogenic effects. This was therefore developed into a drug for treating substance abuse as it can be used as a substitute during rehabilitation (Carod-Artal et al., 2006). Studies have reported significantly lower cancer incidence rates among Native Americans, compared with the general population (Michalek et al., 1990), and their frequent use of peyote also suggest that it is a possible cancer cure, although its potential action against tumor cell growth is still unconfirmed. Interestingly, stimulating mystical experiences, entheogens, and psychedelic drugs have been hypothesized to boost the immune system and, thereby, promoting cancer remission (Roberts, 1999). Mescaline, with its hallucinogenic effect, may therefore be a psychedelic drug that stimulates the immune system. Furthermore, psychedelic drugs may influence the state of mind of individuals to influence health positively; also, religion and the like have been recognized to affect physical health through neuroendocrine and immunologic mechanisms (Koenig,

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2000). These can possibly account for the low cancer incidence among Native Americans.

The methanolic extract of *L. williamsii* can upregulate the immune functions of murine lymphocytes, macrophages, and human peripheral blood mononuclear cells (HPBMC). It could inhibit the growth of both murine and human tumor cells, including murine lymphoma L5178Y-R, fibroblastoma L929, human myeloid U937, and mammary gland MCF7 tumors (Franco-Molina et al., 2003). The underlying mechanism for this is still unclear, and further elucidation of its anticancer effect can lead to the development of new drugs.

Therefore, this study aims to evaluate the cytotoxicity of three different peyote extracts and to determine the possible mechanism of cell death in the HeLa cell-line cervical cancer cells.

## MATERIALS AND METHODS

### Preparation of extracts

*L. williamsii* plant was obtained from China succulent society at age of five and authenticated by senior engineer professor Lee, Department of Life sciences, Nankai University, Tianjin, China. The stems and roots were washed, dried, and chopped finely using a blender. Fifty grams of dried material were exhaustively extracted with methanol, hydrochloric acid, and sodium hydroxide by Soxhlet extraction. The methanol extract was filtered, concentrated, and dehydrated using a rotary evaporator to full dryness. The hydrochloric acid and sodium hydroxide extracts were precipitated by alcohol then washed and aether dried. The recovery content was weighted and recorded for further analysis. All extracts were dissolved in DMSO (Sigma) and subsequently diluted to appropriate working concentrations.

### Determination of polysaccharide contents

The HCl and NaOH extracts were both tested for the polysaccharide contents by Phenol-Sulfuric acid method. Glucose solution was used as control to illustrate the stand curve.

### Cell lines and culture medium

Hela cells (human cervical carcinoma cell line) were purchased from American Type Culture Collection (ATCC, Rockville). Hela cells were cultured in RPMI 1640 medium, and supplemented with 10% (v/v) Fetal Calf Serum (FCS), 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM L-Glutamine, and 1 mM sodium pyruvate (All from Hyclone).

### *In vitro* cytotoxicity assay methyl thiazolyl tetrazolium (MTT) assay

Exponentially growing Hela cell lines were plated at  $5 \times 10^4$  cells/ml in flat-bottomed 96-well plates (Costar) in 100  $\mu$ L of complete RPMI 1640 medium. These tumor cell cultures were then incubated for 72 h in various concentrations (1 – 150  $\mu$ g/ml) of peyote extract in a total volume of 100  $\mu$ L, and absence of extract as the control group. After incubation for 68 h at 37°C in 5% CO<sub>2</sub>, 10  $\mu$ L of MTT (Sigma, 0.5 mg/ml final concentration) was added to all wells followed by

additional 4 h of incubation. Supernatant was then decanted and 150  $\mu$ L of DMSO was added to each well, followed by incubation of the plate for 1 h. Optical densities and percentage viability were then determined by absorption at 540 nm ( $A_{540}$ ). The  $A_{540}$  value through Inhibition Rate (IR) and half-maximal enhancement ( $EC_{50}$ ),  $IR = 1 - (A_{540} \text{ experimental group} / A_{540} \text{ control group})$ , and the analysis of  $EC_{50}$  was calculated by EXCEL.

### Agarose gel electrophoresis to analyze DNA ladder

When the cells reached confluency between 80 - 90%, the medium was replaced with fresh medium containing free-FCS. The cells were then incubated for another 6 h. Subsequently, the cells were stimulated with extracts at  $EC_{50}$  concentration required for 50% growth inhibition of Hela cells for 24 h. Control group was treated with the same final concentration of DMSO [1% (v/v)]. All the stimulated Hela cell's DNAs were extracted for agarose gel electrophoresis. Total cellular DNA products were electrophoresed on a 1% (w/v) agarose gel and visualized with ethidium bromide staining. Analysis was carried out by Gene Genius Super system.

### Western blot analysis

Cells were lysed with loading buffer (50 mM Tris, 150 mM NaCl, 2% SDS, and 0.5% Triton X-100) containing 2 mM EDTA and 1%  $\beta$ -mercaptoethanol. Cells were removed from the dishes by cell scraping. Samples were then subjected to boiling for three 3 min and centrifuged at 12,500 rpm for 30 min. Protein concentration of the samples was determined by Bradford Protein Assay Reagent kit, and whole cell lysates were analyzed by 8 and 10% SDS-PAGE and stained with Coomassie BBR-250 (Sigma) to ensure equal loading. Samples were then transferred to polyvinylidene difluoride membranes (Millipore). The membranes were blocked in Tris-buffered saline containing 0.2% Tween 20 and 5% fat-free dry milk for 1 h at room temperature, and then incubated with rabbit polyclonal antibody  $\beta$ -actin (santa cruz, sc1616-2, 1:150 dilution), caspase-3 (santa cruz, sc-7148, 1:200 dilution), and PARP (Santa cruz, sc-7150, 1:200 dilution) for 1 h at room temperature with agitation, followed by incubation with horseradish peroxidase-conjugated antimouse secondary antibody (Santa cruz, sc-2004, 1:1000 dilution). Blots were developed using an enhanced chemiluminescence detection kit (ECL; Pierce Biotechnology). As for protein loading analysis, the intensity of protein bands was determined by densitometry with Gene Genius Super system (Gene Company limited, EN).

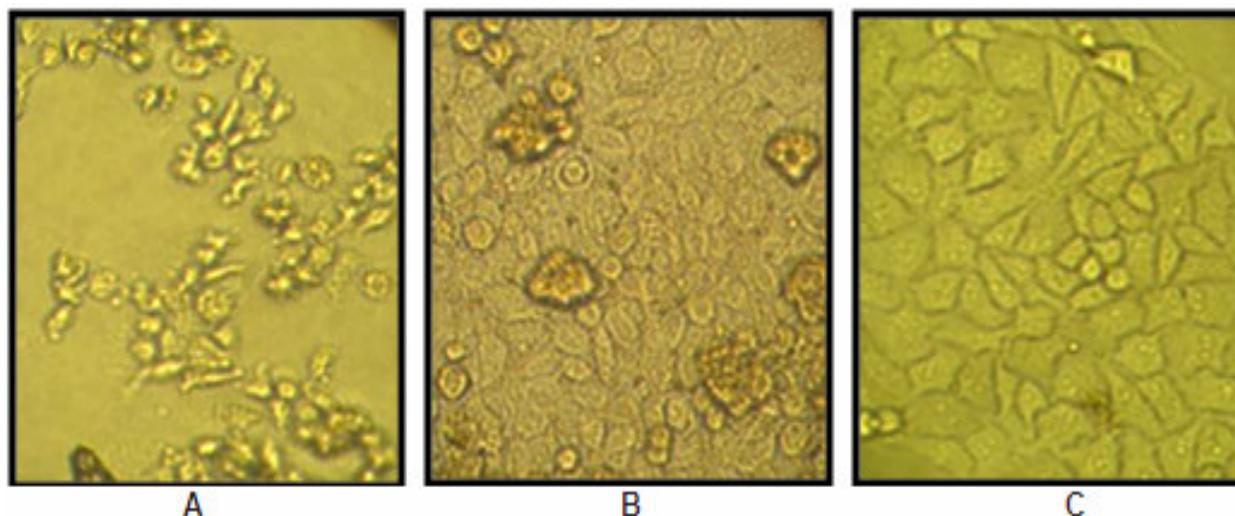
### Statistical analysis

The results were expressed as mean  $\pm$  SD of eight times (n=8) determinations from MTT assay experiment. All experiments were repeated at least three times to confirm similar results. Statistical significance was assessed by one-way analysis of variance. Western blot at each point of time were determined by densitometric scanning using the Gel Analysis Software (Bandscan). The signals from caspase-3 and PARP were standardized to that of  $\beta$ -actin.

## RESULTS

### Weight, appearance, and solubility of the extracts

The methanol extraction yielded 12.5 mg [0.03% (w/w)] of



**Figure 1.** Morphological influences on HeLa cells by *L. williamsii* extracts. (A) HeLa cells treated with methanol extract of 100  $\mu\text{g/ml}$  for 48 h, demonstrating reducing number of cells with shape distortion, plasma shrinkage in size significantly reduced. (B) HeLa cells treated with HCl extract in 100  $\mu\text{g/ml}$  for 48 h, can see accumulation of numerous necrotic cells. (C) HeLa cells treated with NaOH extract in 150  $\mu\text{g/ml}$  for 48 h, no obvious morphological changes.

brown powder from 50 g of *L. williamsii*, which readily dissolved in methanol at room temperature. Extraction with HCl yielded a grayish white powder of 404.3 mg [0.81% (w/w)], which was soluble in HCl at room temperature at a slower rate. NaOH extraction resulted in a dark brownish powder of 126.2 mg [0.25% (w/w)]; the solute was difficult to dissolve in NaOH without increasing the temperature.

#### Determination of polysaccharide content

The polysaccharide content in the HCl and NaOH extracts were 16.2% (w/w) and 4.6% (w/w), respectively. The methanolic extract was negative for polysaccharides.

#### Morphological changes in HeLa cells after treatment with the extracts

Obvious morphological changes in HeLa cells were observed after 48 h of treatment with the methanolic extract. These include a reduction in the number of cells with distorted shapes and condensation of cytoplasm, which resulted in significantly smaller cells. Medium acidification was also observed. These phenomena were most obvious when the concentration of the extract was increased up to 100  $\mu\text{g/mL}$  (Figure 1A). The HCl group exhibited fewer but different changes compared with the methanol group. It involved the accumulation of a large number of necrotic cells. Medium acidification of the HCl extract group was also evident. The changes were most significant at an extract concentration of 100  $\mu\text{g/mL}$

(Figure 1B). The NaOH group showed no obvious morphological changes at 150  $\mu\text{g/mL}$ . The HeLa cell growth appeared better than in the control group, with cluster formation and higher cell density (Figure 1C).

#### Cytotoxicity of the peyote extracts against HeLa cells

In Table 1 and Figure 2, the *in vitro* MTT analysis screening of the extracts of HeLa cells shows that the methanolic and HCl extracts strongly inhibited the HeLa cells in a dose-dependent manner. The NaOH extract had almost no effect on the HeLa cells. At 100  $\mu\text{g/mL}$  and above, the extract killed more than 90% of the cells. The median effective concentrations ( $EC_{50}$ ) of the methanolic and HCl extracts were 4.2 and 7.8  $\mu\text{g/mL}$ , respectively. Both extracts are therefore cytotoxic according to the National Cancer Institute, which states that extracts with  $EC_{50} < 20\mu\text{g/ml}$  are considered cytotoxic against the treated cells. The NaOH extract had little or no cytotoxicity on the HeLa cells.

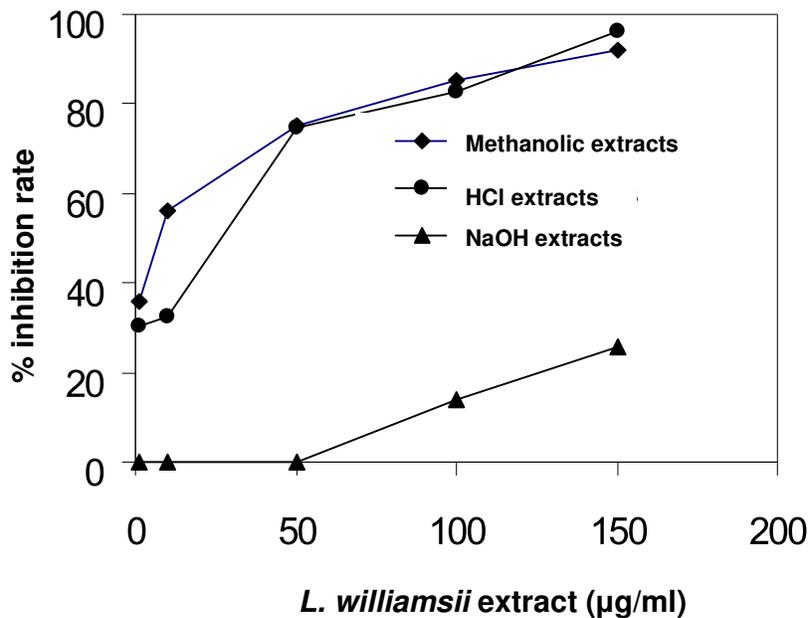
#### DNA ladder induced by *L. williamsii* extracts in HeLa cells

In Figure 3, Band 1 is the DNA marker, and Band 2 is the DNA ladder of the HeLa cells treated with methanolic extract. The HCl extract (Band 3) and the control group (Band 4) showed no DNA ladder bands. Therefore, the methanolic extract induced apoptosis in HeLa cells, whereas the HCl and NaOH extracts had no apparent effect.

**Table 1.** MTT assay analysis of cytotoxic effect of *L. williamsii* extracts on Hela cells (\* P < 0.05 compared with control).

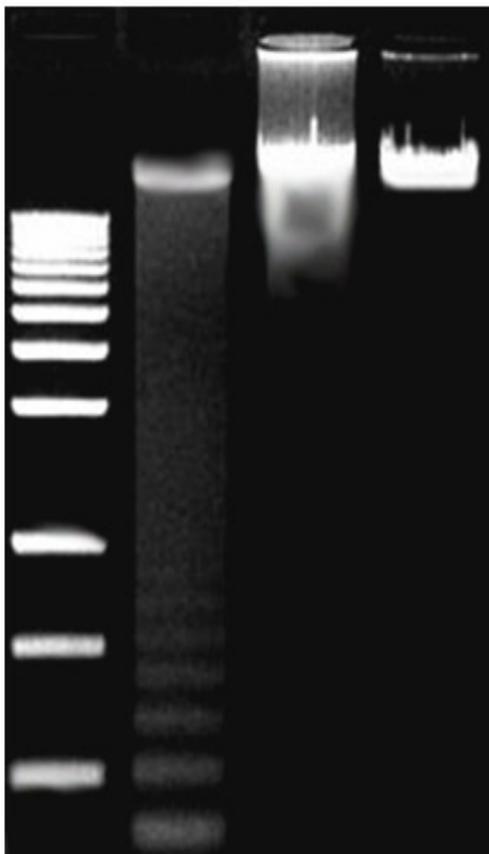
Extracts (µg/ml)	n	A <sub>540</sub>	Inhibition rate (mean %)
<b>Control</b>	8	0.586 ± 0.109	—
<b>Methanolic extracts</b>			
1	8	0.375 ± 0.055*	36.01
10	8	0.257 ± 0.035*	56.14
50	8	0.145 ± 0.037*	75.26
100	8	0.087 ± 0.018*	85.15
150	8	0.047 ± 0.020*	91.98
<b>HCl extracts</b>			
1	8	0.409 ± 0.086*	30.20
10	8	0.395 ± 0.060*	32.60
50	8	0.149 ± 0.047*	74.57
100	8	0.102 ± 0.009*	82.59
150	8	0.021 ± 0.008*	96.41
<b>NaOH extracts</b>			
1	8	0.610 ± 0.106	—
10	8	0.559 ± 0.062	—
50	8	0.564 ± 0.037	—
100	8	0.469 ± 0.068*	14.10
150	8	0.406 ± 0.064*	25.64

\* P < 0.05 compared with control.



**Figure 2.** MTT assay analysis cytotoxicity effect of *L. williamsii* extracts on Hela cells. It shows that methanol extracts has obvious cytotoxicity effect on Hela cells, and was produced a dose-dependent inhibition. The HCl extract showed slightly less inhibition at concentration less than 50 µg/ml than methanol extract, and NaOH group has no effect on Hela cells. (n=8).

Band1 Band2 Band3 Band4



**Figure 3.** DNA ladder induced by *L. williamsii* extracts on HeLa cells. First band depicts DNA marker, second was DNA ladders of methanol extract treated HeLa cells, and HCl extract (third band) and control group (fourth band) showed no DNA ladder bands.

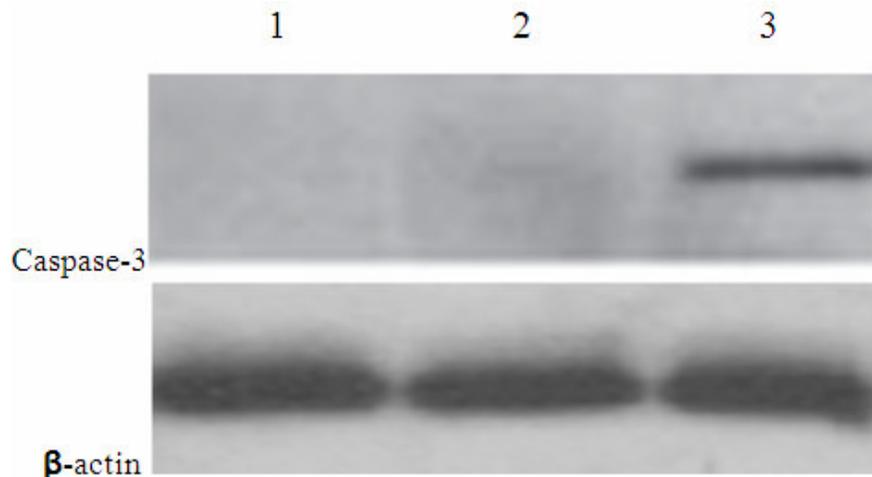
### Western blot analysis of the apoptosis-related cytotoxicity of *L. williamsii* extracts on HeLa cells

Western blot analysis was performed to determine the mechanism of *L. williamsii* extract cytotoxicity in HeLa cells. The 6 h methanolic extract treatment resulted in a significant increase in caspase 3 expression in the HeLa cells. No caspase 3 expression was observed in the HCl and NaOH groups under the same conditions (Figure 4). Aside from measuring the effects of the extracts, determining how apoptosis was induced in the HeLa cells is also important. The levels of caspase-3 and Poly (ADP-Ribose) Polymerase (PARP) were increased drastically when the cells were treated with the methanolic extract for 4 h. The level of caspase 3 at 2 h was half that at 0 h, and it was approximately doubled at 6 h. The levels of PARP increased with time. These results indicate that the apoptotic mechanism in the HeLa cells was mainly

through the activation of caspase 3 and its substrate PARP. The caspase-3 and PARP expression indicates that the apoptosis was stimulated by the methanolic extract (Figure 5).

### DISCUSSION

Ethnopharmacology, since the beginning of medicine, defines ways to discover biologically active compounds from plants (Cordell et al., 1991; Cragg et al., 1994). Methanolic extracts of plants are normally used for anticancer screening because traditional practitioners believe that polar compounds are mostly responsible for the claimed anticancer properties. In this study, the methanolic and HCl extracts of *L. williamsii* showed significant cytotoxicity against HeLa cells. The activities of this plant may be from the highly complex glycosides and



**Figure 4.** Western blotting analysis of caspase-3 expression in HeLa cells after treated with *L. williamsii* extracts for 6 h. 1. NaOH extract group, 2. HCl extract group, 3. methanol extract group. It shows that methanol extract can induce caspase-3 expression on HeLa cells, suggesting occurrence of apoptosis at this time.

saponins (methanolic extract), as well as the polysaccharides (HCl extract).

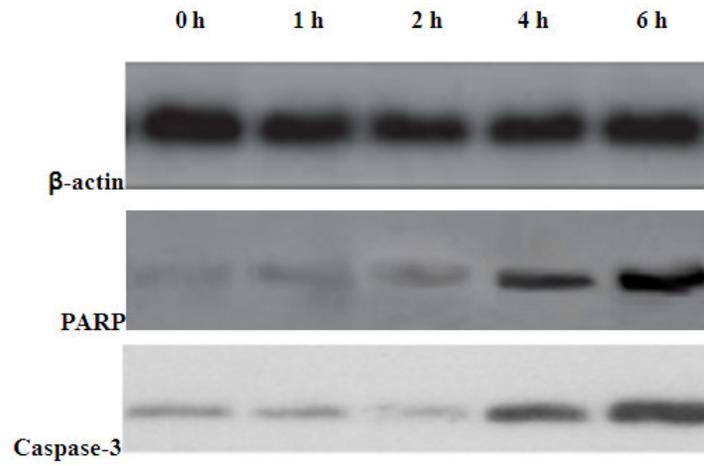
*L. williamsii*, the source of has been used for centuries by the Mexican Indians (Huichol Indians) and the Plains Indian Tribes of the United States and Canada as a ceremonial sacrament (Franco-Molina et al., 2003). It is potentially teratogenic when used in excess (Dorrance et al., 1975). Current studies have shown that the *L. williamsii* extracts are capable of activating nitric oxide and cytokine production in macrophages, lymphocytes proliferation, and is cytotoxic against certain murine and human tumor cells *in vitro*. Moreover, it may directly kill tumor cells (Franco-Molina et al., 2003). Mescaline, the main active compound in peyote, has been shown to inhibit *in vitro* concanavalin A-stimulated murine lymphocyte proliferation *in vitro* (Sissors et al., 1978). In this study, the peyote extract significantly reduced the viability of the HeLa cells, which are sensitive to cytotoxicity. The methanolic extract of peyote had a greater effect on the HeLa cells than the HCl extract; the NaOH extract had little or no effect on the HeLa cells.

The earliest recognized morphological changes during apoptosis are compaction and segregation of the nuclear chromatin, resulting in chromatin margination and condensation of the cytoplasm (Kerr et al., 1972). The progression of the condensation is accompanied by convolution of the nuclear and cell outlines, followed by the breaking up of the nucleus into discrete fragments and by the budding of the cell as a whole to produce membrane-bound apoptotic bodies. The apoptotic bodies are quickly ingested by nearby cells and degraded within their lysosomes (Kerr et al., 1972, 1994). Apparent morphological changes were observed in the HeLa cells treated with the methanolic extract. Cell shrinkage and

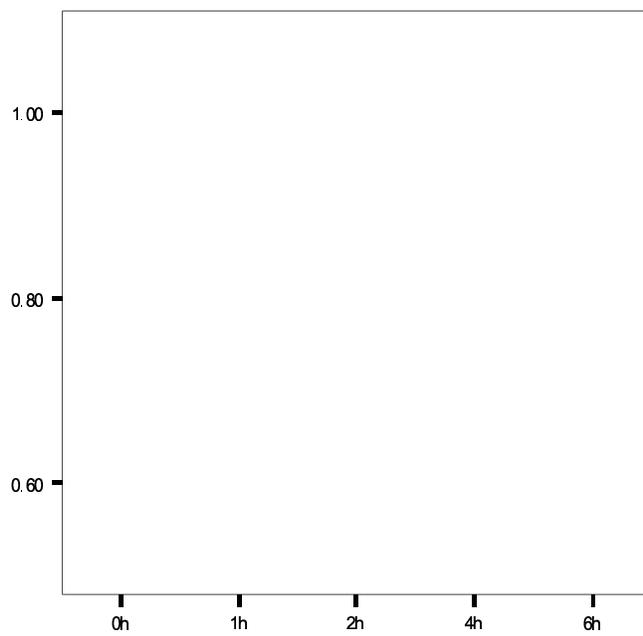
distortion concurrent with cytoplasmic condensation during apoptosis were seen in the cells treated with this extract, whereas those treated with the HCl and NaOH extracts displayed no apoptotic changes. The HeLa cells treated with the methanolic extract clearly demonstrated a DNA ladder (Figure 3).

The family of caspases regulates apoptosis. Caspases are normally present in the cell as proenzymes that require limited proteolysis to activate enzymatic activity (Nunez et al., 1998). Once activated, caspases cleave a variety of intracellular polypeptides, including major structural elements of the cytoplasm and nucleus, components of the DNA repair machinery, and a number of protein kinases. Collectively, these scissions disrupt the survival pathways and disassemble important architectural components of the cell, which contribute to the stereotypic morphological and biochemical changes that characterize apoptosis. Among the caspases, caspase 3 is the most commonly activated in the apoptotic process (Janicke et al., 1998). It mediates the limited proteolysis of the structural protein gelsolin, p21-activated kinase 2 (PAK2), Focal Adhesion Kinase (FAK), and rabaptin 5 (Cosulich et al., 1997; Kothakota et al., 1997; Rudel et al., 1997; Wen et al., 1997), and the cleavage inactivation of DNA fragmentation factors such as DFF45 and ICAD (Liu et al., 1997; Enari et al., 1998; Sakahira et al., 1998).

PARP, or poly (ADP-ribose) glycohydrolase, is an enzyme that cleaves the ribose-ribose bond. It was discovered in 1971 by Masanao Miwa and Takashi Sugimura. It is a 116 kDa nuclear poly (ADP-ribose) polymerase involved in DNA repair, predominantly in response to environmental stress. This protein can be cleaved by many interleukin-1-beta converting enzyme



PARP



ERROR: rangecheck  
OFFENDING COMMAND: .buildcmap

STACK:

-dictionary-  
/WinCharSetFFFF-V2TT9BF4ACCA  
/CMap  
-dictionary-  
/WinCharSetFFFF-V2TT9BF4ACCA