

## RESEARCH ARTICLE

# Bracken-fern Extracts Induce Cell Cycle Arrest and Apoptosis in Certain Cancer Cell Lines

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### Abstract

Bracken fern [*Pteridium aquilinum* (L.) kuhn (Dennstaedtiaceae)] is one of the most common species on the planet. It has been consumed by humans and animals for centuries. Use by some human groups is because they believe bracken fern is good for health as plant medicine. However, it is also one of the few known plants that can cause tumors in farm animals. Many interested groups have focused their attention on bracken fern because of these interesting features. In order to evaluate the biological effects of exposure to this plant in cellular level, human cancer cell lines were treated with the fern dichloromethane extracts and the genotoxic and cytotoxic effects were studied. Anti-proliferative/cytotoxic effects were evaluated by cell count, MTT assay and flow cytometry methods with three different cancer cell lines, TCC, NTERA2, and MCF-7, and two normal cells, HDF1 and HFF3. Pro-apoptotic effects of the extracts were determined by DAPI staining and comet assay, on TCC cancer cells compared to the normal control cell lines. Cellular morphology was examined by light microscopy. Our present study showed that the extract caused DNA damage and apoptosis at high concentrations (200 µg/mL) and also it may induce cell cycle arrest (G2/M phase) at mild concentrations (50 and 30 µg/mL) depending on the cell type and tumor origin. These results indicate that bracken fern extract is a potent source of anticancer compounds that could be utilized pharmaceutically.

**Keywords:** Bracken ferns - cell proliferation - apoptosis - cell cycle arrest

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### Introduction

Search for anticancer drugs throughout the recent decades have gained great deal of attentions (Backorova et al., 2011). Reagents with natural origin continue to be of preferred priority in the drug screening programs (Lee, 2004). Today, the plants are well documented as a good source of biologically active compounds (Iranshahi et al., 2007; 2008). The plant compounds are widely used in treating cancer. Some of these compounds show anti tumor activity (Iranshahi et al., 2009; Sahebkar and Iranshahi, 2010). Bracken fern [*Pteridium aquilinum* (L.) kuhn (Dennstaedtiaceae)] as one of the most popular herbs on the planet (Taylor, 1990), is believed to carry such properties. Some human groups, particularly in the Orient, have been consuming this plant for centuries, because they believe bracken fern is good for health as plant medicine (Sugimura, 2000). Also it is one of the few known plant that can cause tumors in farm animals (Taylor, 1990; Alonso-Amelot and Avedano, 2002). Many interested groups have focused their attention on bracken fern because they believe it is interesting plant. There are

studies showing that DNA damage is also induced by bracken-fern in human cells (Siman et al., 2000). Also, it can induce DNA adduct in animal tissues (Freitas et al., 2001). In the later study, a bracken fern extract (BFE) was used to treat human oral cell lines *in vitro*, leading to DNA damage and apoptosis, as assessed by the comet assay (Pereira et al., 2009). Different active components of the crude extract of bracken fern may cause different responses. In this study we asked whether the BFE would exhibit an anti-proliferative and pro-apoptotic effects on different cancer cell line, by examining the effects of dichloromethane extract of the plant on growth of three human cancer cell lines. The possible mechanism of action for the extract on the cells was also assessed.

### Materials and Methods

#### *Plant material and extracts*

Bracken fern (*P. aquilinum*) samples were collected from savanna of Guilan, Northern Iran in March of 2010, and identified botanically by experts in Ferdowsi University of Mashhad herbarium (FUMH). A voucher

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specimen was deposited in the herbarium of the School of Pharmacy, Mashhad University of Medical Sciences, under accession No. 12562. The dichloromethane and methanol extracts were prepared from aerial parts of the plant. Dried aerial parts were grounded in a Willey mill. Resulting powders (100 g each) were macerated with 1000 mL of each distilled dichloromethane and methanol for 48 h at room temperature separately. The dichloromethane and methanol crude extracts were evaporated and dried out after collection, and the extracts were stored in -20°C until further use. To prepare different concentrations (75, 150, 300, 600 and 1200 µg/mL) of methanol extracts, 10 mg of the powder was first dissolved in 150 µl of dimethyl-sulfoxide (DMSO, Merck, Germany) and diluted with complete culture medium to reach the desired dilution. In order to prepare different concentrations of dichloromethane extract (75, 150, 300, 600, 1200 µg/mL for cell count and 25, 50, 100, 200, 400, 800 µg/mL for MTT assay), 10 mg of the powder was dissolved in 150 µL dimethyl-sulfoxide (DMSO, Merck, Germany), and diluted with complete culture medium before experiments.

Cisplatin, vincristine and doxorubicin (Sobhan, Iran) were considered as positive controls in this study. Different concentrations of cisplatin (4, 12 and 36 µg/mL), vincristine (10, 20, 40, 80 and 160 µg/mL), doxorubicin (2, 4, 8, 16, 32 and 64 µg/mL) were prepared using complete culture medium, before experiments.

#### Cell culture procedures

Three different human cancer cell lines, TCC (Transitional Cell Carcinoma), NTERA2 C1. D1 (Embryonal Carcinoma), and MCF7 (Human Breast Adenocarcinoma) were obtained from Pasteur Institute (Tehran, Iran). All cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, Scotland), supplemented with 10% fetal bovine serum (FBS, Gibco, Scotland). Cells were maintained at 37°C in a humidified 10%CO<sub>2</sub> atmosphere. Two different human normal cell lines, HDF1 (Human Dermal Fibroblast), and HFF3 (Human Foreskin Fibroblast), were a generous gift from Royan Institute (Tehran, Iran). These cells were grown in Dulbeccos modified Eagle's medium (DMEM, Gibco, Scotland), supplemented with 15% fetal bovine serum (FBS, Gibco, Scotland) and incubated at 37°C in a humidified atmosphere of 5% in air. In order to subculture the TCC cells, they were washed with Phosphate Buffer Saline (PBS) and incubated with 0.25% trypsin and 1 mM ethylenediaminetetraacetic acid (EDTA) for 3-5 min. The detached cells were re-suspended in fresh serum-containing medium to inactivate the trypsin, and transferred to new labeled flasks as required.

#### Cytotoxicity assays

The growth rate of the cells was assessed in two different ways of direct cell count and MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. First, cells were counted as an indicator for cell growth. Cells (TCC, NTERA2 and HDF1) trypsinized and seeded at a density of 180×10<sup>3</sup> cells/well in 6-well tissue culture plates (Falcon Becton-Dickinson, USA) in

a total volume of 2 mL of medium. To detect the cytotoxic effects of the methanol and dichloromethane extracts, 24 h after incubation, the TCC cells were treated by methanol and dichloromethane extracts, and the NTERA2 cells were treated by dichloromethane extracts. Both cell lines were treated at variable concentrations of 75, 150, 300, 600 and 1200 µg/mL for 24, 48 and 72 h. the HDF1 cells were also treated by dichloromethane extract at different concentrations of 75, 150 and 300 µg/mL for 24, 48 and 72 h. The half maximal inhibitory concentrations (IC<sub>50</sub>) values of cisplatin was also determined after incubating the TCC and NTERA2 cells with increasing concentrations of 4, 12 and 36 µg/mL for different time periods of 24, 48 and 72 h. Briefly, after incubation with or without plant extracts, the cells were detached by 250 µL trypsin at 37°C for 3-4 min and then re-suspended in 750 µL PBS.

The number of suspended cells were counted with a neobar lam (HBG, Germany). All four corner squares of the neobar lam were counted, and the average number was calculated. The assessment of the cell viability was carried out by the MTT assay method (Mosmann, 1983) using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma, Deisenhofen, Germany). The tetrazolium dye is reduced by living cells, and this reaction is used as the end point in a rapid drug-screening assay. Briefly, TCC, NTERA2, MCF-7 and HFF3 cell lines trypsinized and seeded at a density of 8×10<sup>3</sup> cells/well in 96-well tissue culture plates (Falcon Becton-Dickinson, USA) in a total volume of 200 µl of medium. After 24 h incubation, the cells were treated for 24, 48 and 72 h with increasing concentrations 25, 50, 100, 200, 400 and 800 µg/mL of the dichloromethane extract. The IC<sub>50</sub> values of vincristine and doxorubicin were also determined after incubating the TCC, NTERA2 and MCF-7 cells with increasing concentrations of vincristine (10, 20, 40, 80 and 160 µg/mL) and doxorubicin (2, 4, 8, 16, 32 and 64 µg/mL) for 24, 48 and 72 h. Then the cells were incubated in fresh cell culture medium for 30 min that was again replaced by fresh cell culture medium (Bruggisser et al., 2002).

To assay the cell viability, 5 mg MTT dye (Sigma, Germany) was dissolved in 1 mL phosphate buffered saline (PBS), filtered to sterilize and used freshly before each experiment. 20 µl MTT solution was then added to each well and the plates were incubated for 4 h at 37°C. After this time, the medium was removed and the formazan product in the cells was solubilized by adding 200 µl of DMSO. Absorbance for each well was measured spectrophotometrically at 570 nm (single wavelength) using an ELISA plate reader (Awareness, USA).

All the tests were performed as triplicate. Percentage of cell viability is expressed as [(OD value of treated cells/ mean OD value of control cells)×100%].

#### Morphological studies

The cells were cultured with various concentrations (75, 150, 300, 600, 1200 µg/mL for cell count and 25, 50, 100, 200, 400, 800 µg/mL for MTT assay) of the BFE and their DMSO controls. Their morphology were then monitored under a light inverted microscope (Olympus, Japan).

### Alkaline comet assay

In order to evaluate the bracken fern genotoxicity, the alkaline version of the comet assay, which is a highly sensitive and reliable biochemical method for detection of variety of DNA lesions (Singh et al., 1988) was performed, with modifications. Briefly, the TCC cells were incubated with 200  $\mu\text{g/mL}$  dichloromethane extract for 24 h. At the same time the cells, treated with its equivalent amount of DMSO (0.3%), were used as controls. They were then trypsinized and centrifuged at 3000 rpm (Orto Alresa Digicen 20, Spain) for 15 min. After suspending the cell pellets in 25  $\mu\text{l}$  PBS, they were mixed with 75  $\mu\text{L}$  of 0.75% (w/v) low melting point agarose (LMA, Fermentas, Germany). The cell suspensions were then dispensed onto the glass microscopic slides, precoated with 1% (w/v) normal melting agarose (Helicon, Russia), covered with a coverslip, and kept for 20 min at 4°C. The coverslips were removed, slides were soaked with 100  $\mu\text{l}$  of 0.75% (w/v) LMA, and new coverslips were placed back and kept for another 20 min at 4°C.

Three slides were prepared for each sample. After immersing the slides in freshly prepared ice-cold lysing buffer (2.5 M NaCl, 100 mM Na<sub>2</sub>EDTA, 10 mM Tris, 2% (v/v) triton X-100, pH 10) and incubating for 4 h at 4°C, they were washed with cold distilled water, placed in an electrophoresis chamber [filled with freshly prepared cold alkaline electrophoresis buffer (1mM EDTA, 0.3 N NaOH, pH 13)], and incubated for 30 min at 4°C. Electrophoresis was performed at 4°C for 20 min at 25 V, and 300 mA. Slides were then washed three times with ice-cold neutralizing buffer (0.4 M Tris-HCL buffer, pH 7.5), dried with 96% ethanol, stained with ethidium bromide (20 mg/mL), and analysed using a fluorescent microscope (Olympus, Japan). 60 cells per slide were assayed and the mean of the comet tail moment (the breakage of DNA in tail and DNA migration) was measured using TiTek Cometscore version 1.5 software. This test was performed in triplicates. The DNA damage was expressed as % tail DNA, where % tail DNA=[tail DNA/(head DNA+tail DNA)] $\times$ 100.

### Apoptotic morphology assessment

For semi-quantitative analysis of apoptosis, cells were stained with 4', 6-diamidino-2-phenylindole (DAPI), which detects the DNA in cells. The TCC cells were treated with 200  $\mu\text{g/mL}$  or its equivalent amount of 0.3% DMSO, as control, for 24 h, washed with PBS, and fixed in 4% paraformaldehyde (Sigma, Germany). They were then washed with PBS and centrifuged at 1,500 rpm (Orto Alresa Digicen 20, Spain) for three times, penetrabilised with 0.1% triton X-100 and stained with 2  $\mu\text{g/mL}$  DAPI (Merck, Germany) for 10 min at 37°C. At the end, about 700 cells from each treatment were determined and counted under fluorescent microscope (Olympus, Japan). When bound to double-stranded DNA the absorption maximum for DAPI is 358 nm (ultraviolet) and its emission maximum is at 461 nm (blue). This experiment was performed in 3 separate times. Nuclear fragmentation and chromatin condensation were the standard criteria used as signs of apoptosis.

### Cell cycle analysis by flow cytometry

To examine the effect of dichloromethane extract on cell cycle phase distribution, the TCC and MCF-7 cells were treated with the extract at concentrations of 50 and 30  $\mu\text{g/mL}$ . The adherent cells were harvested 24 h after treatment and washed in PBS separately. The cells were centrifuged at 200 rcf (Orto Alresa Digicen 20, Spain) for 2 times and re-suspended in 500 and 350  $\mu\text{L}$  of cold PBS respectively. Then the cells were stained with staining solution [30  $\mu\text{l}$  of propidium iodide (40  $\mu\text{g/mL}$ ), 50  $\mu\text{L}$  of 0.1% Triton X-100, 50  $\mu\text{L}$  of 0.1% sodium citrate and 15  $\mu\text{L}$  of RNase A (100  $\mu\text{g/mL}$ )]. The samples were kept in dark conditions at 4°C for 1 h and analyzed with a flow cytometer (FACS Calibure, Becton Dickinson, USA). When bound to nucleic acids the absorption maximum for PI is 535 nm and the fluorescence emission maximum is 617 nm. The WinMid software was used to quantify the number of cells in individual phases of the cell cycle and analyze the data. All the tests were performed in duplicates.

### Statistical analyses

Statistical procedures were performed with SPSS, JMP4 and MSTAT softwares. The statistically different groups were identified by one way analysis of variance (ANOVA), followed by Tukey multiple comparison tests. Results were expressed as the mean  $\pm$ SD. A p-value of <0.05 in Tukey test and <0.001 in LSD test was considered significant.

## Results

### Fern extracts show cytotoxic effects on cancer cells

Counting the TCC cells, treated with dichloromethane and methanol extracts of bracken fern, showed the former is more cytotoxic. The half maximal inhibitory concentrations (IC<sub>50</sub>) for the methanol extract on TCC cells were 850 and 600  $\mu\text{g/mL}$  after 48 and 72 h, respectively, while that of the dichloromethane extract on TCC cells were determined as 210, 140 and 112  $\mu\text{g/mL}$  after 24, 48 and 72 h, respectively. Therefore in the following experiments the effects of the dichloromethane extract (BFE) was the focus of investigation. This extract significantly reduced the proliferation rate of the NTERA2

**Table 1. IC<sub>50</sub> values ( $\mu\text{g/mL}$ ) Obtained from Direct Cell Count Experiments Representing the Cytotoxic Effects of the Dichloromethane Extract of Bracken Fern on Different Cell Lines, Compared with the Effect of Standard Drug Cisplatin for Different Time Periods after Administration**

Treatment	Time (h)	IC <sub>50</sub> ( $\mu\text{g/mL}$ )		
		NTERA2	TCC	HDF1
BFE*	24	115	210.00	>300
	48	90	140.00	>300
	72	<75	112.00	>300
Cisplatin	24	6	11.95	
	48	<4	4.95	
	72	<4	<4	

\*Bracken Fern Extract

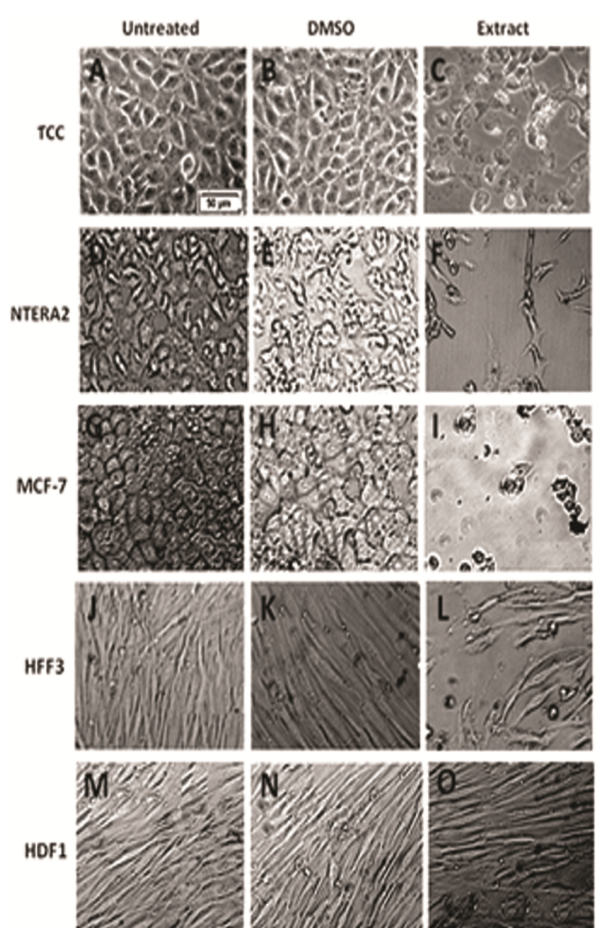


cells in concentration and time dependent manner too. Analyses of cell viability by cell count showed different  $IC_{50}$  values for BFE on the NTERA2 cells (Table 1). These values were compared with the effects of known therapeutic drug of cisplatin. The  $IC_{50}$  values of cisplatin on TCC and NTERA2 are also shown in Table 1. While the extract did not reach the  $IC_{50}$  on HDF1 cells even in 300  $\mu\text{g/mL}$ . Examination of the BFE on the various cell lines demonstrated a time and concentration dependent action as evaluated by the MTT assay. Similar to the results of cell count, the data obtained by MTT assay in this study showed that the  $IC_{50}$  values of the extract on TCC cells were 200, 180 and 100  $\mu\text{g/mL}$  after 24, 48 and 72 h of its administration, respectively. The  $IC_{50}$  of the extract on the NTERA2 and MCF-7 cell lines are shown in Table 2. To compare this cytotoxic efficiency with the effects of known therapeutic drugs, the TCC and NTERA2 cells were also treated with different concentrations of

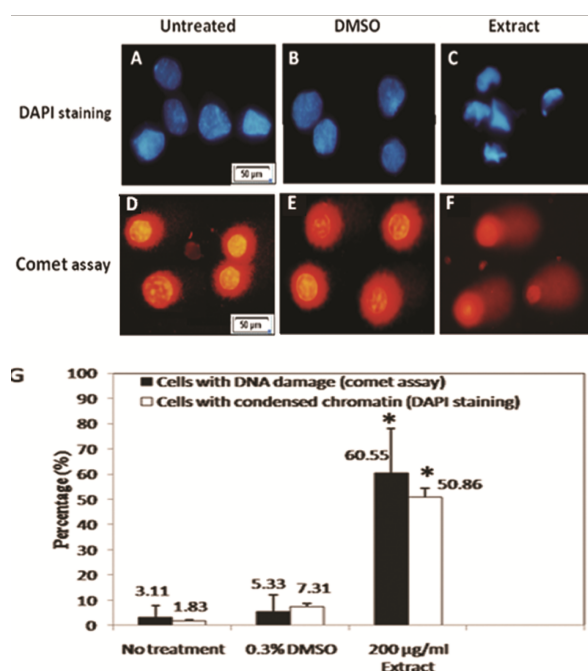
vincristine for 24, 48 and 72 h. The MCF-7 cells were also treated with different concentrations of doxorubicin for the same period of time. The  $IC_{50}$  values of vincristine on the TCC and NTERA2 cells are shown in Table 2. Furthermore, the different  $IC_{50}$  values of doxorubicin on the MCF-7 cells were obtained (Table 2). Treatment of the normal cells (HFF3) with BFE for 24, 48 and 72 h also showed concentration-dependent growth inhibition but with a higher  $IC_{50}$  values (Table 2). In our study the sensitivity of the cell lines to BFE, from the most sensitive to the most resistant, was as follows: MCF-7 > NTERA2 > TCC > HFF3/HDF1. These results demonstrate that growth inhibition of the BFE is more effective in cancer cell lines than that in the normal examined cells.

#### Morphological alterations in the cells treated by the fern extracts

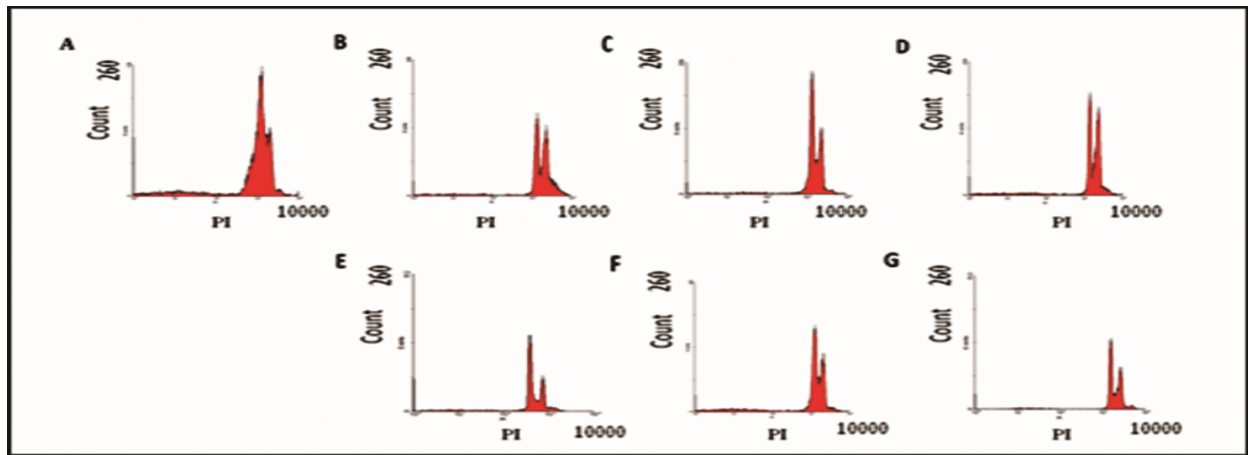
The cytotoxic effects of the BFE on the cancer cell lines (TCC, NTERA2, MCF-7) were also confirmed by morphological observations. The TCC, NTERA2, MCF-7, HFF3 and HDF1 cells, treated with 100, 50, 230 and 300  $\mu\text{g/mL}$  of the BFE were monitored after 72 h of its administration. A large number of the cells became spherical and deformed with granulated cytoplasm. Cell populations were greatly decreased in comparison to those of the untreated and control cultures (Figure 1). The morphological analyses showed that BFE was cytotoxic to cancer cells more effectively than the normal cells.



**Figure 1. Morphological Examination of Normal and Cancer Cells Treated with Dichloromethane Extract.** Cancer and normal cells were monitored for 72 h. The TCC cells treated with 100  $\mu\text{g/mL}$  dichloromethane extract (C) and 15% DMSO (B), NTERA2 cells treated with 50  $\mu\text{g/mL}$  dichloromethane extract (F) and 0.075% DMSO (E), MCF-7 cells treated with 50  $\mu\text{g/mL}$  dichloromethane extract (I) and 0.075% DMSO (H), HFF3 cells treated with 230  $\mu\text{g/mL}$  dichloromethane extract (L) and 0.34% DMSO (K), HDF1 cells treated with 300  $\mu\text{g/mL}$  dichloromethane extract (O) and 0.45% DMSO (N). The morphological analyses showed that the extract is cytotoxic to cancer cells. Magnification: 50 X; BFE: Bracken Fern Extract

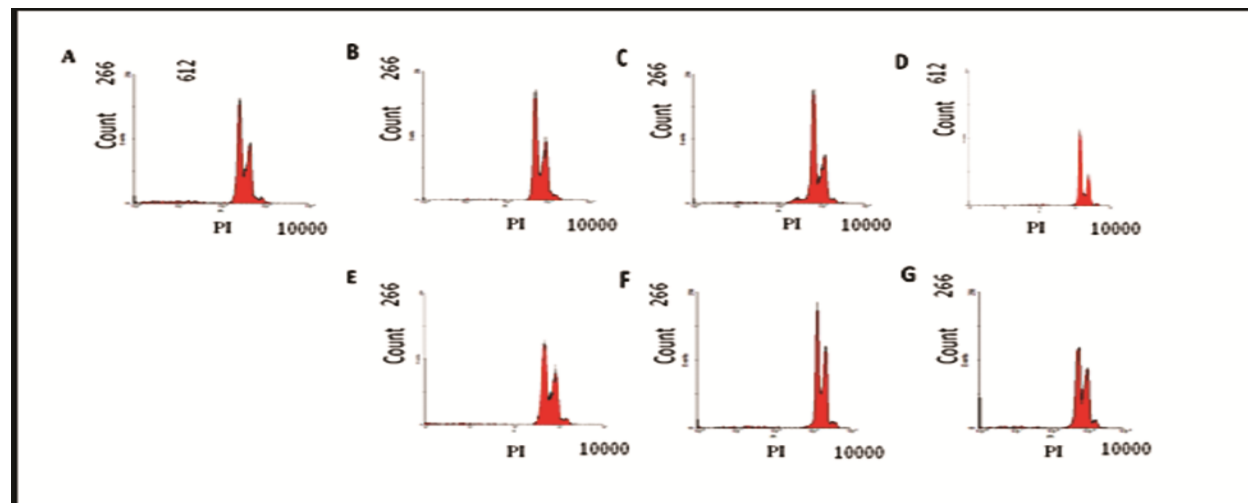


**Figure 2. DNA Damage and Chromatin Condensation Examined by DAPI Staining (A-C) and Comet Assay (D-F) in Untreated, DMSO and Bracken fern Dichloromethane Extract (BFE) Treated TCC Cells.** TCC cells in control group were treated with 0.3% DMSO and in the test group with 200  $\mu\text{g/mL}$  of the bracken extract for 24 h. The Cells with condensed chromatin and DNA in tail nuclei were calculated and histograms prepared as a percentage of the total nuclei of the cells (G). Data are shown as mean  $\pm$  SD. The Asterisks indicate the P value ( $P < 0.05$ ) of differences between the treated and untreated cells. BFE: Bracken Fern Extract



Time: 24 h		Dichloromethane bracken extract		Vincristine		DMSO		Not treated
Concentration (µg/mL)		50	30	50	30	0.075%	0.045%	
Percentage	G0/G1	49 ± 12.7	47.7 ± 5.1	35.2 ± 3.8	46 ± 4.2	50.5 ± 2.6	45.9 ± 3.8	63.2 ± 2.4
	S	5 ± 2.8	5.3 ± 1.8	3.0 ± 1.2	6.2 ± 2.1	12.6 ± 2.1	10.9 ± 2.4	7.2 ± 1.5
	G2	32.5 ± 9.1	30.4 ± 3.2	47.5 ± 3.5	29.7 ± 8.1	23.3 ± 2.6	29.1 ± 6.3	18.0 ± 1.5

**Figure 3. Propidium Iodide Staining of TCC Cells Indicating the Cell Population in Each Cell Cycle Stage, Examined by Flow Cytometry.** The TCC cells are arrested in G2 phase of the cell cycle after treating with dichloromethane extract of bracken fern and vincristine for a period of 24 h. The treatments are as follows: untreated TCC cells (A), Cells treated with 50 and 30 µg/mL of BFE (B and E respectively), 0.075% and 0.045% of DMSO (C and F respectively), and 50 and 30 µg/mL of vincristine (D and G respectively). Distribution of the cells (in percentage) are compared between treatments of two concentrations, 50 and 30 µg/mL (mean±SD)



Time: 24 h		Dichloromethane Bracken extract		Vincristine		DMSO		Not treated
Concentration (µg/mL)		50	30	50	30	0.075%	0.045%	
Percentage	G0/G1	57.3 ± 1.4	48.0 ± 2	47.1 ± 16.2	51.5 ± 3.2	50.7 ± 7.6	41.9 ± 7.5	57.6 ± 0.3
	S	3.8 ± 0.1	8.35 ± 0.82	7.38 ± 1.6	5.59 ± 0.0	9.1 ± 2.2	8.0 ± 3	5.61 ± 1.9
	G2	33.2 ± 2.9	32.0 ± 1.3	18.7 ± 8.8	35.3 ± 1.9	28.9 ± 2.6	34.6 ± 5.5	32.3 ± 4.9

**Figure 4. Propidium Iodide Staining of MCF-7 Cells Indicating the Cell Population in Each Cell Cycle Stage, Examined by Flow Cytometry.** Dichloromethane extract (BFE) at selected concentrations did not alter cell cycle distribution in MCF-7 cell line. The MCF-7 cells arrest in S phase following 50 µg/mL doxorubicin treatment. The cell treatments are as follows: Untreated (A), 50 and 30 µg/mL of BFE (B and E respectively), 0.075% and 0.045% of DMSO (C and F respectively), and 50 and 30 µg/mL doxorubicin (D and G respectively) for 24 hours. Distribution of the cells (in percentage) are compared between treatments of two concentrations, 50 and 30 µg/ml (mean±SD)

### *Fern extract is genotoxic to the TCC cell line*

In order to determine the mechanisms involved in anti-proliferative/cytotoxic effects of the BFE, the extent of DNA damage and chromatin condensation were analyzed by comet assay and DAPI staining on TCC lines (Figure 2). Figure 2G represents photomicrographs of DNA lesions of untreated cells in comparison with the cells treated with the extract. It is obvious that the extract is genotoxic and that it induced cellular degeneration by apoptosis in the TCC cell line.

### *Cell cycle distribution by flow cytometry analysis*

Impact of the BFE on different phases of cell cycle in TCC and MCF-7 cells was evaluated. TCC and MCF-7 cells were treated with the BFE at 50 and 30  $\mu\text{g/mL}$  for 24 h and were subjected to the flow cytometric analysis after PI staining. These concentrations were chosen based on the results from MTT assay. Percentage of the cells at G2/M phase was remarkably increased after treatment of the TCC cells with 50 and 30  $\mu\text{g/mL}$  of the extract for 24 h (Figure 3). These results indicate that the extract induces G2/M phase arrest in the TCC cells. However, the selected concentrations did not alter the cell cycle distribution in the MCF-7 cell line after treatment with the extract (Figure 4). These effects were also compared with the cell cycle distribution of the cells treated with known therapeutic reagents. The TCC and MCF-7 cells were treated with 50 and 30  $\mu\text{g/mL}$  of vincristine and doxorubicin for the same period, respectively. Similar to the results obtained from the extract on TCC cell line, vincristine seemed to arrest the TCC cells at the G2/M phase in both concentration (Figure 3). The treated MCF-7 cells with doxorubicin leads to cell cycle arrest at the S phase at 50  $\mu\text{g/mL}$  (Figure 4).

## **Discussion**

In present study, we investigated the inhibitory effects of the BFE on cell proliferation and cell cycle distribution in different cancer cell lines. The results indicated that this extract caused growth inhibition or even death in the cancer cells in dose and time dependent manners. As the  $\text{IC}_{50}$  values of the extract on TCC, NTERA2 and MCF-7 cells were very close to those of very well known drugs vincristine and doxorubicin. Furthermore, it was revealed that the BFE induces its cytotoxic effects on the cancerous cells in lower concentrations compared to that on the cells of normal HDF1 and HFF3 cells. In order to determine the mechanism of such effects, DAPI staining and comet assay methods were used.

Our findings indicate that the BFE has genotoxic effects at high concentrations (Table 2) on cancer cells and it induces cellular degeneration through apoptosis in TCC, NTERA2, and MCF-7 cell lines. It was previously shown that bracken fern aqueous extract induces DNA damage and apoptosis in the oral cell lines of HSG and OSCC-3 (Pereira et al., 2009). Other studies have indicated that extracts made from spore of bracken fern induced a very high frequency of DNA damage (Siman et al., 2000). Our results also confirm the reports of bracken fern induction of DNA strand breaks. Flow cytometric analysis for the DNA content of the TCC cells, which had received the

BFE, in our experiments showed occurrence of G2/M phase arrest (Figure 3). However, it did not induce such changes in the MCF-7 cells. While doxorubicin, standard drug, induced S phase arrest in the MCF-7 cells (Figure 4). Therefore it is possible, as expected, that the mechanism of action for our extract is different from that of doxorubicin. We found however that the pattern of flow cytometric histograms, obtained from the TCC cells treated with BFE, were very similar to the vincristine treated cells. Thus it is likely that the fern extracts work in the same way as the anti-microtubule reagents, including vincristine. These reagents normally induce disassembly of the G2-phase microtubules array or cause depolymerization of the microtubules.

Numerous reports have indicated that both the microtubule stabilizing agents (like Taxol) and the microtubule depolymerizing agents (like vincristine, vinblastine, and colchicine) (Checchi et al., 2003; Honore et al., 2005; Li et al., 2006) show strong anticancer activity through inhibiting the cell cycle progression and inducing programmed cell death (Li et al., 2006). Therefore our study clearly shows that BFE at mild concentrations (50 and 30  $\mu\text{g/mL}$ ) has cytotoxic effects on bladder cancer cells as well as inhibition of the cell growth through cell cycle arrest. The variation in responses could be due to the presence of several differently active constituents in the crude BFE. The BFE, used in this experiment, may contain different components (Wilson et al., 1998) for example Quercetin (Qu), the major dietary flavonoid, which present in bracken fern (Gibellini et al., 2011) has anti-proliferative and pro-apoptotic effects. We tend to suggest that the BFE may contain high amounts of Qu. Qu has been accepted as pro-apoptotic flavonoid with a special and nearly exclusive activity on tumor cell lines rather than normal cells (Lugli et al., 2009). Therefore, based on its major effects, this component is generally known as pro-apoptotic and cell cycle regulator (Gibellini et al., 2011). These properties are believed to be connected to the ability of Qu in targeting tubulins, which lead to depolymerization of the cellular microtubules (Gupta and Panda, 2002).

Depending on the cell type and tumor origin, Qu is capable to arrest the cell cycle at G2/M or at the G1/S transition (Gibellini et al., 2011). Based on the fact, that BFE caused DNA damage and apoptosis at high concentrations (200  $\mu\text{g/mL}$  on TCC cells) and also it probably induce cell cycle arrest at mild concentrations (50 and 30  $\mu\text{g/mL}$ ) depending on the cell type and tumor origin. Further bracken fern extract can be developed as a botanical drug initially in cancer patients before it can be thoroughly analyzed for the isolation, characterization and testing of anticancer compounds.

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