

## ORIGINAL ARTICLE

# Antioxidant, anticancer and apoptotic effects of the *Bupleurum chinense* root extract in HO-8910 ovarian cancer cells

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## Summary

**Purpose:** The purpose of this study was to evaluate the anticancer, apoptotic and antioxidant properties of *Bupleurum chinense* (B.C) root extract against human epithelial ovarian cancer cells (HO-8910) in vitro.

**Methods:** MTT assay was used to evaluate the cell viability of HO-8910 cells after treatment with different B.C extract doses. Apoptotic and morphological effects induced by the extract were demonstrated by inverted phase contrast microscopy and fluorescence microscopy. The percentage of apoptotic cells was quantified by Annexin V/PI double staining assay. Flow cytometry using rhodamine-123 dye was used to measure disruption of mitochondrial membrane potential ( $\Delta\psi_m$ ). Gel electrophoresis was used to study the effects of the extract on DNA fragmentation. The antioxidant activity of the extract using 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) and 2,2'-azino-bis (3-ethylbenzothiazolin-6-sulphonic acid) (ABTS) radical scavenging assays was also evaluated.

**Results:** The results showed that B.C extract could induce potent and dose-dependent cytotoxic effects on the HO-

8910 cells as demonstrated by MTT assay. The extract also induced cell shrinkage, chromatin condensation and membrane blebbing which are the hallmark of apoptosis. The average proportion of Annexin V-staining positive cells (total apoptotic cells) significantly increased from 9.4% in control cells to 18.5, 28.2 and 50.5% in 20, 80 and 120  $\mu\text{g/ml}$  B.C extract-treated cells respectively. Different doses of the extract (20, 80 and 120  $\mu\text{g/ml}$ ) after 48 hrs exposure led to a substantial increase in DNA fragmentation. The number of cells with disrupted  $\Delta\psi_m$  increased from 6.6% in untreated (control cells) to 14.2, 42.1 and 73.4% in 20, 80 and 120  $\mu\text{g/ml}$  in extract-treated cells, respectively

**Conclusion:** The anticancer effects of *Bupleurum chinense* extract were mediated through the induction of apoptosis, DNA fragmentation and disruption of mitochondrial membrane potential.

**Key words:** antioxidant, apoptosis, *Bupleurum chinense*, mitochondria, ovarian cancer

## Introduction

Epithelial ovarian cancer is the leading cause of death among gynecological cancers and close to 60-70% of the patients with advanced-stage disease will experience recurrence [1,2]. Patients with advanced ovarian cancer are firstly treated by a combination of surgery and chemotherapy [3]. Regardless of an initial 65–75% response rate, most of the cases will relapse within 1–2 years and develop resistance to chemotherapy. In fact, the overall 5-year survival rate is less than 30%

[4]. The recurrence primarily occurs due to acquired resistance to current chemotherapeutic regimens, creating thus the need to design and develop new therapeutic strategies and innovative mechanism of action with less chances of developing resistance. Despite recent developments in our understanding of the biological processes leading to cancer, there is still a necessity for new and active agents to help bring this disease under control [5,6]. One of the old and most effective

approaches for developing novel chemotherapeutics is the isolation and evaluation of molecules of natural origin. The utility of molecules from natural sources for drug discovery has been remarkable. This is evident from the number of clinically active drugs that are used as chemotherapeutic agents in cancer treatment, most of which are either natural products or natural product derivatives or semisynthetic analogues of natural products. Combination of natural products with known anticancer drugs has been reported to show additive or synergistic effects in eliminating cancer cells. This combination approach has the advantage to exert a better therapeutic effect and at the same time allow very low doses of drugs to be used. For instance, paclitaxel, a chemotherapeutic agent first isolated from the bark of the Pacific yew tree. Its combinations with platinum produced much more clinical success in ovarian cancer patients than platinum alone and is the standard chemotherapeutic strategy in treating ovarian cancer patients [7-9].

The objective of the present study was to demonstrate the antioxidant, anticancer and apoptotic activities of the ethanol extract of B.C roots in epithelial ovarian cancer (HO-8910) cells. We also evaluated its effect on cellular morphology, DNA fragmentation and mitochondrial membrane potential using microscopy as well as flow cytometry techniques.

## Methods

### *Plant material and preparation of the extract*

The roots of *B.C* were collected during May-June 2014 from Jiuzhaigou, Chengdu, China. The plant material was confirmed by a well-known taxonomist. The roots were thoroughly washed with tap water, shade-dried and then chopped into small pieces. Ethanol (95%) was used for hot extraction which was carried out for 3 hrs using a soxhlet extraction apparatus. The ethanol extract usually extracts polar compounds from the roots. The extract was then concentrated under reduced pressure in a rotary evaporator at 40°C and was then kept in a refrigerator at 4°C prior to use.

### *Cell line, culture conditions and extract treatment*

HO-8910 cell line was purchased from Guangdong Medical College (Zhanjiang, China). The cells were cultured in RPMI 1640 (Gibco BRL, Grand Island, NY, USA), supplemented with 10% (v/v) fetal bovine serum (Gibco BRL), penicillin 100 U/ml and streptomycin 100 U/ml and maintained in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37°C.

### *MTT assay for cell viability*

HO-8910 cell density was adjusted to 1x10<sup>6</sup> cells per 100 µl. Cells were seeded onto 96 well plates, which were placed in an incubator overnight to allow for attachment. Briefly, cells were pretreated with 10, 20, 40, 80 and 120 µg/ml extract for 48 hrs and MTT was then dissolved to a concentration of 10 mg/ml in assay medium. A total of 20 µl MTT solution was transferred to each well to yield a final volume of 120 µl/well. Plates were incubated for 4 hrs at 37°C in 5% CO<sub>2</sub>. Following incubation, supernatants were removed and 150 µl DMSO were added. Plates were then placed on an orbital shaker for 20 min and the absorbance was recorded using the ELISA plate reader (Perkin Elmer, Inc., Waltham, MA, USA) at 595 nm.

### *Inverted light microscopic study of cellular morphology of HO-8910 cells*

HO-8910 cells were seeded in 6-well plates at 2x10<sup>5</sup> cells per well in 5 mL of complete growth medium, incubated for 24 hrs and treated with extract at various concentrations (0, 20, 80 and 120 µg/ml). Control cells treated with 0.1% DMSO alone were also included. The morphological changes, were observed under an inverted light microscope (Olympus, Center Valley, PA, USA) after 48 hrs. The images were captured at a magnification of x200.

### *Morphological study of apoptosis using fluorescence microscopy*

HO-8910 cells were seeded into 12-well plates at a density of 1x10<sup>6</sup> cells/well in 1 ml medium. After treatment with different concentrations (0, 20, 80 and 120 µg/ml) of the extract for 48 hrs, cell apoptosis was determined by the Hoechst staining kit according to the manufacturer's instruction. After treatment, cells were fixed with 4% polyoxymethylene and then incubated in Hoechst solution for 10-15 min in the dark. The stained images were recorded using a UV fluorescence microscope (Olympus, Olympus Optical Co., LTD, Tokyo, Japan) using UV filter at 200x magnification to detect morphological evidence of apoptosis. The extract-treated cells were also stained with acridine orange and propidium iodide and visualized under the fluorescence microscope.

### *Quantification of cell apoptosis by Annexin V-FITC/PI assay*

Apoptotic cells were quantified using an Annexin V fluorescein isothiocyanate (FITC)/PI kit (BD Biosciences, San Jose, CA, USA) and detected using flow cytometry using a FACS Calibur flow cytometer (Becton, Dickinson and Co, Franklin Lakes, NJ, USA) and analyzed using Modfit and CellQuest™ software. HO-8910 cells were plated at a density of 1x10<sup>5</sup> cells/well into 12-well plates and incubated overnight. The cells were then treated with extract at varying doses (0, 20,

80 and 120 µg/ml) for 48 hrs. Cells grown in media containing an equivalent amount of 0.11% DMSO without any drug served as control. Cells were then collected and resuspended in binding buffer and were incubated with Annexin V-fluorescein isothiocyanate and PI for 30 min in the dark, before flow cytometric analysis. Annexin V positive cells were considered to be in the early stage of apoptosis, whereas Annexin V and PI-positive cells were considered to be in the late stage of apoptosis.

#### *DNA fragmentation analysis by gel electrophoresis*

After subjecting HO-8910 cells to B.C extract treatment for 48 hrs at various concentrations (0, 20, 80, and 120 µg/ml), both adherent and floating cells were collected and washed with PBS. Pellets were then lysed with DNA lysis buffer (50 mM EDTA, 150 mM Tris, pH 7.6, 0.8% SDS) at room temperature for 20 min. After centrifugation for 20 min at 12 000×g, the supernatants were collected and treated with RNase A (final concentration, 300 µg/ml) for 20 min at 37°C, followed by digestion with proteinase K (final concentration 300 µg/ml) for 2.5 hrs at 55°C. The DNA was extracted using the phenol/chloroform/isoamylol (25:24:1), precipitated with ethanol, dissolved in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA), and subjected to 2% agarose gel electrophoresis for DNA fragmentation analysis.

#### *Measurement of mitochondrial membrane potential ( $\Delta\Psi_m$ ) disruption*

The fluorescent dye rhodamine-123 (Rh-123) is a specific probe for the detection of alterations in mitochondrial membrane potential in living cells. Mitochondrial membrane potential ( $\Delta\Psi_m$ ) in HO-8910 ovarian cancer cells was measured by Rhodamine-123 dye, which preferentially partitions active mitochondria based on the highly negative MMP. HO-8910 cells were seeded in 96-well plates at density of  $1 \times 10^6$  cells/ml. The cells were treated with B.C extract at 0, 20, 40, 80 and 120 µg/ml for 48 hrs. Depolarization of MMP results in the loss of Rhodamine 123 from mitochondria and a decrease in intracellular fluorescence was observed. Rhodamine 123 (final concentration of 20 µM) was added to the harvested cells and analyzed using FACS Calibur instrument (BD Biosciences, San Jose, CA, USA) equipped with Cell Quest 3.3 software.

#### *DPPH radical scavenging assay*

DPPH radical scavenging assay was done as previously reported. The extract was dissolved in DMSO and stock solution of the extract was prepared as 10 mg/ml. 100 µl of the solution of the extract was pipetted into a 96-well microtitre plate and mixed with 400 µl of 0.2 nM DPPH methanolic solution. The plate was incubated in the dark up to 1h. Absorbance was read at 540 nm using Microplate reader (Thermo Molecular Devices Co., Union City, USA). Ascorbic acid was used

as standard and analyses were carried out in triplicate. Percent inhibition of DPPH radical was calculated using the following formula:

$$\text{Percent inhibition (\% I)} = \frac{\{\text{Abs}_{\text{control}} (\text{DPPH}) - \text{Abs}_{\text{sample}} (\text{DPPH} + \text{Sample})\}}{\text{Abs}_{\text{control}}} \times 100$$

#### *ABTS radical cation scavenging assay*

Oxidation of 5 mM ABTS with 2.5 mM potassium persulphate followed by overnight incubation in the dark led to the generation of ABTS radical cation. The ABTS radical cation solution was then diluted with ethanol to obtain an absorbance of  $0.50 \pm 0.03$  at 734 nm spectrophotometrically. The extract (100 µl) was mixed with 900 µl of diluted ABTS solution and incubated for 20 min at room temperature. The absorbance was measured again at 734 nm. All the measurements were carried out in triplicate. Ascorbic acid was used as standard and the percentage of ABTS radical cation decolorization inhibition was calculated using the following formula:

$$\text{Percent inhibition (\% I)} = \frac{\{\text{Abs}_{\text{control}} (\text{ABTS}) - \text{Abs}_{\text{sample}} (\text{ABTS} + \text{Sample})\}}{\text{Abs}_{\text{control}}} \times 100$$

#### *Statistics*

All statistical analyses were done using SPSS® software (version 19.0) and were conducted by one-way analysis of the variance (ANOVA) and Tukey's test. Data were expressed as the mean±SEM and a p value <0.05 was considered statistically significant.

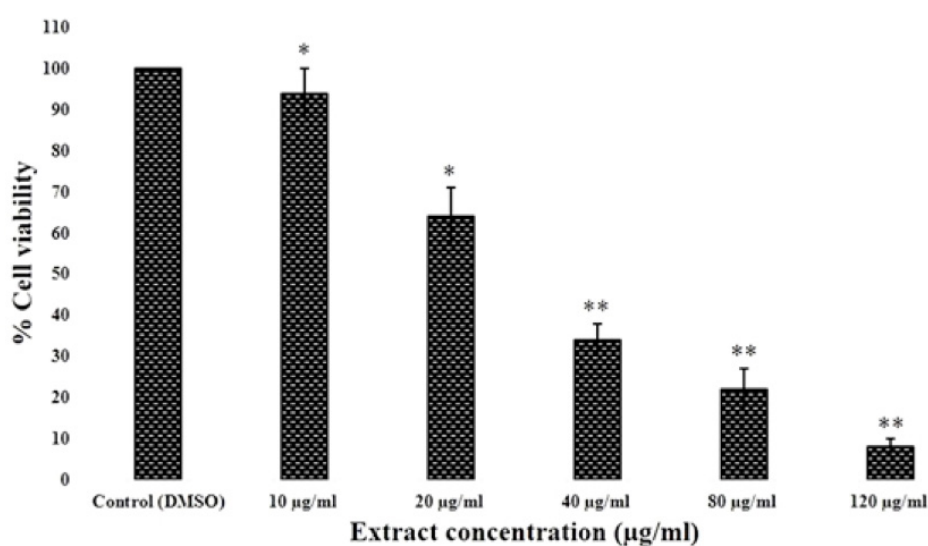
## **Results**

#### *Effect of the extract on the viability of HO-8910 cells*

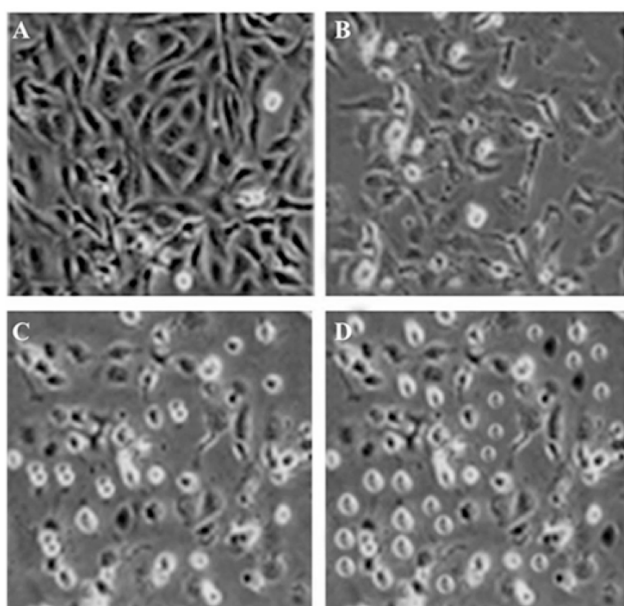
HO-8910 cells were treated with different concentrations (0, 10, 20, 40, 80 and 120 µg/ml) of the extract for 48 hrs and cell viability was evaluated using an MTT assay. Figure 1 shows the dose-dependent growth inhibitory effects of the extract on the cell viability of HO-8910 cells. The percentages of growth inhibition at various concentrations in ovarian cancer cells were determined as the percentage of viable treated cells in comparison with viable cells of untreated controls. At higher doses of the extract, significant cell death was observed and at 120 µg/ml, 92% growth inhibition was observed.

#### *Cellular morphological study using inverted phase contrast microscopy*

In this study, the morphological alterations HO-8910 cells untreated and treated with B.C extract were observed under an inverted light microscope. The most conspicuous changes characteristic of apoptosis were observed in the treated



**Figure 1.** Cytotoxic effect of the Bupleurum chinense extract on the viability of human epithelial ovarian cancer (HO-8910) cells. Cell viability was determined by MTT assay. Results are the mean±SD in triplicates. \*  $p < 0.01$  vs control, \*\*  $p < 0.001$  vs control.



**Figure 2.** Bupleurum chinense (B.C.) extract-induced morphological changes in human epithelial ovarian cancer cells HO-8910 as identified by phase contrast microscopy (x200). Cellular shrinkage was observed in B.P extract-treated cells. **A** represents control (untreated cells), **B**, **C** and **D** represent effect of 20, 80 and 120 µg/ml extract on cell morphology of HO-8910 cells.

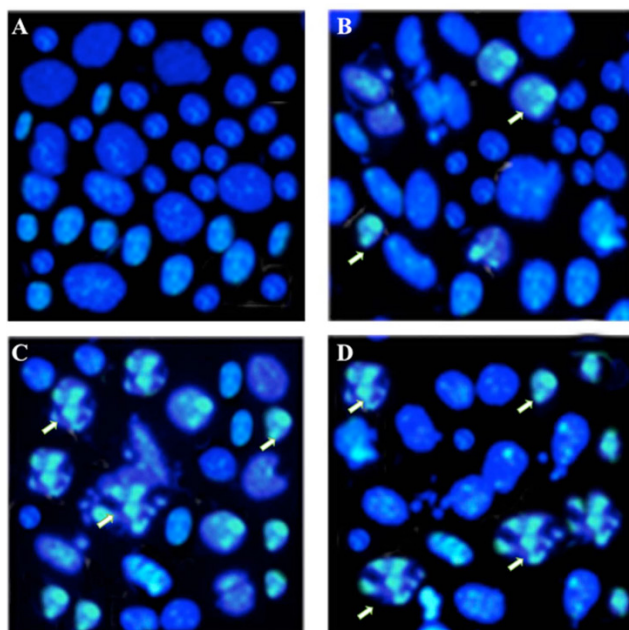
cells and included detachment of the cells from the substrate, cell shrinkage and membrane blebbing. As revealed by inverted light microscopy, the untreated control cells were evenly distrib-

uted on the substrate. Decrease in the cell population was seen with the increase in the extract concentration. As can be seen in Figure 2 A-D, the cells with higher doses of extract revealed cellular shrinkage and overall morphological alterations.

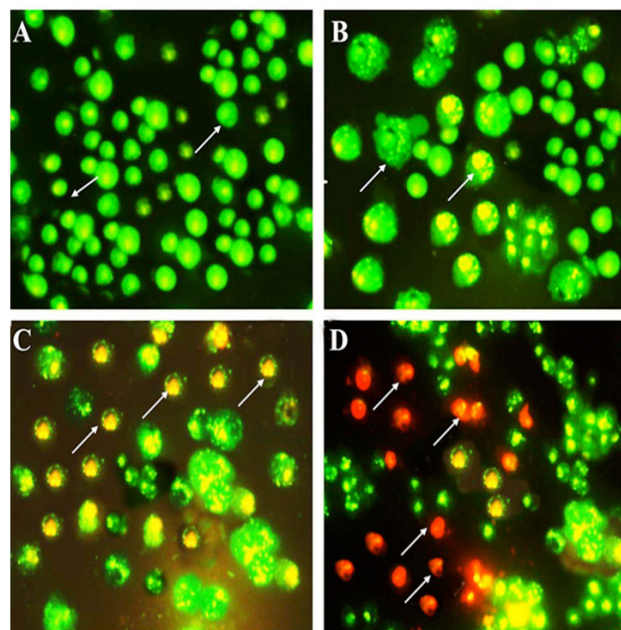
#### Cellular morphological study using fluorescence microscopy

In case of fluorescence microscopy, HO-8910 cells were stained and evaluated for nuclear shape using a fluorescence microscope with Hoechst 33342 staining (Figure 3 A-D). The results revealed that B.C extract-treated cells showed substantial chromatin condensation, fragmented nuclei and nuclear shrinkage, which implied an early apoptotic event. The appearance of such features were related with the extract dose. HO-8910 cells underwent morphologic changes typical of apoptosis after treatment with the extract.

The morphological alterations in HO-8910 cells, like early and late apoptosis, chromatin condensation and membrane blebbing were also investigated by using acridine orange and propidium iodide as staining dyes using fluorescence microscopy. After treatment with 20, 80 and 120 µg/ml of the B.C extract for 48 hrs, HO-8910 cells showed early apoptotic features at low dose and late apoptotic features at higher doses of the extract. Bright green fluorescence indicated chromatin condensation detected by the use of acridine orange (Figure 4 B,C). Similarly, light green



**Figure 3.** Morphological examination of *Bupleurum chinense* (B.C) extract-induced apoptosis with Hoechst 33258 staining at actual magnification  $\times 200$ . Human epithelial ovarian cancer cells HO-8910 were treated without (A) and with B.C extract 20  $\mu\text{g/ml}$  (B), 80  $\mu\text{g/ml}$  (C), and 120  $\mu\text{g/ml}$  (D) for 48 hrs. Arrows show apoptotic cells exhibiting chromatin condensation and nuclear fragmentation.



**Figure 4.** Apoptotic morphological changes in ovarian cancer cells HO-8910 were observed by fluorescent microscope ( $\times 200$ ). (A) Untreated HO-8910 cells showed normal morphology without noticeable apoptosis after 48 hrs. (B,C) Early apoptotic features, including chromatin condensation and cell blebbing were witnessed after treatment with 20  $\mu\text{g/ml}$  (B) and 80  $\mu\text{g/ml}$  (C) of B.C extract (bright green fluorescence). (D) Characteristic features of late apoptosis (red fluorescence) were seen after treatment with 120  $\mu\text{g/ml}$  of B.C extract. The arrows indicate apoptotic cells.

fluorescence showed intact nuclear structure in healthy cells (Figure 4 A). Propidium iodide binding to denatured DNA indicated by red color established the late apoptosis stage induced by the extract (Figure 4 D).

#### *Apoptosis quantification by Annexin V-FITC/PI assay*

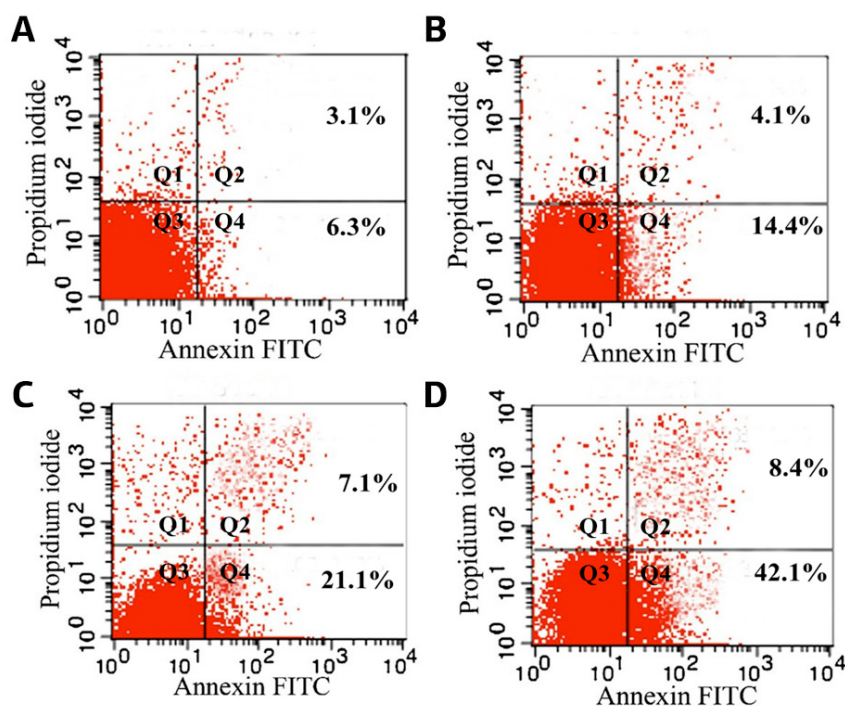
Annexin V/PI double staining was used to detect apoptosis in HO-8910 cells (Figure 5 A-D). HO-8910 cells were treated with different concentration (0, 20, 80 and 120  $\mu\text{g/ml}$ ) of B.C extract for 48 hrs. The extract induced both early and late apoptosis in a concentration-dependent manner (Figure 5 B-D) as compared to the untreated control cells (Figure 5 A). The average proportion of Annexin V-staining positive cells (total apoptotic cells) significantly increased from 9.4% in control cells (A), to 18.5, 28.2 and 50.5% in 20  $\mu\text{g/ml}$  (B), 80  $\mu\text{g/ml}$  (C) and 120  $\mu\text{g/ml}$  (D) in B.C extract-treated cells, respectively. The extract showed dose-dependent apoptosis inducing effect in these cells.

#### *Effect of B.C extract on DNA fragmentation in HO-8910 cells*

In addition to the morphological changes of apoptosis in B.C extract-treated HO-8910 cells, DNA fragmentation of was also studied by observation of the formation of DNA ladder. As shown in Figure 6, DNA ladder appeared to be more evident with increasing extract concentration, however, no DNA fragments were observed in the control groups (Figure 6, 0  $\mu\text{g/ml}$ ). However, 20, 80 and 120  $\mu\text{g/ml}$  of the extract after 48 hrs exposure led to a substantial increase in DNA fragmentation (Figure 6, right panel).

#### *Disruption of mitochondrial membrane potential ( $\Delta\psi_m$ ) induced by B.C extract in HO-8910 cells*

To evaluate the role of B.C extract on the mitochondrial apoptotic pathway, mitochondrial membrane potential ( $\Delta\psi_m$ ) in HO-8910 cells treated with different concentrations of the B.C extract was examined and measured making use of the rhodamine-123 cationic dye as a fluorescent probe. As can be seen from the Figure 7 A-D, the population of cells with depolarized mitochondria



**Figure 5.** *B.C* extract-induced apoptosis in human epithelial ovarian cancer cells HO-8910 subjected to different doses of extract (0, 20, 80 and 120 µg/ml) for 48 hrs and analyzed by flow cytometry with annexin V-FITC/PI staining. The different quadrants Q1, Q2, Q3 and Q4 represent necrotic cells, late apoptotic cells, viable cells and early apoptotic cell population, respectively. The percentage of apoptotic cells increased from 9.4% in control cells (A), to 18.5, 28.2 and 50.5 in 20 µg/ml (B), 80 µg/ml (C) and 120 µg/ml (D) *B.C* extract-treated cells respectively.

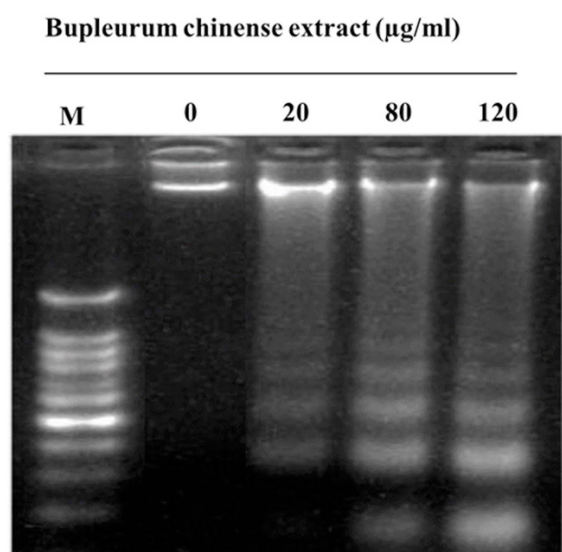
increased with increasing extract concentration. The number of cells with disrupted  $\Delta\psi_m$  increased from 6.6% in untreated (control cells) to 14.2, 42.1 and 73.4% in 20, 80 and 120 µg/ml extract-treated cells, respectively.

#### Radical scavenging activity

*B.C* extract showed a concentration-dependent scavenging of DPPH radicals and was found to be an active radical scavenger. The percent DPPH radical scavenging activity was found to be 7.2, 18.1, 34.5, 52.1 and 88.4% at concentrations of 10, 20, 40, 80 and 120 µg/ml, respectively (Figure 8). Proton radical scavenging is an important attribute of antioxidants. The extract was also found to be an effective scavenger of ABTS radicals. The percent ABTS scavenging activity was found to be 15.1, 26, 36.2, 65.4 and 91.6%, respectively at concentrations of 10, 20, 40, 80 and 120 µg/ml of the extract (Figure 8). The higher concentrations of the extracts were more effective in quenching free radicals in the system. The scavenging of the ABTS radical by the extracts was found to be much higher than that of DPPH radical.

#### Discussion

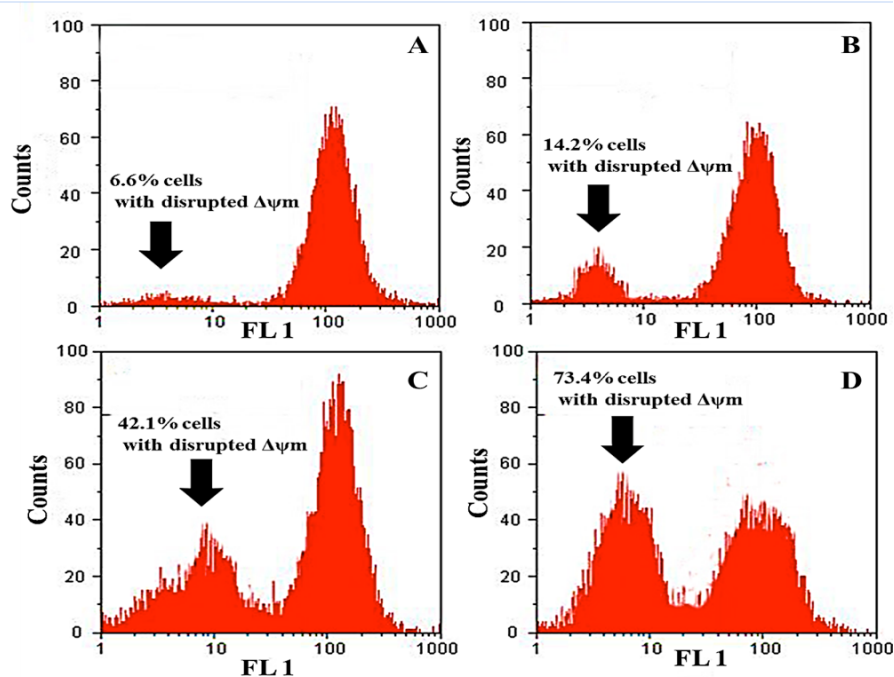
*Bupleurum species* (Apiaceae family) represent one of the most successful and widely used herbal drugs for the treatment of many diseases over the past 2000 years. Dried roots of *Bupleurum spp.* have been used medicinally in China for over 2000 years. The roots, which are called *Bupleuri Radix*, are used in at least 66% of the formulations/prescriptions in traditional Chinese medicine and Kampo medicine. *Bupleurum chinense* (Chinese name: Chai Hu) is one of the important medicinal plants in Chinese Pharmacopoeia. *B.chinense* along with *B. falcatum L.*, have been extensively used in Chinese and Japanese herbal medicines for treating kidney diseases, chronic hepatitis, inflammatory diseases, and ulcers of the digestive system. *Bupleurum* is a primary ingredient in ancient Chinese medicinal formula known as Xiao Chai Hu Tang, first recorded in the Treatise on Cold Induced Febrile Disease [10-12]. Recent research has shown the pharmacological actions of *Bupleurum* and its active components (saikosaponins) revealed immunomodulatory



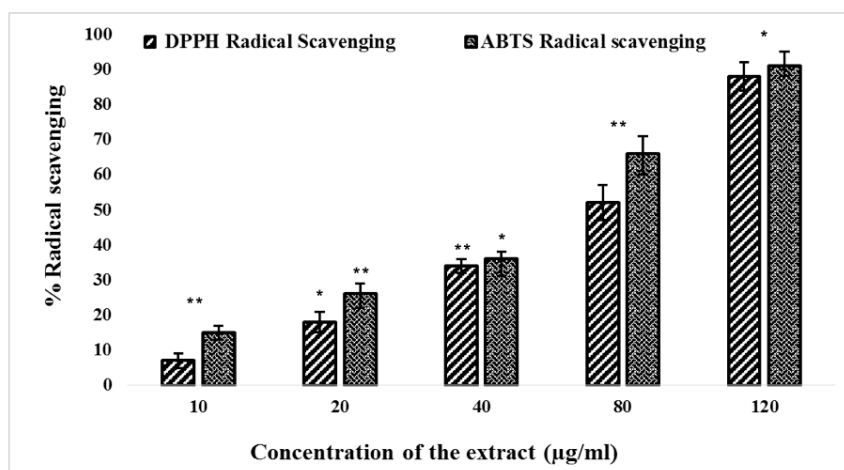
**Figure 6.** B.C extract induces DNA fragmentation in human epithelial ovarian cancer cells HO-8910. Cells were treated with 0, 20, 80 and 120  $\mu\text{g/ml}$  of B.C extract for 48 hrs. Cells from each sample were harvested for DNA gel electrophoresis as described in Methods. The Figure shows the appearance of DNA fragments which increased with the increase of B.C extract concentration. No such fragments were seen in controls

[13,14], hepatoprotective [15], anticancer [16,17], and analgesic [18] effects.

To the best of our knowledge anticancer and apoptotic effects of B.C against ovarian cancer cells have not been reported so far. Therefore, the objective of the current study was to evaluate the antioxidant, anticancer and apoptotic effects of the root ethanol extract of B.C medicinal herb by studying its effect on cellular morphology using phase contrast and fluorescence microscopy. The effect of extract on apoptotic induction and mitochondrial disruption was studied by flow cytometry while as gel electrophoresis was used to evaluate the effect of extract on DNA fragmentation. The results showed that the extract exhibited potent and dose-dependent anticancer effects against HO-8910 cells. The extract induced morphological changes characteristic of apoptosis in these cells in a dose-dependent manner. Lower doses of the extract led to early apoptotic cells while late apoptotic phenomena were seen at higher doses of the extract. Different doses of the extract (20, 80 and 120  $\mu\text{g/ml}$ ) after 48 hrs of exposure led to a substantial increase in DNA fragmentation. Further, the population of cells with depolarized mitochondria increased with increasing extract concentration. The number of cells with disrupted  $\Delta\psi\text{m}$  increased from 6.6% in untreated (control cells) to 14.2, 42.1 and 73.4% in 20, 80 and 120  $\mu\text{g/ml}$



**Figure 7.** Disruption of mitochondrial membrane potential ( $\Delta\psi\text{m}$ ) in human ovarian cancer cells HO-8910 after treatment with B.C extract using Rh123 staining. (A) Treatment with 0  $\mu\text{g/ml}$  extract; (B) treatment with 20  $\mu\text{g/ml}$  extract; (C) treatment with 80  $\mu\text{g/ml}$  extract; (D) treatment with 120  $\mu\text{g/ml}$  extract. The experiments were performed in triplicate and representative graphs are shown



**Figure 8.** DPPH and ABTS radical scavenging activities of the B.C extract. Data are expressed as mean  $\pm$  SD along with regression and correlation ( $n=3$ ). Results are the mean $\pm$ SD in triplicate. \* $p<0.01$  vs control, \*\* $p<0.001$  vs control

ml extract-treated cells, respectively. B.C extract showed a concentration-dependent scavenging of DPPH radicals and was found to be an active radical scavenger. The extract was also found to be an effective scavenger of ABTS radicals.

In conclusion, B.C extract showed anticancer and apoptotic effects against human ovarian cancer cells and these effects were mediated through DNA fragmentation, chromatin condensation and loss of mitochondrial membrane potential.

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