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Induction of Apoptosis and Cytotoxic Activities of Iranian Orthodox Black Tea Extract (BTE) Using in vitro Models

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ABSTRACT

Purpose: Plant-derivate therapeutic agents can perform cancer chemotherapeutic activity through triggering apoptotic cell death. Our aim was to investigate the cytotoxic effects, induction of apoptosis, and the mechanism of cell death of Iranian orthodox black tea extracts (BTEs) and hydro methanolic purified fractions (40, 60, 80 and 100%) in KB cells (oral squamous cell carcinoma).

Methods: In order to analyze the cytotoxic activity of the BTEs, MTT (3-(4, 5-dimetylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide) and Trypan-blue assays were performed in oral squamous cell carcinoma (KB). Furthermore, the apoptosis inducing action of the extracts was determined by TUNEL, DNA fragmentation and cell death detection analysis.

Results: Dichloromethane BTE and hydro methanol fractions (40 and 60%) extract showed no cytotoxic effects; however, hydro methanol crude and hydro methanol fractions of BTE (80 and 100%) significantly inhibited cell growth and viability in a dose and time dependent manner. In addition, Cell death assay, TUNEL, and DNA fragmentation indicated induction of apoptosis by hydro methanol 80 and 100% fractions of BTE in KB cells. Statistical significance was determined by analysis of variance (ANOVA), followed by Duncan test and p value ≤ 0.05 was considered significant.

Conclusion: The results from the present study suggests that the hydro methanol crude and hydro methanol fractions of BTE (80 and 100%) are significant source of compounds with the anti proliferative and cytotoxic activities, and this may be useful for developing potential chemo preventive substances.

Introduction

Oral squamous cell carcinoma (OSCC), which is a knotty health setback, leads to a wide range of mortality and morbidity in developing countries.[1] Conventional therapeutic modalities (e.g., surgery, radiotherapy, chemotherapy and drugs) have been utilized for tackling OSCC.In spite of advances in surgery and radiotherapy, these approaches cause unwanted side effects by the non-specific targeting on both normal and cancer cells.[2] Currently, chemotherapy has been applied for oral cancer patients. For example, cisplatin-based chemo radiation has been used for loco regionally advanced head and neck SCC.[3]

One of the approaches used in drug discovery, is herbal medicine as an alternative cancer therapy due to their low toxicity or damage to normal cells. Most therapeutic agents exert their cancer chemotherapeutic activity by triggering apoptotic cell death.[4] Therefore, induction of apoptosis in tumor cells has become an indicator of the tumor treatment response in employing a plant derived-bioactive substance to reduce and control human mortality resulting from cancer. Individual cells in apoptosis, as a programmed cell suicide, are destroyed, while the integrity and architecture of surrounding tissue is preserved.[5]

Black Tea from the young tender leaves of *Camellia sinensis* (L) is one of the most popular non-alcoholic beverages in the world. In the recent decades, therapeutic effects of various type of tea has been revealed in many studies.[6] The chemical composition of Black tea includes poly phenols, alkaloids (caffeine, theophyllineand theobromine), amino acids, carbohydrates, proteins, chlorophyll, volatile

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compounds, minerals, and trace elements.[7] The toxicity of Iranian orthodox black tea extract (BTE) has not been intensively studied yet. Accordingly, the toxicity of BTE was investigated in the present research. The objective of this study was to examine the *in vitro* cytotoxic activities of a wild Iranian orthodox black tea extracts (BTEs) and hydro methanolic purified fractions (40, 60, 80 and 100%) using a MTT cytotoxicity assay. The study also tested whether the mechanism of action involves induction of apoptosis. Cell death ELISA, TUNEL and DNA fragmentation gel agarose were employed to quantify the nucleosome production resulting from nuclear DNA fragmentation during apoptosis.

Materials and Methods

Preparation of extracts

For preparation of the hydro methanol extract of Iranian black tea, *Camellia sinensis*, Var sinensis leaves were collected from North of Iran, in April 2012. A voucher specimen was deposited at the Herbarium of the Faculty of Pharmacy, Tabriz University of Medical Sciences and processed in orthodox method. The leaves were washed, dried and ground to get powder using a blender. Extractions were performed in a Soxhlet apparatus with hydro methanol. The black tea extract (BTE) was concentrated by rotary evaporator (Heildolph, Germany) at about 45 ºC and then dried in very low pressure. The dried extracts were stored at –20 ºC. In order to localize the active fraction, hydro methanolic extract of Iranian orthodox BTE was purified using C18 cartridges (Sep-pack, Supelco), by gradient elution with Hydro methanol mixture (40%, 60%, 80 and 80% Hydro methanol) to give 4 fractions. Hydro methanol solvent was removed from fractions by using rotating evaporator at 35°C and distilled water was then added to the residues and the aqueous phases were lyophilized. The powdered fractions were stored at −20°C until use. Twenty mg of each extract and purified fractions (40, 60, 80 and 100%) were dissolved in 100 µL dimethyl sulfoxide (DMSO) and were diluted with RPMI-1640 medium. Then, test solutions were sterilized using 0.22 µm Syringe filters (Nunc, Denmark) and used as stock solution for further experiments.

Cell culture

KB cell (oral squamous cell carcinoma cell line and HUVEC (Human umbilical vein endothelial cells)) were purchased from National Cell Bank of Iran (Pasteur Institute, Tehran, Iran). Cells were grown in RPMI- 1640 medium (Sigma, Germany) supplemented with 10% fetal bovine serum (FBS) (Sigma, Germany), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma, Germany). The cells were then incubated in a humidified incubator containing 5% CO2 at 37 ºC. At 80% confluence, cells were rinsed with PBS/0.5% EDTA and harvested from 25 cm^2 flasks using 0.25 % trypsin/ EDTA solution (Gibco, U.K). Then, the cells were sub cultured into 75cm2 flasks, 96-well plates or 6-well plates (Nunc, Denmark) according to our researches. The experiments were performed in triplicate.

MTT assay

Cytotoxicity of hydro methanolic extract from Iranian orthodox black tea extracts (BTEs) and hydro methanolic purified fractions (40, 60, 80, and 100%) were assessed in KB cells as well as HUVEC by measuring the amount of insoluble formazan formed in live cells based on the reduction of 3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) salt (Roche Diagnostics GmbH, Germany) according to the manufacturer's protocol. The cells were seeded in 96-well plates with a density of 10^4 cells/well incubated for 24 h at 37°C and 5% CO2. The cells were treated with different concentrations of BTE extracts (50, 100, 150, 200, 300, 400, 500, 600 µg/ml) and 0.2 % (v/v) DMSO (Merck, Germany) as a negative control. After 24 h treatment, 50 µl of MTT labeling reagent (2µg/ml) was added to each well. The plates were incubated at 37°C in a humidified atmosphere with 5% CO2 for 4 hours. Thereafter, 100 µl of the solubilization solution was added to each well and followed by incubation overnight at 37°C to dissolve formazan crystals. Absorbance was ultimately read using an ELISA plate reader (Bio Teck, Germany) at a wavelength of 570 nm.[8] The percentage of cytotoxicity was calculated using the following equation:

$$\%\ Cytotoxicity = 1 - \frac{AB_T}{AB_N}$$

Where, AB_T and AB_N are mean absorbance of treated cells and negative control, respectively.

The dose-response curve was plotted and concentration which gave 50% inhibition of cell growth (IC50) was calculated. Concentration that inhibits 50% of cell viability was used as a parameter for cytotoxicity.

Trypan blue assay

Cell membrane integrity and direct counting of living and dead cells were evaluated by trypan blue dye exclusion. This dye does not enter living cells, but it passes through the membranes of dead cells. KB cells (10^4) in 96 well-plates were exposed to same different concentrations of Iranian orthodox black tea extracts (BTEs) and hydro methanolic purified fractions (40, 60, 80 and 100%) and 0.2 % (v/v) DMSO for 24 h. The medium was then removed from the wells, and the cells were washed with 200 µL of PBS. The cells were detached by adding100 µL of 0.5 % trypsin/EDTA. RPMI-1640 medium supplement with 10% FBS (50 µL) and 0.5 % trypan blue (50 µL) (Merck, Germany) were added to each well, and the plates were incubated for 5 min. Subsequently, a 20 µL aliquot was removed and placed on a Neubauer hemacytometer. The numbers of viable and nonviable cells were finally counted under a microscope. The number of viable cells was calculated according to the following formula:

$$\frac{\text{viable cell count} \times \text{dilution} \times 104}{n}$$

Where n is the number of hemacytometer squares that were counted. The percent viability was calculated as:

$$\frac{\text{viable cell count}}{\text{total cell count}} \times 100$$

Morphological changes of cells

After treating of cells with BTE, cells morphological appearance was observed under inverted microscopy. Morphologic alteration was considered including detachment, cell shrinkage, nuclear condensation, fragmentation, margination, cell blebbing and presence of apoptotic bodies.

Assessment of necrosis and apoptosis

Apoptosis and necrosis of cells were measured using the Cell Death Detection ELISA and a kit (Roche Diagnostics GmbH, Germany) that quantified histone associated DNA fragments (mono and oligonucleosomes). KB cells (10^4) were treated with the same different concentrations of hydro methanol and 80 and 100% fractions of BTE and 0.2 % (v/v) DMSO at 37°C for 24 hrs. The procedure was performed according to the manufacturer's protocol. Briefly, the culture supernatants and lysate of cells were prepared and incubated in the microtiter plate coated with anti-histone antibody. Subsequent to color development, the results were analyzed spectrophotometrically using an ELISA plate reader at 405 nm.[9]

TUNEL assay

DNA fragmentation was detected by terminal deoxy transferase (TdT)-mediated dUTP nick- end labeling (TUNEL) with the In Situ Cell Death Detection Kit, POD (Roche Diagnostics GmbH, Germany) as described by the manufacturer's protocol. Briefly, (1.5×10^5) KB cells were sub-cultured into 6 well-plates and incubated for 24 h at 37°C and 5% CO2. The cells were treated with hydro methanol 80 and 100% fractions of BTE at concentrations required for 50% inhibition of growth of KB cells (IC50) for 24h. Negative control cells were treated with the same final concentration of DMSO present in treated wells [0.2% (v/v)]. Having treated, the cells were fixed with 4% (w/v) paraformaldehyde in PBS (pH 7.4) for 1 h at room temperature and rinsed twice with PBS. Then, the fixed cells were incubated with blocking solution (3% H2O2 in methanol) for 10 min and rinsed with PBS. The cells were then incubated in permeabilisation solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice. Subsequently, 50 µl of reaction mixture containing TdT enzyme and nucleotide was added to the cells and they were all incubated for 1 h at 37°C. After washing three times with PBS, the slides were incubated with 50 µl converter-POD sterptavidin HRP solution for 30 min, and rinsed three times with PBS. Finally, the cells incubated with DAB and stained cells were analyzed with the light microscopy.[10]

DNA Fragmentation assay

Apoptosis has been characterized biochemically by the activation of a nuclear endonuclease that cleaves the DNA into multimers of 180-200 base pairs and can be visualized as an oligosomal ladder by standard agarose gel electrophoresis. KB cells were seeded in 6 wells plates and kept in CO2 incubator. KB cells were treated by hydro methanol and 80 and 100% fractions of BTE in IC50 concentrations (µg/ml) for 24 h. At the end of incubation period, the cells were centrifuged for 1000 rpm for 3 mins at 14°C. The pellet was re suspended in a lysis buffer (10 mM Tris-HCI, pH 8.0, 10 mM NaCl, I0 mM EDTA, 20mg/ml Proteinase K, 10% SDS), and incubated at 37°C. The pellet was dissolved in TE buffer (0.1 M Tris-HCl, pH 8.0, 10 mM EDTA). DNA samples were electrophoretically separated on 1.8 % agarose gel containing ethidium bromide (0.4µg/mL). DNA was visualized by a UV (302 nm) transilluminator. Untreated cells were used as control.[11]

Statistical analysis

All the data represented in this study were based on means ± SEM of three identical experiments made in three replicates. Statistical significance was determined by analysis of variance (ANOVA), followed by Duncan test. P value ≤0.05 was considered statistically significant. LC50 values were derived from prohibit analysis. All analyses were conducted using the SPSS 20.

Results

The cytotoxic effects of Iranian orthodox black tea extracts (BTEs) on the growth of oral squamous cell carcinoma (KB cell line) were determined by MTT and trypan blue assays shown in Figure 1 and 2. As illustrated in Figure 1, the treated cells with hydromethanol and 80 and 100% fractions of BTE exhibited significant decline in viability, in comparison with the untreated control cells. Moreover, the dichloromethane BTE and hydromethanol fractions (40 and 60%) extract had no cytotoxic effects on KB cell.

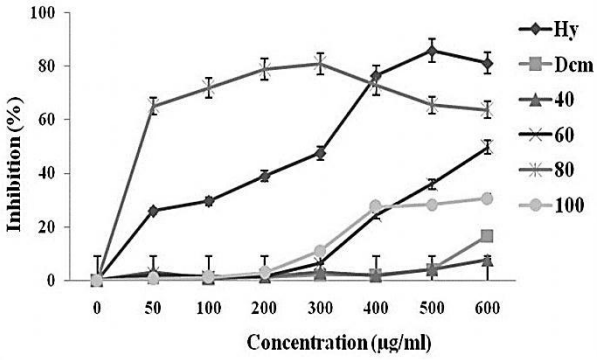

Figure 1. Effects of Iranian orthodox black tea extracts (BTEs) and hydromethanolic purified fractions (40, 60, 80 and 100%) with increasing concentrations (50–600 µg/mL) on proliferation of KB cells (b) for 24 h, the proliferative response was assessed by MTT assay.

Moreover, treatment of KB cells with hydro methanol crude and hydro methanol fractions of BTE (80 and 100%) showed cell growth inhibition in a time and dose dependent response. In the higher concentrations and longer time of the hydro methanol crude and hydro methanol 80% fractions of BTE treatment on KB cells, higher significant cytotoxicity was observed. Data analysis of cytotoxicity assay showed that IC50 (dose required for 50% inhibition) of hydro methanol crude and hydro methanol 80% fractions of BTE on KB cells were 446.08± 12.4 and 280.4 ± 33.1 µg/ml for 24 h, respectively.

Direct counting for viable cells using the trypan blue exclusion test showed that 86% hydro methanol BTE-treated cells with the highest concentration (600 µg/ml) absorbed the dye at 24 h (Figure 2).

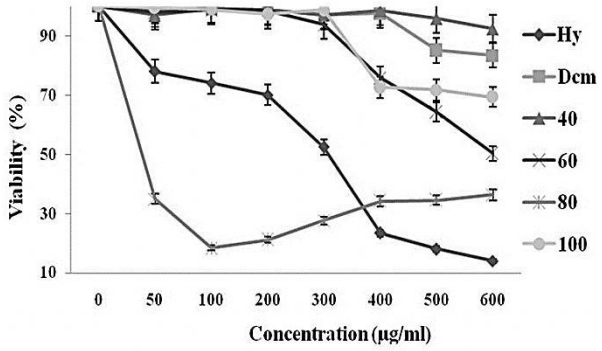

Figure 2. Results are expressed as the mean percentage of viable cells with 3 wells each. Percentage of viable cells was calculated from the ratio of viable cells to total number of cells using trypan blue exclusion test.

After incubation with IC50 of hydro methanol crude and hydro methanol 80 and 100% fractions of BTE, morphological alteration in KB cells were illustrated compared to the control cells (Figure 3). Sensitive cells were detached roundly from the surface. In order to determine the mechanism of the cytotoxic effects of hydro methanol and purified fractions (80 and 100%), apoptosis of cells were measured by cell death detection ELISA kit.

The ratio of apoptotic effect in hydro methanol and 80 and 100% purified fraction was 48%, 73% and 89%, respectively. In comparison with the hydro methanol and their fractions of BTE and the purified fractions extract induced the greatest apoptotic activity in KB cells.

One of the hallmarks of apoptotic cell death confirmed the presence of nucleosomal DNA fragments in cells treated with Iranian orthodox black tea extracts (BTEs) and hydro methanolic purified fractions (80 and 100%) by TUNEL assay. As shown in Figure 4, after the treatment of KB cells with 24 h IC50 concentration of hydro methanol BTE and their fractions, the apoptotic cells produced dark brown stained nuclei, whereas the non-apoptotic cells were not stained with similar observation was found in the negative control cells treated with 0.2% (v/v) DMSO.

As shown in agarose gel electrophoresis in Figure 5, increased DNA fragmentation was apparent in KB cells after treatment with 300 µg/ml (near to IC50) of BTE. Fragmented DNA was clearly observed in KB cells, whereas untreated cell did not provide ladders. Thereby, hydro methanol BTE and hydro methanolic purified fractions (80 and 100%) possibly cause apoptosis in KB cells.

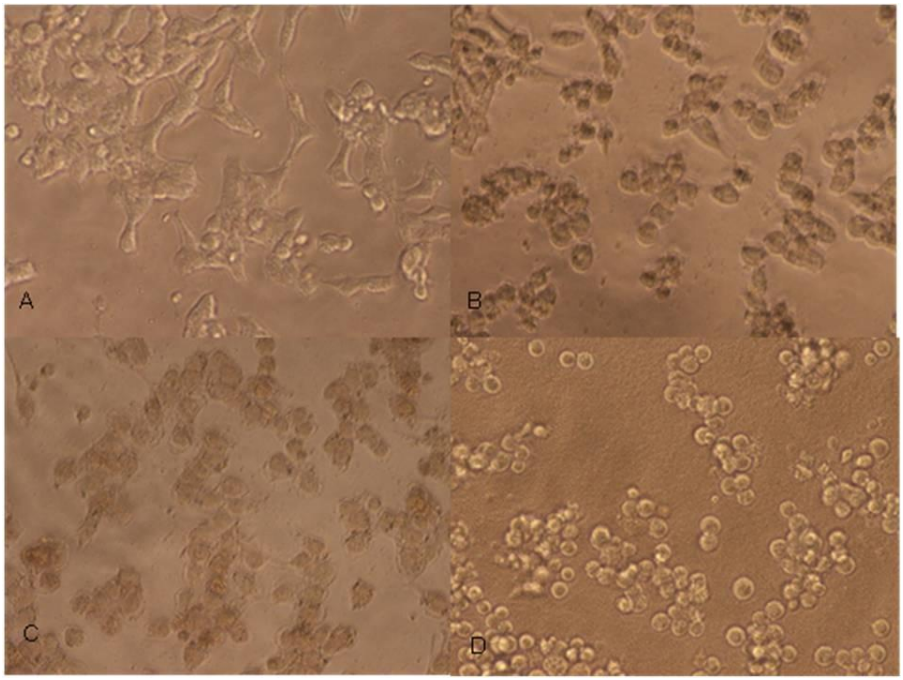

Figure 3. Morphological changes induced after incubation with IC50 of hydro methanol crude and hydro methanol 80 and 100% fractions of BTEon KB cells during 24 h treatment. (A) Untreated controls, (B) hydro methanol crude, (C) hydro methanol 80 and (D) 100% fractions of BTE.

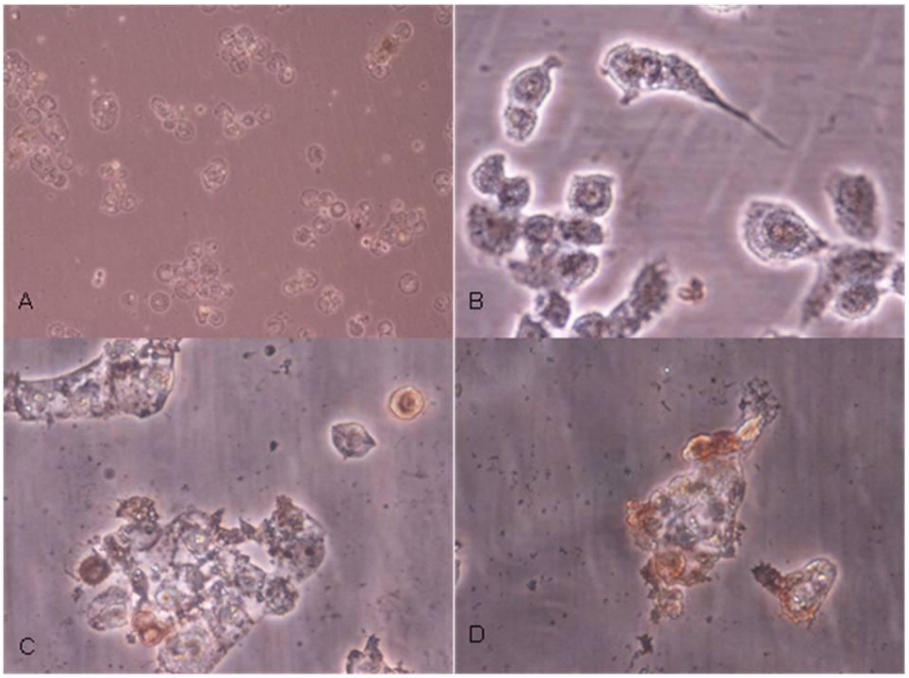

Figure 4. Nuclei morphological changes during hydro methanol BTE and hydro methanolic purified fractions (80 and 100%) induced apoptosis in KB cells detected by TUNEL assay. For KB cells, (a) shows negative control (without treatment) and (b) and (c, d) treated with extract (IC50) for 24 h (*n*: 3). (b), (c) and (d) indicate representative apoptotic cells with nuclei morphological changes.

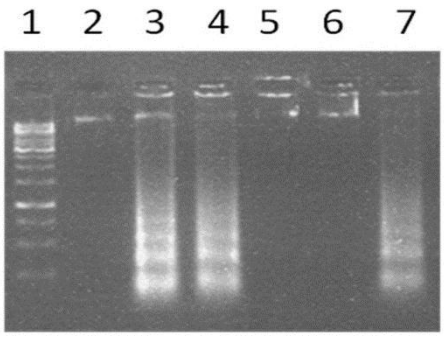

Figure 5. Analysis of DNA fragmentation using agarose gel electrophoresis. **KB** cells were incubated in the presence of IC50 of hydro methanol crude and hydro methanol 80 and 100% fractions of BTE on KB cells during 24 h treatment. Genomic DNA was prepared and analyzed by 2% agarose gel electrophoresis followed by ethidium bromide staining. Lanes show results from 1000 bp marker , Left side(lane 1), untreated sample (lane 2), hydromethanol100% fraction (lane 3), 80% fraction (lane 4), 60% fraction (lane 5), 40% fraction (lane 6), and hydro methanol crude (lane 7) of BTE. The figure is a representative of the results from three independent experiments.

Discussion

Recent in vitro studies have shown that many constituents from Iranian orthodox black tea extracts have a wide range of biological action including antibacterial and antifungal activities. The inhibitory action of the tea against experimental carcinogenesis has been demonstrated in many animal models, including those involving cancers of the lung, skin, esophagus, liver and stomach.[12] For example, the biological activities of purified tea polyphenols, strong growth inhibitory effects were investigated using human lung adenocarcinoma cell lines (NCI-H661, NCI-H441 and NCIH1299) and a human colon cancer cell line (HT-29).[13] Growth inhibition was measured by [3H] thymidine incorporation after 48 h of treatment. In addition, the induction of apoptosis was investigated using the Apo Alert TM Annexin V and TUNEL methods.[13]

In the present study, the cytotoxic effects, induction of apoptosis, and the mechanism of cell death of Iranian orthodox black tea extracts (BTEs) and hydro methanolic purified fractions (40, 60, 80 and 100%) were investigated in KB cells (oral squamous cell carcinoma) in vitro. Hydro methanol crude and hydro methanol 80 and 100% fractions of BTE significantly inhibited oral squamous cell carcinoma cells after an incubation period of 24 h by MTT reduction assays. The dye exclusion assay showed a concentration-dependent decrease in percentage of cell viability and a 300 µg/ml concentration of BTE was sufficient to effectively inhibit the cell proliferation. To investigate whether apoptosis is involved in the cell death caused by hydro methanol crude and 80 and 100% fractions of BTE on KB cells, cell death detection ELISA, morphological changes, TUNEL, DNA ladder patterns on agarose gel electrophoresis were done. Morphological changes were observed by convert microscopy which exhibited cytoplasmic membrane, loss of contact with neighboring cells and membrane belbbing. TUNEL assay based on labeling of DNA strand breaks generated during apoptosis revealed that hydro methanol crude and 80 and 100% fractions of BTE induces apoptosis in KB cells. Due to degradation of DNA that resulted from the activation of Ca/ Mg-dependent endonucleases in apoptotic cells, DNA cleavage occurred and led to breaking of strand within

the DNA. In addition, oligonucleosomal DNA fragment (ladders) from cells were exhibited by 1.8% agarose gel electrophoresis after incubation with IC50 of hydro methanol crude and 80 and 100% fractions of BTE. These hallmark features of morphological changes suggest that hydro methanol crude and 80 and 100% fractions of BTE caused apoptosis of KB oral squamous cell carcinoma cells.[14,15]

These probable properties of hydro methanol crude and 80 and 100% fractions of BTE need further detailed evaluation. In order to elucidate the cytotoxic activities of hydro methanol crude and 80 and 100% fractions of BTE extract on the growth of different cell lines may become the target cells used in our future studies. In addition, mechanistic studies on cell cycle arrest and early apoptotic events may be conducted to delineate other possible anti-tumor mechanisms of the hydro methanol crude and 80 and 100% fractions of BTE extract. Besides, future in vivo anti-tumor studies are suggested in order to confirm these in vitro results.

Conclusion

In conclusion, the present study, perhaps for the first time, showed cytotoxicity of Iranian orthodox black tea extracts in oral squamous cell carcinoma in which apoptosis or programmed cell death plays an important role. In addition, mechanisms underlying this cytotoxicity were further clarified. Iranian orthodox black tea extracts could be also considered as a promising chemotherapeutic agent in cancer treatment.

Conflict of Interest

The authors report no conflicts of interest.

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