Evaluation of cytotoxic and necrotic effect of *Beberistina multifida* alcoholic extracts on MCF-7, Hela and A2780 cell lines

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Abstract:

Conventional treatments for cancer treatments are costly and with different serious side effects on patients. Natural herbal treatments have wide acceptance among people because of their minimal side effects, although there are little scientific knowledge about them. One of these remedy, is the root of *Beberistina multifidi* that in Iran, have been used for years in treatment of different genital chronic disease. In current study, methanolic and ethanolic extracts of *B.multifida* effect (induction of necrosis and apoptosis) on Breast cancer (MCF-7), ovarian cancer (A2780), human cervix cancer (Hela) cell lines in comparison with normal breast cells was studied. These effects were determined as morphological alterations in cell light microscopy and by flow cytometry (staining with annexin V and propidium iodide) and measurement of live cells and inhibition concentration by MTT assay. IC50 of *B.multifida* on MCF-7 cell line (methanolic extract) was 400 ug/ml and for A2780 was 250 ug/ml. The IC50 amount of *B.multifida* on MCF-7 cell line (ethanolic extract) was 750ug/ml and 1500 for A2780. Results demonstrated that apoptosis and necrosis occurred in MCF-7 and A2780 following addition of ethanolic and methanolic extracts of *B.multifida* to the medium. These findings were confirmed the anti-cancer effects of *Biebersteinia multifida* root alcoholic extracts and their safety for normal cells, so it can be applied in cancer therapy as a novel medication.

Key words: *Biebersteinia multifida*, traditional medicine, extract, Mcf-7, Hela, A2780, cancer, cytotoxicity

Introduction:

Cancer is a hyperproliferative disease, that causes dysregulation of apoptosis, invasion, proliferation and metastasis in involved patients. In 2018, the global cancer burden was 18.1 milion and 9.6 million death. 1 in every 5 men and 6 in every 6 women globally develop cancer in their life course. Lung, female breast and colorectum cancers are the most occurring types of cancer (Organization, 2018). Treatment of cancer have been researched for many years and novel drugs and different
vaccines (as peptide vaccines against cancer) was being designed and represented to the world (Majidi et al., 2020). The patients are involving in an advanced cancer, commonly face this fact that chemotherapy and chemical medications only can affect a cure for tiny minority of many such cases and many patients and researchers are looking for alternative treatment options as traditional and herbal medicine.

For a long time, natural and herbal products have been considered as precise sources of treatment used in traditional medicine to treat a variety of diseases including infections and malignant diseases (Tavakoli et al., 2012). According to the results of different performed researches on different herbal plants, anticancer activity of some of them have been demonstrated. In these cases they act through enhancing immune system in patients, induction of cell differentiation and apoptosis induction in cancer cells (Lian et al., 2003). The advantage of applying these medications are lack of significant side effects and less dependency to the drug (Fong, 2002).

According to a national research in 2005 in Australia, about 68.9% of general population in this country, had used at least 1 of complementary alternative medications for health enhancement (Jones et al., 2019). In a Canadian research was observed that 20% breast cancer patients was used at least 1 herb traditional medication for cancer therapy or as complementary medication. In America the rate of applying these medications was more than 65% (Tavakoli et al., 2012). Although these alternative medications are growing globally, most of knowledge are anecdotal and not being clinically studied and are rarely with clinical trials needed for a drug to be applied by population (Wachtel-Galor et al., 2004). About 20% of plants are being used in pharmacological studies such as treating of cancer. Plants have the ability to produce diverse bioactive compounds. Plants contain different phytochemicals as natural antioxidants. Many plants are rich of antioxidants as tannins, flavonoids, and lignins. Antioxidants reduce oxidative damages caused by reactive oxygen species and so increase foods safety.

*Biebersteinia multifida* DC (Geraniaceae) is a local herb in Iran that known as Adamak. It has long stems (about 20-20–70 cm, with yellow flowers dark brown roots. The main part of plant as e herbal medicine is its roots (Farsam et al., 2000). *B. multifida* had been applied rarely in novel biological investigations. The plant had been applied as a medication for problems of skeleton-muscles and disorders in bones (Naghibi et al., 2005) and as a drug for easing pain and anti-inflammatory medicine (Khakpour et al., 2013). There are some reports in application of *B. multifida* in treatment of anxiety and phobia (Monsef-Esfahani et al., 2013). In *B. multifida* extract, there are some bioactive compounds as polysaccharides, peptides, flavonoids (apigenin and luteolin), alkaloids and essential oils (Golshan et al., 2016). There are different methods for isolation of bioactive compounds. Solvent based methods are consisting of application of different solvents as methanol, water, ethyl alcohol, hexane, *N,N* dimethylformamide (DMF) and acetone (Altemimi et al., 2017) and a variety of bioactive compounds are released in each solvent. Selection of appropriate solvent is essential and crucial for extraction method and different parameters as cost of solvents and method, solubility, safety and selectivity should be considered in all steps (Cragg and Newman, 2013). The aim of the present study was to evaluate the cytotoxicity effect of ethanolic and methanolic extracts of *Beberistina multifidi* on MCF-7, HeLa, A2780 and human normal breast cells.

**Materials and methods:**

**Plant material**

*Beberistina multifidi* root was collected from Khorasan Razavi province, Iran, and its validation was performed by Mashhad Ferdowsi University, pharmacological research center for medicinal plants (registry
code: 28592). The roots was cleaned, air-dried, chopped to small pieces and after all it was stored in light protected containers (-20°C).

**Alcoholic extract preparation**

Both methanolic and ethanolic extracts were prepared by perulation method. Root dried, chopped pieces were stored in a alcohol containing container (methanol or ethanol) for 24 hours at room temperature. Wet roots with alcohol were moved to perculator instrument and after obtaining extracts, concentrated by evaporation method. Concentrated extracts were dried by lyophilization method and obtained methanolic and ethanolic powders were stored at -20°C.

**Cell culture**

Cell lines consisting of MCF-7 (breast cancer cell line), Hela (human cervix epithelial cell line) and normal breast cells were obtained from Razi Vaccine and serum researches institute and A2780 (human ovarian cancer cell line) was purchased from Buali (Avicenna) research institute of Mashhad (University of Medical science). Cell lines were cultured in 25cm² flasks (in Dulbecco’s Modified Eagle Medium (DMEM)-high glucose, 10% fetal bovine serum (FBS) (Gibco) and 4% penicillin/streptomycin) and stored at 37°C in a humidified 5% CO2 atmosphere. After growth and reaching to 80% confluency, cells were transferred to 96 well plates and stored in incubator for treatment with extracts.

**Cytotoxicity assay and determination of the IC50**

Both methanolic and ethanolic extracts of Beberistina multifidi were subjected for cytotoxicity evaluation test on MCF07, A2780, Hela and Normal breast cells (NB). After reaching of cell confluency to 80% in plates, different concentrations of alcoholic extracts (methanol and ethanol) were prepared (final concentration: 30, 50, 100, 250, 400, 500, 1000, 2000, 5000 ug/ml of extract). Cells were treated with prepared concentrations and incubated for 48 hours and after incubation time, morphology of cells was examined by microscope. 100 ul of MTT (4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) containing DMEM (2.5mg of MTT in 10ml DMEM), was added to each well. The plates were incubated for 4 hours and the medium was removed and 100ul of dimethyl sulfoxide (DMSO) was added to each well. After 10 minutes, the absorbance was measured at 570nm in micro titer plate reader (Biotek®-ELx808). Finally, the function of mitochondrial metabolism was expressed as the percentage of viable treated cells to viable cells in untreated control. The results were analyzed by excel.

**Flow cytometry analysis**

Annexin V conjugated to fluorescein isothiocyanate (FITC) and Propidium iodide (PI) (Annexin V-FITC kit, IQ Products, Netherland) were used for flow cytometry staining and analysis of cells in different steps of growth and death. MCF-7, A2780, Hela and normal breast cells were cultured in 24 well plates (80% confluency) and treated with 500, 750 and 1000 ug/ml concentrations of methanolic and ethanolic extracts of B. multifidi for 24 hours in 37°C. After incubation time, the media was collected (floating cells were in collected medium) and the cells were trypsinized and cells were added to floating cells before. After centrifugation of cells, the pellet was washed with phosphate buffer saline (PBS) and 500ul of binding buffer (mentioned Annexin V Kit) was added to each tube (cell numbers about 5×10⁵). To each test tube, 5 ul of both annexin V/FITC and PI reagents were added and vortexed. Cells were incubated in darkness (4°C- 15 minutes). Finally cells were analyzed by flow cytometer instrument (FACS Calibur, BD, USA). Cell alterations of annexin/PI stained cells was observed by Nikon fluorescent microscope (Nikon, Tokyo, Japan) equipped with a digital camera (Nikon, Tokyo, Japan).

**Antioxidant and poly phenolic content measurement**
Anti-oxidant activity of *B. multifida* alcoholic extracts was determined by DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) method (Chu et al., 2000). Phenolic compounds determination and measurement of both methanolic and ethanolic extracts of *B. multifida* was performed by Folin-Ciocalteu assay (Ainsworth and Gillespie, 2007).

**Results**

**MTT assay results**

For each concentration, dose response curve (DRC) against all of the cell lines was plotted. Positive control (mitomycin) response, was considered as 100% cell inhibition and negative control with DMED was considered as 100% cell viability. The results are presented in figure 1 and 2.

**Evaluation of morphological alteration upon *Beberistina multifidi* extract treatment**

All observations for morphological alteration study was performed using invert microscope. Cytoplasmic granulation and about 50% cell rounding were observed in MCF-7 cell line after 36hrs. Whereas, there was no observable alteration in other cell lines (table 2 and figure 3).

**Poly phenol and antioxidant content of *B. multifida***

According to DPPH and spectrophotometric results, the antioxidant content of *B. multifida* in 100g of ethanolic powdered extract was 465 mg and the amount of polyphenols in extract was 0.8g in 100 grams. For methanolic powdered extract, antioxidant content was 480 mg and polyphenol amount was 0.9 in 100 grams.

**Discussion:**

Recently, application of herbal medicines has been increased globally because of the miraculous therapeutic effects of these drugs and fewer side effects on patients in comparison with modern medicines. Previously, application of herbal and traditional medicines was in a great doubt due to the lack of a clear knowledge about processing, standardizing, extracting and ingredients. These days’ scientists, are increasingly applying different herbs in treatment of a variety of diseases, and performing investigations on their effects and ingredients in vitro and in vivo.

According to high prevalence of cancer in human population, and different side effects of chemical medications on patients, a significant percent of patients prefer to use traditional methods instead of novel medicine treatments. Numerous studies have shown different applications of *Beberistina multifidi*, in traditional treatment and as a medications.

*B. multifida* have been traditionally and for decades in medication of women genital chronic and incurable diseases and according to the previous studies that mentioned before and in this part, we had decided to investigate its effects on cancer cell lines.

Figures 1-3 and tables 1 and 2, are presenting and showing the effect of *Beberistina multifidi* on cell lines applied in current study. As can be seen in these presented data, MCF-7 (in ethanolic extract and methanolic extract respectively) and A2780 (in methanolic) had been affected more than the other cell lines by alcoholic extracts of *B. multifida*. IC50 of *B. multifida* on MCF-7 cell line (methanolic extract) was 400 ug/ml and for A2780 was 250 ug/ml. The IC50 amount of *B. multifida* on MCF-7 cell line (ethanolic extract) was 750ug/ml and 1500 for A2780. Ethanolic
and Methanolic extracts are complexes of different ingredients and effect of these extracts on cell lines is because of association of these components. In this study, the levels of antioxidants and poly phenols were analyzed and the amount of poly phenolic and antioxidants was relatively high. This measured parameters were indicated that methanolic extract is richer in comparison with ethanolic and these results are in confirmation of IC50 results. It is worth mentioning that the difference level of antioxidants and polyphenols measured here is ignorable. The main effective ingredients of *B.multifida* were hexadecanoic acid, phytol and trimethyl pentadecanone and Nerolidol (Omurkamzino et al., 1991b, Omurkamzina et al., 1991a, Greenham et al., 2001, Golshan et al., 2016). According to Greenham et al., *B.multifida* is highly distinctive in the content of flavonoids (Greenham et al., 2001).

Cells in the cases of oldness or damage, die by some mechanisms as necrosis, apoptosis and a combination of these two. The immortality of cancer cells is because of their resistance to apoptosis. In medications by chemicals and other pharmaceuticals, the apoptosis and necrosis induces to cancer cells (Mahassni and Al-Reemi, 2013). There are different methods to recognize apoptosis, as the main mechanism of action of cytotoxic agents (herbal or chemical). The role of natural compounds in as a pharmacological regulator of cell proliferation and differentiation specially in cancer cells, has been recently been appreciated (Lombardi et al., 2017). The induction of necrosis and apoptosis in MCF-7, A2780, Hela and normal breast cells by alcoholic (ethanolic and methanolic) extracts was monitored by analysis of morphological changes (indicated by microscopic observations) and by using fluorescent stains (PI and Annexin V) with flow cytometry assay and fluorescent microscopy. Results demonstrated that apoptosis and necrosis occurred in MCF-7 and A2780 following addition of ethanolic and methanolic extracts of *B.multifida* to the medium. Although in current study, the concentrations applied was relatively high (for flow cytometric assay in figures 5-8, about 750 ug/ml), the rate of necrosis was ignorable and the extracts didn’t cause necrosis in cell lines. According to the lack of caspase 3 pathway in MCF-7 (Blanc et al., 2000), we can suggest that the effect of *B.multifida* extract on cell apoptosis was not correlated to this pathway. In current study, because of effectiveness of *B.multifida* on cell death rate of MCF-7 and A2780 cell lines, it can be a candidate in treatment of these cancer types. In Golshan *et al*, study, the cytotoxic effects of *Beberistina multifidi* extract (hydro-ethanolic) on human prostate cancer cells was studied and the results indicated that *B.multifida* extract decreased the viability of PC3 cell lines (Golshan et al., 2016). In another study, Hashem Dabaghian *et al* demonstrated the anticancer effects of *Beberistina multifidi* on human leukemia cells and suggested it as a good medication candidate for cancer treatment. (Dabaghian et al., 2014).

### Conclusion:

In the present study, our findings demonstrated that alcoholic extracts of Beberisteina multifidi exert satisfactory cytotoxic effects on MCF-7 and A2780 cancer cell lines. Based on chemical analysis of *B.multifida* performed in current study, these alcoholic extracts have high contents of antioxidants and phenolic compounds and according to these achievements this herbal compound can be a useful in finding new medications for cancer therapy.

### Acknowledgement:

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Conflicts of interest:

The authors declare that they have no conflict of interest.

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**Figures and Tables:**

![Figure1](image_url)

**Figure1**. Dose Response chart of ethanolic extract of *Beberistina multifidi* on different cell lines. IC50 is determined by threshold line.
Figure 2. Dose Response chart of methanolic extract of *Beberistina multifidi* on different cell lines. IC50 is determined by threshold line.

Figure 3. IC50 values of methanolic and ethanolic extracts of *B. multifidi* in Hela, MCF-7, A2780 and NB cells.

<table>
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<th>Cell type</th>
<th><em>Beberistina multifidi</em> extract concentration (ug/ml)</th>
<th>Positive control (mitomycin)</th>
<th>Negative control</th>
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<tbody>
<tr>
<td></td>
<td></td>
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Table 1. Morphological changes of different cell lines in response to *Beberistina multifidi* ethanolic extract effect in 3 concentrations (500, 1000 and 2000 ug/ml) after 36 hrs.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Beberistina multifidi extract concentration (ug/ml)</th>
<th>Positive control (mitomycin)</th>
<th>Negative control</th>
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<td><img src="image10" alt="Image" /></td>
<td><img src="image11" alt="Image" /></td>
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Table 2. Morphological changes of different cell lines in response to *Beberistina multifidi* methanolic extract effect in 3 concentrations (500, 1000 and 2000ug/ml) after 36 hrs.

Figure 4. FITC/PI staining of different states of apoptotic cells and necrotic cells of MCF-7 breast cancer cell line. Necrotic cells (Annexin V−,PI+) are orange (A), early apoptotic cells (Annexin V+,PI−) are green (B), late apoptotic cells (Annexin V+,PI+) are green and orange (C) and viable cells do not take any color (Annexin V−,PI−)(D).
Figure 5. Table of representing the percentages of both apoptotic (late and early) and necrotic cells induced by the *B. multifida* methanolic extract in MCF-7, Hela, A2780 and NB cells stained with annexin V/propidium iodide as observed by flow cytometry (in 750ug/ml concentration of extract).

Figure 6. Table of representing the percentages of both apoptotic (late and early) and necrotic cells induced by the *B. multifida* ethanolic extract in stained cell lines.

Figure 7. Qualitative Flow Cytometric Analysis of Apoptosis/Necrotic Cell Death using Annexin-V-FITC/PI Staining of MCF-7 Cells Treated with *B. multifida*. Quadrant 1, represents Necrotic cells; Quadrant 2 represents late apoptotic cells and necrotics; Quadrant 3 represents early apoptotic cells and Quadrant 4 represents live MCF-7 cells. Effect of 750 ug/ml of ethanolic extract on different cell lines.
Figure 8. Qualitative Flow Cytometric Analysis of Apoptosis/Necrotic Cell Death using Annexin-V-FITC/PI Staining of MCF-7 Cells Treated with *B. multifida*. Quadrant 1, represents Necrotic cells, Quadrant 2 represents late apoptotic cells and necrotics; Quadrant 3 represents early apoptotic cells and Quadrant 4 represents live MCF-7 cells. Effect of 750 ug/ml of Methanolic extract on different cell lines.