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Original Article

Cytotoxic Effect of *Biebersteinia multifida* Alcoholic Extracts on MCF-7, HeLa, and A2780 Cell Lines

Irvani, Z[1, 2, 3], Mehrbani, M[4 *], Farzin, H[3], Majidi, B[3], Mohammadi, A[3], Toroghi, R[3], Motevasselian, M[5]

1. Neuroscience Research Center, Institute of Neuropharmacology, Kerman University of Medical Sciences, Kerman, Iran
2. Department of Traditional Medicine, Faculty of Traditional Medicine, Kerman University of Medical Sciences, Kerman, Iran
3. Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Tehran, Iran
4. Herbal and Traditional Medicines Research Center, Kerman University of Medical Sciences, Kerman, Iran
5. Herbal and Traditional Medicines Research Center, Mashhad University of Medical Sciences, Mashhad, Iran

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Corresponding Author: mzmehrbani@yahoo.com

Abstract

Conventional cancer treatments are costly and have different serious side effects for patients. Natural herbal treatments are widely accepted among people because of their minimal side effects, although there is little scientific knowledge about them. One of these remedies utilizes the root of *Biebersteinia multifidi* that has been used for years in Iran to treat different chronic genital diseases. The current study examined the effects of methanolic and ethanolic extracts of *B. multifida* (induction of necrosis and apoptosis) on breast cancer (MCF-7), ovarian cancer (A2780), and human cervix cancer (HeLa) cell lines in comparison with normal breast cells. These effects were determined to be morphological alterations in cell light microscopy, by flow cytometry (staining with annexin V and propidium iodide), and by measuring live cells and inhibition concentrations by MTT assay. IC50 of *B. multifida* on the MCF-7 cell line (methanolic extract) was 400 µg/ml and for A2780 was 250 µg/ml. The IC50 amount of *B. multifida* on the MCF-7 cell line (ethanolic extract) was 750 µg/ml and 1500 for A2780. Results demonstrated that apoptosis and necrosis occurred in MCF-7 and A2780 following the addition of ethanolic and methanolic extracts of *B. multifida* to the medium. These findings confirmed the anti-cancer effects of mehthanolic extracts of *Biebersteinia multifida* root and its safety for normal cells; thus, it can be applied in cancer therapy as a novel medication.

Keywords: *Biebersteinia multifida*, traditional medicine, extract, MCF-7, HeLa, A2780, cancer, cytotoxicity

Effet Cytotoxique et Nécrotique des Extraits Alcooliques de *Biebersteinia multifida* sur les Lignées Cellulaires MCF-7, HeLa et A2780

Résumé: Les traitements conventionnels du cancer sont coûteux et ont différents effets secondaires graves pour les patients. Les traitements naturels à base de plantes sont largement acceptés par les gens en raison de leurs effets secondaires minimes, bien qu'il y ait peu de connaissances scientifiques à leur sujet. L'un de ces remèdes utilise la racine de *Biebersteinia multifidi* qui est utilisée depuis des années en Iran pour traiter différentes maladies génitales chroniques. La présente étude a examiné les effets des extraits méthanoliques et éthanoliques de *B. multifida* (induction de nécrose et d'apoptose) sur le cancer du sein (MCF-7), le cancer de l'ovaire (A2780) et le cancer du col de l'utérus humain (HeLa) en comparaison avec des cellules mammaires normales. Ces effets ont été déterminés comme étant des altérations morphologiques en microscopie optique cellulaire, par cytométrie en flux (coloration à l'annexine V et à l'iodure de propidium) et en mesurant les cellules vivantes et

les concentrations d'inhibition par dosage MTT. La CI50 de *B. multifida* sur la lignée cellulaire MCF-7 (extrait méthanolique) était de 400 ug/ml et pour A2780 était de 250 ug/ml. La quantité de CI50 de *B. multifida* sur la lignée cellulaire MCF-7 (extrait éthanolique) était de 750 ug/ml et de 1500 pour A2780. Les résultats ont démontré que l'apoptose et la nécrose se sont produites dans MCF-7 et A2780 suite à l'ajout d'extraits éthanoliques et méthanoliques de *B. multifida* au milieu. Ces résultats ont confirmé les effets anticancéreux des extraits méthanolique *de racine deBiebersteinia multifida* et leur sécurité pour les cellules normales; ainsi, il peut être appliqué dans le traitement du cancer en tant que nouveau médicament.
Mots-clés: *Biebersteinia multifida*, médecine traditionnelle, extrait, Mcf-7, HeLa, A2780, cancer, cytotoxicité

1. 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 million, and 9.6 million cancer cases resulted in death. One in every five men and one in every six women globally develop cancer. Lung, female breast, and colorectum cancers are the most prevalent types of cancer (1). Treatments for cancer have been researched for many years, and novel drugs and different vaccines (as peptide vaccines against cancer) have been designed and presented to the world (2). Patients involved in an advanced cancer commonly face the fact that chemotherapy and chemical medications only can affect a cure for a tiny minority of cancer cases, and many patients and researchers are looking for alternative treatment options, such 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 (3). The results of research on different herbal plants had demonstrated that some of them have anticancer activity. Such plants act through enhancing the immune system in patients, inducing cell differentiation, and inducing apoptosis in cancer cells (4). The advantage of applying these medications are the lack of significant side effects and less dependency on drugs (5).

According to a national research in 2005 in Australia, about 68.9% of the general population had used at least one complementary alternative medication for health enhancement (6). In a study in Canada, it was observed that 20% of breast cancer patients had used at least one herbal traditional medication for cancer therapy or as a complementary medication. In America, the rate of applying these medications was more than 65% (3). Although the use of such alternative medications is growing globally, most of our knowledge of them is anecdotal. Most of them have not been clinically studied, and even more rarely have they been tested in the clinical trials needed for a drug to be applied by the population (7). About 20% of plants are being used in pharmacological studies on topics such as the treatment of cancer. Plants can produce diverse bioactive compounds and contain various phytochemicals as natural antioxidants. Many plants are rich in antioxidants, such as tannins, flavonoids, and lignins. Antioxidants reduce oxidative damage caused by reactive oxygen species, thus increasing food safety.

Biebersteinia multifida DC (Geraniaceae) is a local herb in Iran known as Adamak. It has long stems (about 20-70 cm), yellow flowers, and dark brown roots. The main part of this plant used in herbal medicine are its roots (8). *B. multifida* has rarely been applied in novel biological investigations, yet it has been applied as a medication for skeletal-muscular issues and bone disorders (9) and as a pain reliever and anti-inflammatory medicine (10). There are some reports of *B. multifida* being applied for the treatment of anxiety and phobia (11). *B. multifida* extract has some bioactive compounds, such as polysaccharides, peptides, flavonoids (apigenin and luteolin), alkaloids,

and essential oils (12). There are different methods for isolating bioactive compounds. Solvent-based methods consist of applying different solvents, including methanol, water, ethyl alcohol, hexane, *N*,*N*dimethylformamide (DMF), and acetone (13), and a variety of bioactive compounds are released in each solvent. Selection of the appropriate solvent is essential in extraction methods, and different parameters such as the costs of solvents and methods, solubility, safety, and selectivity should be considered in all steps (14). The aim of the present study was to evaluate the cytotoxicity effects of ethanolic and methanolic extracts of *Beberistina multifidi* on MCF-7, HeLa, A2780 and human normal breast cells.

2. Material and Methods

2.1. Plant Material

Biebersteinia multifidi root was collected from Khorasan Razavi province, Iran, and validated by the Mashhad Ferdowsi University, Pharmacological Research Center for Medicinal Plants (registry code: 28592). The roots were cleaned, air-dried, chopped finely, and then stored in light protected containers at -20 ℃).

2.2. Alcoholic Extract Preparation

Both methanolic and ethanolic extracts were prepared by percolation. Chopped and dried root pieces were stored in a container containing alcohol (methanol or ethanol) for 24 hours at room temperature. Roots wet with alcohol were moved to a percolator, and after extracts were obtained, they were concentrated through evaporation. Concentrated extracts were dried by the lyophilization method, and the methanolic and ethanolic powders were stored at -20 ℃.

2.3. Cell Culture

MCF-7 (breast cancer cell line), HeLa (human cervix epithelial cell line), and normal breast cells were obtained from Razi Vaccine and Serum Research Institute. A2780 (human ovarian cancer cell line) was purchased from the Buali (Avicenna) Research Institute of Mashhad (University of Medical Science). Cell lines were cultured in 25 cm^2 flasks (in Dulbecco's Modified Eagle Medium (DMEM)-high glucose, 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin/streptomycin) and stored at 37 ℃ in a humidified 5% CO2 atmosphere. After growth and reaching 80% confluency, cells were transferred to 96-well plates and stored in an incubator for treatment with extracts.

2.4. Cytotoxicity Assay and Determination of IC50

Both methanolic and ethanolic extracts of *Beberistina multifidi* were subjected to cytotoxicity evaluation tests on MCF-7, A2780, HeLa, and normal breast cells (NB). When cells reached about 80% confluence, different concentrations of alcoholic extracts (methanol and ethanol) were prepared (final concentrations: 30, 50, 100, 250, 300, 400, 500, 750, 1000, 2000, and 5000 µg/ml extract). Cells were treated with the prepared concentrations and incubated for 48 hours. After incubation, the morphology of cells was examined by a microscope. 10 µl of MTT (4, 5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide) was added to each well, and the plates were incubated for 4 hours. Then the medium was removed, and 100 µl of dimethyl sulfoxide (DMSO) was added to each well. After 10 minutes, the absorbance was measured at 570 nm in a microtiter plate reader (Biotek®-ELx808). Finally, the function of mitochondrial metabolism was expressed as the percentage of viable treated cells to viable cells in the untreated control.

2.5. 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 µg/ml concentrations of methanolic and ethanolic extracts of *B. multifidi* for 24 hours at 37 ℃. After incubation, the media was collected (floating cells were in collected medium) and the cells were trypsinized and

added to floating cells collected before. After centrifugation of cells, the pellet was washed with phosphate buffer saline (PBS), and 500 µl of binding buffer (Annexin V Kit) was added to each tube (cell numbers about 5×10^5). To each test tube, 5 µl of both annexin V/FITC and PI reagents were added, and the tubes were vortexed. The cells were incubated in darkness (4 ℃- for 15 minutes) and then analyzed by flow cytometry (FACS Calibur, BD, USA). Cell alterations of annexin/PI-stained cells were observed with a Nikon fluorescent microscope (Nikon, Tokyo, Japan) equipped with a digital camera (Nikon, Tokyo, Japan).

2.6. Polyphenolic Content Measurement

Phenolic compounds of both methanolic and ethanolic extracts of *B. multifida* were determined and measured by Folin-Ciocalteu assay (15). To examine the type of cell death, all cell lines were stained with annexin V-FITC/PI and analyzed by flow cytometry. The result of flow cytometry showed that both extracts had higher toxicity at a concentration of 750 µg/ml, especially on MCF-7 and A2780 cell lines, so that the cell population shifted from viable to apoptotic."

3. Results

3.1. MTT Assay Results

For each concentration, the dose response curves (DRCs) against all cell lines were plotted. The 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 Figures 1 and Figure 2.

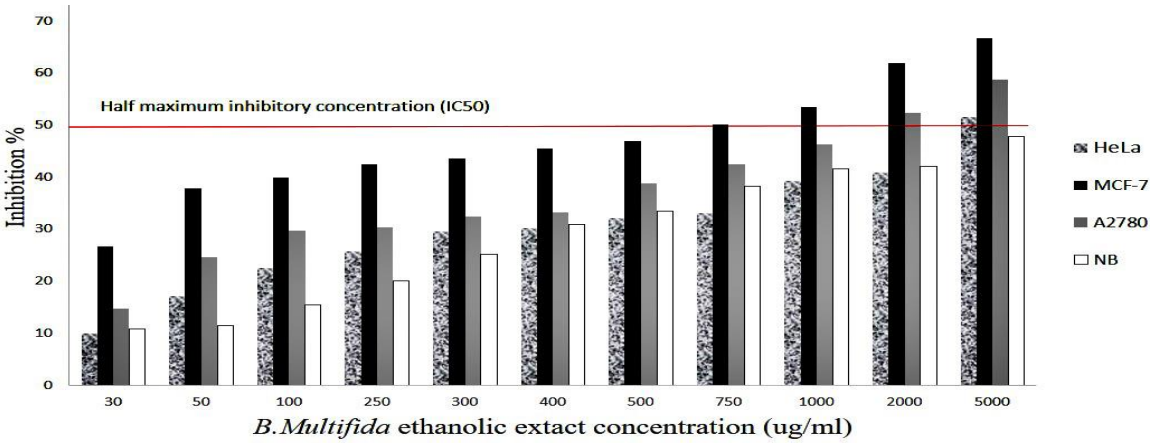


Figure 1. Dose response chart of ethanolic extract of *Beberistina multifidi* on different cell lines. IC50 is determined by threshold line.

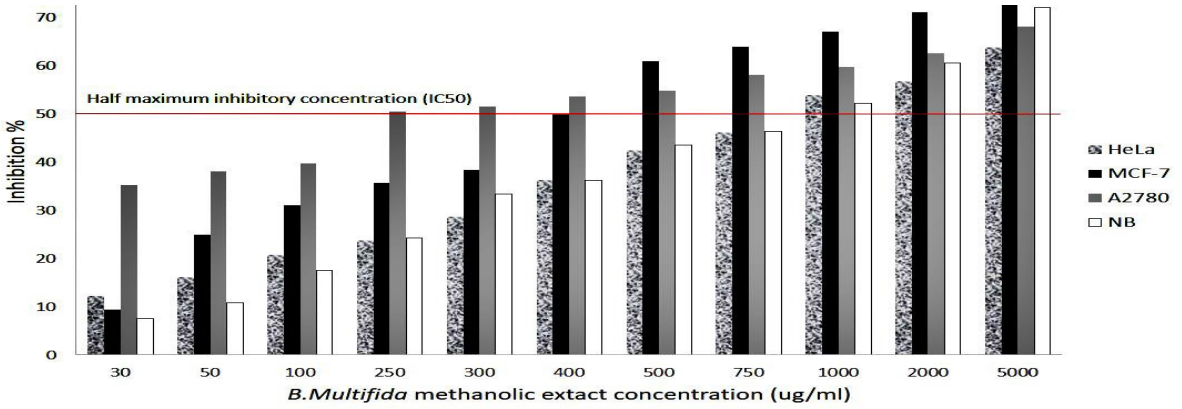


Figure 2. Dose response chart of methanolic extract of *Beberistina multifidi* on different cell lines. IC50 is determined by threshold line.

3.2. Evaluation of Morphological Alteration upon *Beberistina multifidi* Extract Treatment

All observations for morphological alteration study were made using an inverted microscope. Cytoplasmic granulation and about 50% cell rounding were observed in the MCF-7 cell line after 36 hrs, whereas there were no observable alterations in other cell lines (Table 2 and Figure 3).

3.3. Polyphenolic Content of *B. multifida*

The content of polyphenols in the extract was 0.8±0.04 g in 100 grams. For the powdered methanolic extract, polyphenol amount was 0.9±0.02 in 100 grams.

3.4. Apoptotic Rate Measurement

To examine the type of cell death, all cell lines were stained with annexin V-FITC/PI and analyzed by flow cytometry. The result of flow cytometry showed that both extracts had higher toxicity at a concentration of 750 µg/ml, especially on MCF-7 and A2780 cell lines, so that the cell population shifted from viable to apoptotic (Figures 4-8).

Table 1. Morphological changes of different cell lines in response to *Beberistina multifidi* ethanolic extract effect at three concentrations (500, 1000, and 2000 µg/ml) after 36 hrs

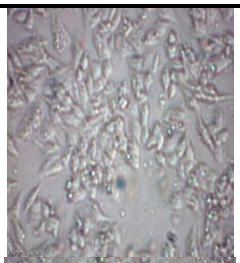
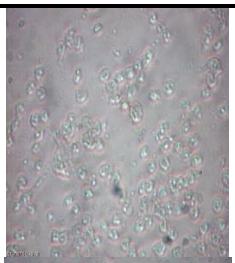
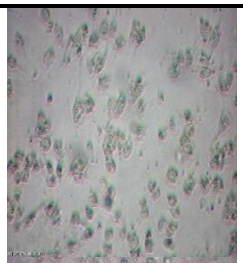
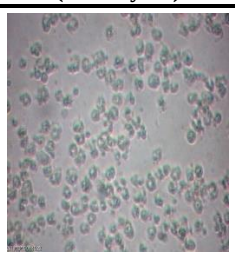
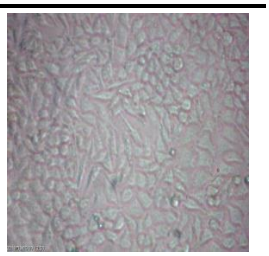
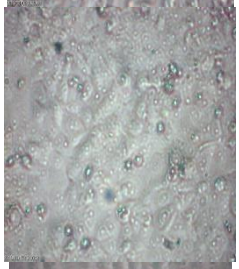
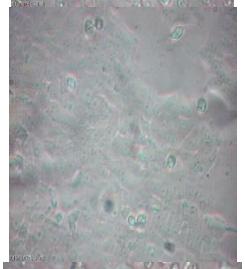
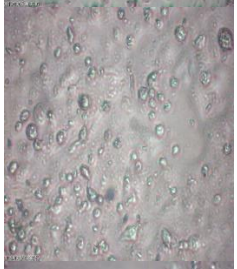
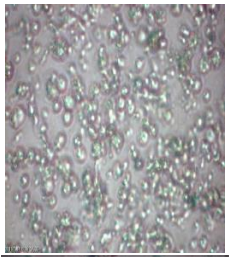
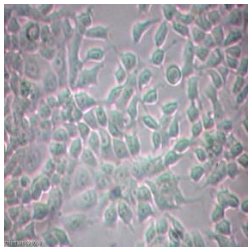
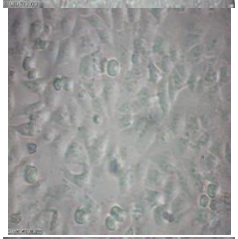
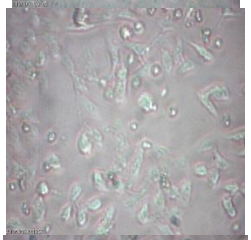
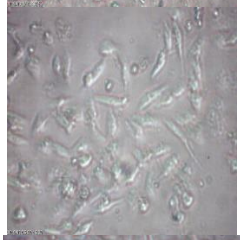
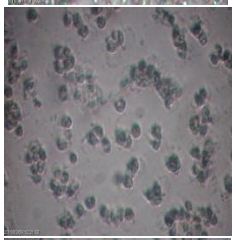
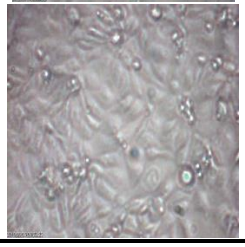
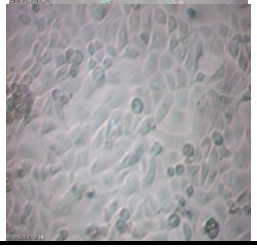
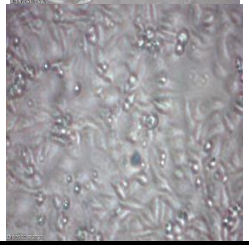
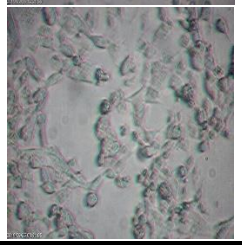
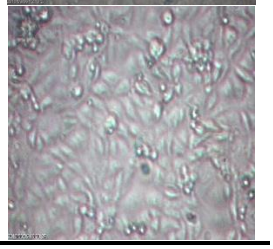
Cell type	*Beberistina multifidi* extract concentration (ug/ml)			Positive control (mitomycin)	Negative control
	500	1000	2000		
A2780					
MCF-7					
HeLa					
Normal breast cell (NB)					

Table 2. Morphological changes of different cell lines in response to *Beberistina multifidi* methanolic extract effect in 3 concentrations (500, 1000, and 2000 ug/ml) after 36 hrs

Cell type	*Beberistina multifidi* extract concentration (ug/ml)			Positive control (mitomycin)	Negative control
	500	1000	2000		
A2780					
MCF-7					
HeLa					
Normal breast cell (NB)					

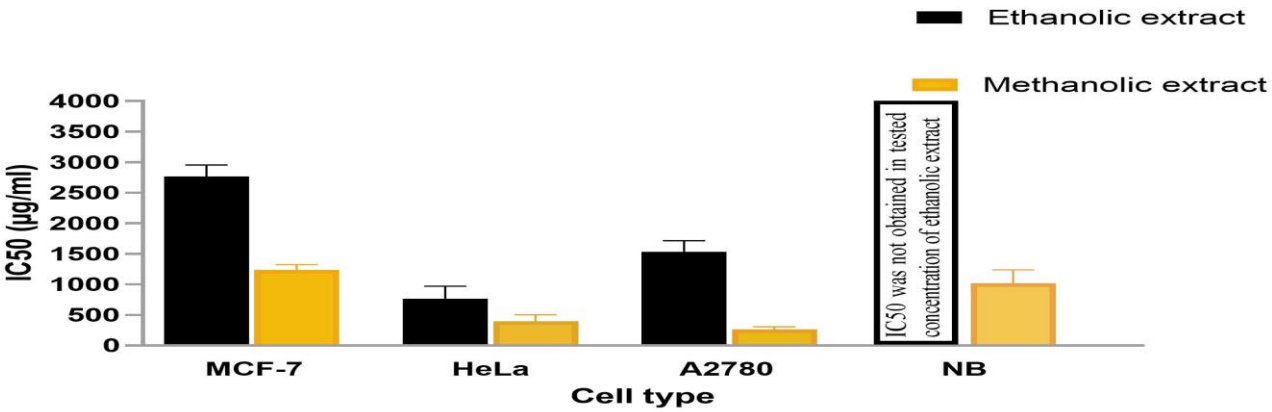


Figure 3. IC50 values of methanolic and ethanolic extracts of *B. multifidi* in HeLa, MCF-7, A2780, and NB cells. Data are presented as Mean ± SEM (n=3).

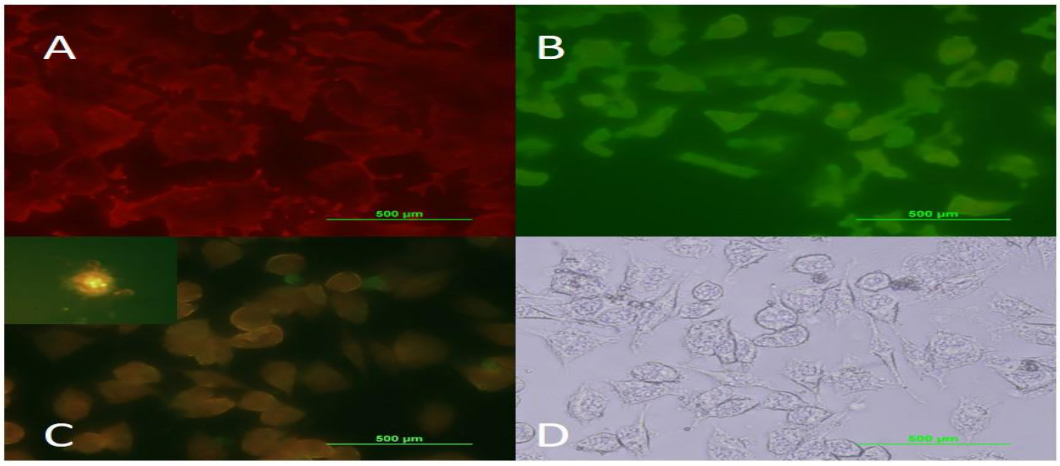


Figure 4. FITC/PI staining of different states of apoptotic cells and necrotic cells of MCF-7 breast cancer cell line. Necrotic cells (Annexin V−, PI+) are orange **(A)**, early apoptotic cells (Annexin V+, PI−) are green **(B)**, late apoptotic cells (Annexin V+, PI+) are green and orange **(C)**, and viable cells do not take any color (Annexin V−, PI−) **(D)**.

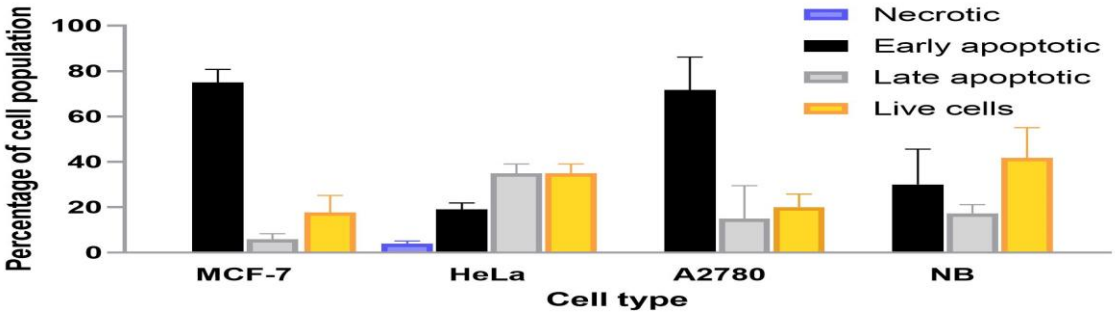


Figure 5. Percentages of both apoptotic (late and early) and necrotic cells induced by *B. multifida* methanolic extract in MCF-7, Hela, A2780, and NB cells stained with annexin V/propidium iodide as observed by flow cytometry (in 750 µg/ml concentration of extract). Data are presented as Mean ± SEM (n=3).

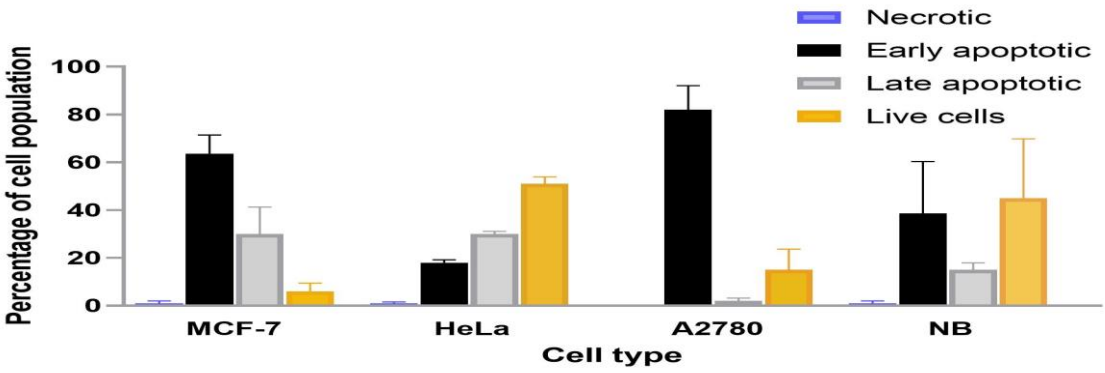


Figure 6. Percentages of both apoptotic (late and early) and necrotic cells induced by *B. multifida* ethanolic extract in MCF-7, HeLa, A2780, and NB cells stained with annexin V/propidium iodide as observed by flow cytometry (in 750 µg/ml concentration of extract). Data are presented as Mean ± SEM (n=3).

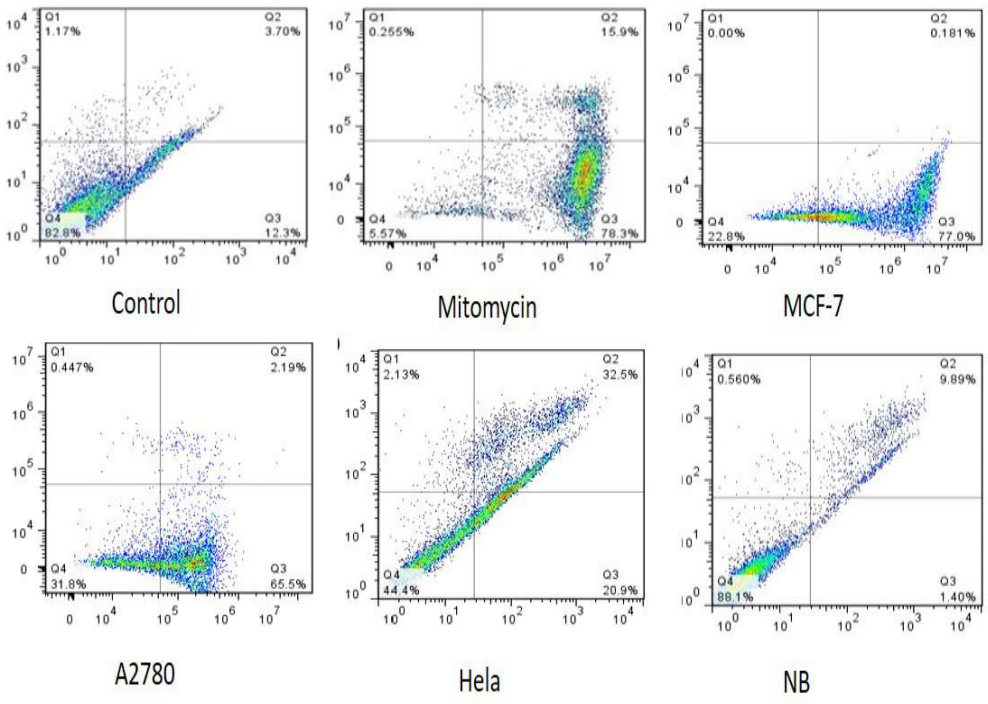


Figure 7. Qualitative flow cytometric analysis of apoptosis/necrotic cell death using annexin-V-FITC/PI staining of all cell lines treated with *B. multifida*. Quadrant 1 represents necrotic cells; quadrant 2 represents late apoptotic cells and necrotics; quadrant 3 represents early apoptotic cells; and quadrant 4 represents live cells. Effects of 750 µg/ml of ethanolic extract on different cell lines

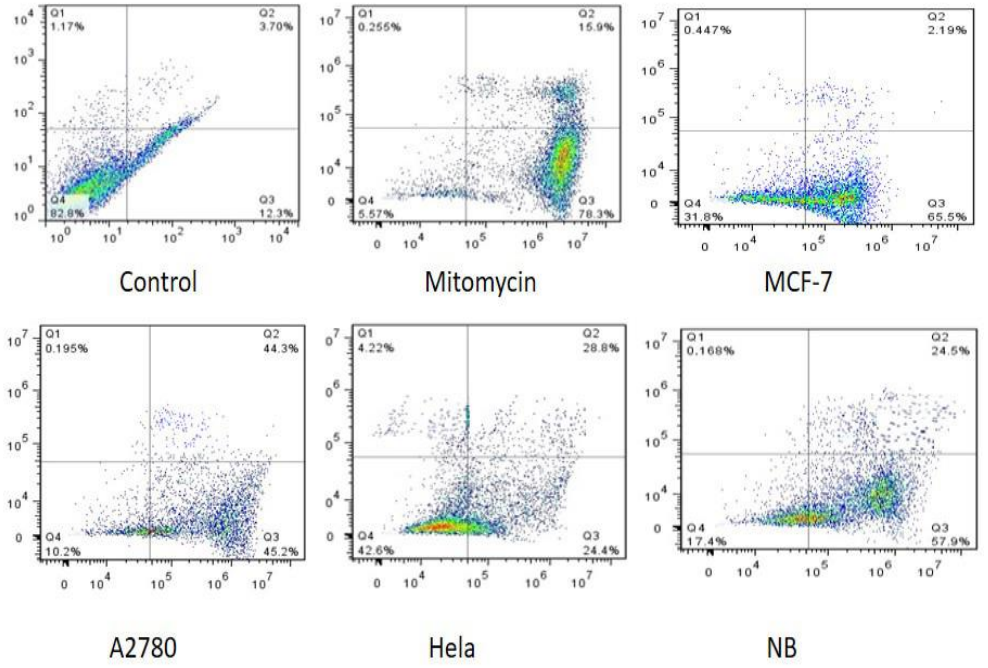


Figure 8. Qualitative flow cytometric analysis of apoptosis/necrotic cell death using annexin-V-FITC/PI staining of all cell lines treated with *B. multifida*. Quadrant 1 represents necrotic cells; quadrant 2 represents late apoptotic cells and necrotics; quadrant 3 represents early apoptotic cells; and quadrant 4 represents live cells. Effects of 750 µg/ml of methanolic extract on different cell lines

4. Discussion

Recently, the application of herbal medicines has increased globally because of the miraculous therapeutic effects and fewer side effects of these drugs on patients in comparison with modern medicines.

Because of the high prevalence of cancer in the human population and the various side effects of chemical medications on patients, a significant percentage of patients prefer to use traditional methods instead of chemical treatments. Some studies have demonstrated different applications of *Beberistina multifidi* in traditional treatments and as a medication.

B. multifida has been used in traditional medicine for decades for women with genital chronic and incurable diseases. Based on that and the studies previously mentioned, the current research investigated the effects of *B. multifida* on cancer cell lines.

Figures 1-3 and Tables 1 and 2 show the effects of *Beberistina multifidi* on the cancer cell lines applied in the current study. As can be seen in the presented data, MCF-7 (in ethanolic and methanolic extracts, respectively) and A2780 (in methanolic extract) were more affected than the other cell lines by the alcoholic extracts of *B. multifidi*. The IC50 of *B. multifida* on the MCF-7 cell line (methanolic extract) was 400 ug/ml and for A2780 was 250 ug/ml. The IC50 amount of *B. multifida* on the MCF-7 cell line (ethanolic extract) was 750ug/ml and 1500 for A2780. Ethanolic and methanolic extracts are complexes of different ingredients, and the effects of these extracts on cell lines are due to the association of these components. In this study, the levels of polyphenols were analyzed and found to be relatively high. The measured parameters indicated that methanolic extract is richer in comparison with ethanolic extract, and these results confirm the IC50 results. It is worth mentioning that the difference in polyphenol levels measured here is ignorable. The main effective ingredients of *B. multifida* were hexadecanoic acid, phytol and trimethyl pentadecanone, and nerolidol (12, 16-18). According to Greenham et al., *B. multifida* is highly distinctive in flavonoid contents (16).

In cases of old age or damage, cells die by mechanisms such as necrosis, apoptosis, or a combination of these two. The immortality of cancer cells is due to their resistance to apoptosis. In chemical medications and other pharmaceuticals, apoptosis and necrosis induce cancer cells (19). There are different methods for recognizing apoptosis as the main mechanism of the action of cytotoxic agents (herbal or chemical). The role of natural compounds as a pharmacological regulator of cell proliferation and differentiation specially in cancer cells, has recently been appreciated (20). 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 using florescent stains (PI and Annexin V) with flow cytometry assay and fluorescent microscopy. The results demonstrated that apoptosis and necrosis occurred in MCF-7 and A2780 following the addition of ethanolic and methanolic extracts of *B. multifida* to the medium. Although in current study, the concentrations applied were relatively high (for flow cytometric assay in figures 5-8, about 750 µg/ml), the rate of necrosis was ignorable, and the extracts caused no necrosis in the cell lines. Based on the lack of caspase 3 pathway in MCF-7 (21), we can suggest that the effect of *B. multifida* extract on cell apoptosis was not correlated to this pathway. Because of the effectiveness of *B. multifida* on the cell death rate of the MCF-7 and A2780 cell lines seen in the current study, it can be a candidate for use in treatment of these cancer types. In a study by Golshan et al., the cytotoxic effects of *Beberistina multifidi* extract (hydro-ethanolic) on human prostate cancer cells reported that *B. multifida* extract decreased the viability of PC3 cell lines (12). 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 (22).

5. Conclusion

In the present study, the findings demonstrated that methanolic extracts of *Biebersteinia multifidi* exert satisfactory cytotoxic effects on MCF-7 and A2780 cancer cell lines. Based on the chemical analysis of *B. multifida* performed in the current study, the

methanolic extract has high contents of phenolic compounds, and accordingly, this herbal compound might be a useful in finding new medications for cancer therapy.

Authors' Contribution

Study concept and design: M. M., H. F. and M. M.
Acquisition of data: Z. I., R. T. and B. M.
Analysis and interpretation of data: Z. I. and H. F.
Drafting of the manuscript: Z. I.
Critical revision of the manuscript for important intellectual content: M. M. and H. F.
Statistical analysis: Z. I. and H. F.
Administrative, technical, and material support: M. M.

Conflict of Interest

The authors declare that they have no conflict of interest.

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