The Quarterly Scientific Journal of Applied Biology
Vol 35, No. 4, Winter 2023, P: 17-20
Journal homepage: https://jab.alzahra.ac.ir
10.22051/JAB.2021.34043.1392

Cytotoxic Effect of Hydroalcoholic Extract of *Berberis vulgaris* Fruit Extract on MCF-7 Human Breast Cancer Cells

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Introduction

Cancer is a significant public health problem worldwide and the second leading cause of death in the United States. In 2020, cancer diagnosis and treatment were affected by the 2019 coronavirus disease (COVID-19) pandemic (Yabroff *et al*., 2022). Thirteen million deaths from cancer and more than 21 million new cancer cases are predicted worldwide by 2030 (Ashta *et al*., 2020). Breast cancer is the most common cancer in women and the most common cancer in general. In 2020, more than 2 million new breast cancer cases were registered in women (WHO, 2022).

Immunohistochemistry detects oestrogen receptor expression in at least 1% of tumour cells in more than 70% of breast tumours. Early menarche, late menopause, and late pregnancies impact breast cancer risk via 17-estradiol (E2) and progesterone (P4) alterations (Colditz *et al*., 2004).

The use of external hormone receptor agonists is also related to the risk of breast cancer and tumor progression (Scabia et al., 2022). Because breast cancer acquires multiple mutations, each tumor is likely to have a different signaling and epigenetic context, which can influence the outcome of hormone receptor signaling (Scabia *et al*., 2022).

Breast cancer is a malignant illness that begins in the cells of the breast. As with other malignant tumors, there are several factors that can increase the risk of developing breast cancer. Damage to deoxyribonucleic acid (DNA) and hereditary changes can lead to breast cancer. Some patients inherit defects in deoxyribonucleic acid (DNA) and genes such as P53, BRCA1, and BRCA2. People with a history of breast or ovarian cancer in their family are more likely to get breast cancer themselves (Scabia *et al*., 2022).

There are two types of breast cancer: those that spread to other parts of the body (invasive) and those that don't (Akram *et al*., 2017). For stage I to III disease, invasive breast cancer treatment includes breast surgery, radiotherapy, and adjuvant/neoadjuvant systemic therapy (cytotoxic chemotherapy, endocrine therapy, and targeted agents), as well as the right kind of supportive care. However, cancer-related pain and drug toxicity are always a problem. There are treatments for healthy cells and effects on the body as a whole (Mutebi *et al*., 2020). Because of this, it is important to find and introduce agents or compounds that don't hurt or kill healthy cells.

Programmed cell death or apoptosis is usually characterized by various structural changes and energy-dependent biochemical mechanisms. Apoptosis is a distinct and essential type of "programmed" cell death that affects the specific genetic elimination of cells (Elmore, 2007).

Many genes, enzymes, proteins, and biochemical signaling pathways have been identified or introduced in the process of apoptosis. One of the most critical apoptosis genes is the P53 gene. P53-induced apoptosis stimulates early DNA repair (Elmore, 2007).

Genetic findings show that murine double minute-type 4 (MDM4), like MDM2, is essential for controlling p53 activity during embryonic development (Marine & Jochemsen, 2016). On the other hand, apoptosis is a coordinated and regular energy-dependent mechanism that activates a group of cysteine proteases called caspases and a multifaceted cascade of actions that connect the initiating stimuli to cell death (Elmore, 2007).

It is believed that caspase-3 is the most essential and effective caspase that is stimulated by initiator caspases such as caspase-8 and caspase-9 (Elmore, 2007).

As a member of the family Berberidaceae, barberry (*Berberis vulgaris* L., Var. Asperma Don.) find in Eurasia This plant is well known in Iran and is widely used as a medicinal plant in traditional Iranian medicine (Motalleb *et al*., 2008). All parts of the barberry plant have medicinal properties, including antimicrobial, antiemetic, antipyretic, and anti-itching properties, and have been used in cases of cholecystitis, cholelithiasis, jaundice, dysentery, leishmaniasis, malaria, and gallstones (Motalleb *et al*., 2006).

In 2005, Motallab, for the first time, confirmed the anti-tumor properties of barberry fruit extract against induced liver cancer in an animal model (Motalleb, 2006). In this study, the cytotoxic effect of barberry fruit extract on MCF-7 cancer cells was investigated for the first time. Also, the present study provided a proof of concept on the mechanism of expression of *P53* and *MDM4* genes and caspase-3, -8 and -9 and their activities in the apoptosis pathway after treatment with barberry fruit extract.

Methods and Materials

Barberry plant

The utterly organic barberry fruit was prepared from Fal village in Sarbisheh city in South Khorasan province and was identified by the biology department of the Faculty of Basic Sciences of Zabul University.

Cell culture

MCF-7 human breast cancer cells were used in this research. These cells were purchased from the cell bank located in Pasteur Iran Institute (Tehran). The cells were cultured in 25 cm3 flasks in an incubator with 5% carbon dioxide, 37°C temperature, 95% humidity, DMSO, and 10% FBS culture medium.

Preparation of barberry fruit extract

The hydroalcoholic extract of barberry fruit was prepared according to the method of Motalleb *et al*. (2005).

MTT test

5 x 103 cells per milliliter were transferred to 96 well plates. One hundred microliters of culture medium were added to each well. Then the cells are incubated for 24 hours (Kutlu *et al*., 2018) until they stick to the bottom of the dish. In the next step, different concentrations of barberry fruit extract (31.25, 62.5, 125, 250, 500, 1000, and 2000 micrograms/ml) were added to the treatment and control groups in a volume of 100 microliters. Then the MTT solution with a 5 mg/ml concentration was read at a wavelength of 570 nm by ELISA MicroPlate Reader StatFax 2100 (USA). In order to determine the percentage of cell viability, the average light absorbance of cells treated with barberry fruit extract was calculated over the average light absorbance with DMSO. IC50 of barberry fruit extract was calculated by GraphPad Prism 6.07 software (USA).

Changes in the expression of *P53* and *MDM4* genes after treatment with IC50 of barberry fruit extract for 24 hours were performed for treated MCF-7 cells and the control group. The specific primers of *P53* (NC_000017.11),

MDM4 (NC_000001.11) and *GAPDH* (Endogenous control, NC_000012.12) genes were designed by Primer3 software, and then the specificity of the primers was predicted using the Primer-BLAST online tool. The expression levels of P53 ، MDM4 and GAPDH genes were measured using Real-Time PCR SYBR Green I kit (Sigma-Aldrich, USA) and 2-ΔΔCt. cDNA synthesis was done by cDNA Takara Kit (Takara Bio, USA). The specificity of the primers was predicted using the Primer-BLAST online tool. Genes were expressed using ABI One Step (ABI, USA). cDNA was synthesized for fifteen minutes at 37°C, five seconds at 85°C, and ten minutes at 4°C. qRT-PCR reactions were performed at 94°C for 30 seconds and 60°C for one minute (40 cycles) for all genes. qRT-PCR results and desired parameters were calculated and checked by Beacon Designer software (PREMIER Biosoft, USA).

Flow cytometry test

Flow cytometry was performed by FITC Annexin V Apoptosis Detection Kit PI (Biolegend, USA) according to manufacturer protocol.

Microscopic fluorescence

Morphological changes of MCF-7 cells under the influence of barberry fruit extract (IC50) were performed using a fluorescence microscope (Nikon Eclipse Ti-S, USA), according to Heidarzadeh *et al*. (2019).

Investigating the activity of caspases 3, - 8 and -9

The activity of caspases was performed by a Caspase assay kit (Colorimetric, Abcam) according to the method of Zhang *et al*. (2018).

Statistical analysis

In order to analyze the data, one-way ANOVA and non-parametric Post Hoc Tukey test were used. The data were presented as Mean SEM, and significance was defined as a value of $P<0.05$.

Results & Discussion

In this study, the cytotoxic activity of Berberis vulgaris fruit extract was investigated on MCF-7 cancer cells. The results showed that the effect of barberry fruit extract on MCF-7 cells is dose-dependent, and the highest effect of the extract occurred at a concentration of 2000 µg/ml in 24 hours (IC50 = 1681 µg/ml). The results showed a significant increase in the relative expression of *P53* and *MDM4* genes compared to the control group (untreated) ($P < 0.001$). The percentage of apoptotic cells increased more than 81 times compared to the control group (untreated). The activity of caspases 3, -8, and -9 increased in treated MCF-7 cells ($P < 0.001$). After treatment with barberry fruit extract, apoptotic cell nuclei were condensed and fragmented. On the other hand, treated MCF-7 cells were separated from the bottom of the flask containing barberry fruit extract and were observed floating.

Targeting cancer cells while avoiding harmful effects on non-cancerous cells is the golden key to cancer treatment (Nounou *et al*., 2015). Complementary and alternative medicines (Complementary and alternative medicines) represent various sources that complement or replace conventional treatments. The WHO's 2014-2023 strategy intends to enhance traditional medicine by promoting and incorporating medicinal plants in member nations' health systems (Sánchez *et al*., 2020).

Berberis vulgaris is traditionally cultivated in Iran (especially in Birjand and Qaen), known as "barberry". It also grows in southern Europe and the northeastern United States. This plant is widely used in traditional medicine to treat cardiovascular and metabolic disorders. Medicinal properties have been reported for all parts of the barberry plant, including antimicrobial, antioxidant, anti-inflammatory, and anticholinergic effects (Kapitonova *et al*., 2022).

The strongest impact of barberry fruit extract on MCF-7 cells was at 2000 g/ml in 24 hours (IC50=1681 g/ml). This is consistent with the results of El Khalki and his colleagues in 2018 (Gogal *et al*., 2018). They reported that the pronounced cytotoxicity of the barberry root extract in vitro against the MCF-7 cell line was dose-dependent. On the other hand, they reported that barberry root extract had no toxic effect on normal cells and that this cytotoxic effect on MCF-7 cells may be due to its main composition, berberine. For this critical reason, we used only

negative control in our research. Changes in the expression of *P53* and *MDM4* genes after treatment with IC50 of barberry fruit extract for 24 hours were performed for treated MCF-7 cells and the control group.

Our data demonstrated that the expression level of *P53* and *MDM4* genes increased significantly compared to the control group (non-treated) ($P < 0.001$. The increase in *P53* gene expression in our study with the results of Suk Choi in 2008, which showed that berberine in barberry caused the significant increase in the P53 gene, is consistent (Myoung *et al*., 208).

In this study, the activity of caspases 3, -8, and -9 in MCF-7 cells treated with 1681 µg/ml of barberry fruit extract increased after 24 hours ($P < 0.001$). This is consistent with the results of Yung-Tsuan and colleagues (Ho *et al*., 2009). They showed that berberine stimulates gene and protein expression of caspases 3, -8, and -9 and apoptosis-inducing factors in SCC-4 human tongue squamous cancer cells.

Conclusion

The extract of barberry fruit in specific doses decreased cell growth and increased the induction of apoptosis in MCF-7 cancer cells. The barberry fruit extract has anti-cancer ability in breast cancer treatment. Therefore, it is suggested that our significant results be seriously evaluated in the preclinical and clinical phases.

Keywords: *Apoptosis, Berberis vulgaris, Breast cancer, Cytotoxicity*

Acknowledgment: *We would like to thank University of Zabol and Javid Biotechnology Laboratory, and Dr. Askari.*

Declaration of conflict of interest: *There is no conflict of interest between the authors*