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Original Article

# Cytotoxic and Cell progression Effects of *Mentha pulegium L* Extract on Selected Cancer Cell Lines

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## Abstract

*Mentha pulegium L*. is one of the *Mentha* (Labiatae) species that is grown in many different parts of the earth including northern Iran. The total ethanolic extract of the aerial parts of this plant is used in this work to investigate on the possible cytotoxicity on two different cell lines. Human lung carcinoma cells (A549) and human breast cancer (MCF7), have been used to show the clonogenic count of cells, as well as their cell cycle phases progression pattern after the exposure to the different concentrations of this extract. The cytotoxicity of the total extract of *Mentha pulegium L*. is 59 ± 1.73 µg/ml for the MCF7, and 48.86 ± 2.5 µg/ml for the A549 cell lines, respectively. Flow cytometry analysis have shown two different adventures for these cell lines after 24 hours exposure to this extract. While the MCF7 cells presented a dose-dependent arrest at the $G_2$/M phase of the cell cycle, the A549 cells are more intended to accumulate in the so-called $G_0$ resting phase after the exposure to this extract. These promising initial results might bring hope for the introduction of a tissue discriminating anticancer agent in future.

*Keywords*: Mentha pulegium L, Cancer, MCF7, A549, Clonogenic assay, Flow Cytometry.

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## 1. Introduction

Traditionally, medicinal herbs are well known candidates for the treatment of different malignancies with promising results. *Mentha pulegium L*. is one of the *Mentha* (Labiatae) species commonly known as pennyroyal. It is the native species of Europe, North Africa, and Asia Minor and near East [1]. It also grows naturally in Golestan and Gilan Provinces in the north of Iran [2] with

popular name of "Khalvash". The flowering aerial parts of *Mentha pulegium L*, have been commonly used as an antiseptic for treatment of cold, sinusitis, cholera, food poisoning, bronchitis and tuberculosis, antiflatulent, carminative, antispasmodic, expectorant, antitussive, diuretic, menstruate [3], insect repellent and anti-inflammatory [4]. Traditionally, total decoction of this herb was used for the treatment of different kinds of cancers such as gingival [5], colon, pudenda, spleen, belly, stomach and uterus [6]. In spite of most publications being on the *Mentha pulegium L* essential oil, we have published on the cytotoxicity of total and fractionated extract of Iranian *Mentha pulegium L* on three human carcinoma cell lines [7]. Here, we are examining the cytotoxic and cell cycle phase alteration effects of *Mentha pulegium L* total extract on two different human tissues of breast (MCF7) and lung (A549) cell lines.

Breast cancer is the second leading cause of death among women worldwide, bringing a huge financial and human burden to communities [8]. A variety of factors affect the incidence and response to treatment of this disease, including family history and the prevalence of receptors, the status of receptors is one of the most important indicators of malignancy and response to treatment [9]. There are several treatments available for breast cancer, one of the most common of which is chemotherapy. Over time, various chemotherapies have been used against breast cancer, some of which, including tamoxifen, has affected sex-receptors, some, such as cisplatin and the platinum family, have alkylated DNA and newer therapies are monoclonal antibodies like trastuzumab have been used [10]. Targeted therapy is one of the new therapies because it facilitates the treatment and reduces the side effects of the drug. Among the drugs used in the treatment of breast cancer in this field are trastuzumab and lapatinib[11]. These drugs are effective on HER2 receptors and to prevent metastasis and the spread of disease, which shows the importance of this receptor.

Lung cancer is a malignant lung tumor characterized by uncontrolled cell growth in tissues of the lung. More than 80% of cases of lung cancer are due to long-term tobacco smoking. About 10–15% of cases occur in people who have never smoked. These cases are often caused by a combination of genetic factors and exposure to radon gas, asbestos, second-hand smoke, or other forms of air pollution. This is one of the most resistance cancers to the present treatment protocols [12, 13]. Variety of receptors and mechanisms are involved in the promotion of this disease including genetic factors, smoking, air pollution and the radon gas [14, 15]. Chemotherapy, although not fully successful alone in most cases, is a combination of different agents including carboplatin, gemcitabine, paclitaxel, and topotecan [16]. One of the main mechanism involved in occurrence, as well as targeted for the treatment of this cancer is the epidermal growth factor receptor (EGFR) with drugs like Erlotinib, osimertinib and afatinib were developed to improve patients' survival [17, 18].

The aim of this study was to investigate the cytotoxic effects of the total extract of *Mentha pulegium L* collected from northern Iran on these two tumor cells survival and cell cycle behavior.

## 2. Materials and Methods

### *2.1. Plant Materials and Preparation of the Extracts*

Aerial parts of *Mentha pulegium L* were collected from Bandar Anzali (Province of Gilan, Iran) during May 2019, dried in a laboratory environment with proper ventilation, then 100 gr of the dried plant was weighed and 700 ml of ethanol 50% was added to it and shaken for 48 hours at room temperature, in the next step extract was filtered and transferred to the Rotary Evaporator (Hiedolph,Germany) to be contracted in vacuo at 40 °C [19]. The dried extract is dissolved in water and DMSO (1:1) and various dilutions of it are made in RPMI culture medium for cytotoxicity tests.

### *2.2. Cell Culture*

The cancer cell lines of MCF7 human breast carcinoma, and the human lung adenocarcinoma of A549 were obtained from Pasture Institute of Iran (Tehran, Iran), and were grown in RPMI 1640 (Gibco BRL, USA) media supplemented with 10% fetal bovine serum, 50 unit's/ml penicillin, and 50 µg/ml streptomycin in an incubator with 5% $CO_2$ at 37 $^{\circ}$c.

### *2.3. Clonogenic Assay*

Clonogenic assay is one of the in-vitro methods to determine the toxicity of substances based on the ability of a single cell to become a colony. The colony is defined to consist of at least 50 cells. After preparing the cell lines, cell counting is performed with trypan blue and 100 cells are seeded into each well of a 6-well plate. For each concentration, three replicates are considered, in order to normalize the resulting data, three controls were set by exposing the cells to compounds-free solvent in each set of experiment. After 24 hours, different concentrations of the total ethanolic extract of *Mentha pulegium L* are added to the cells in a concentration range of 0-150µg/ml and the cells are exposed to the extract for 7 days. The incubation period varies from 1 to 3 weeks depending on the cell line. For these cell lines, it takes about a week for the colonies formation. After observing the colonies, first, wash twice with normal saline. In the next step, the colonies are fixed with 70% ethanol for 15 minutes and then stained with trypan blue [20].

### *2.4. Flow Cytometry*

$1\times10^6$ cells were harvested from a flask, then seeded into a T25 $cm^2$ culture flask and were allowed to attach overnight, then three concentrations of 30, 60, and 120 µg/ml of the total extract of *Mentha pulegium L* were added to it, and the cells were exposed to the total extract of *Mentha pulegium L* for 24 hours and the staining steps were performed for them. Cells were trypsinized, then centrifuged (2000 rpm, 5 minutes) and washed twice with cold

phosphate-buffered saline (PBS), aliquoted into tubes and labeled with annexin V and propidium iodide using an apoptosis detection kit (APOAF, Sigma, St. Louis, MO). Annexin V and PI staining were performed following the manufacturer's recommendations [21]. Flow cytometry analysis was performed during one hour after staining.

*2.4. Statistics*

All statistical analyzes were performed by GraphPad PRISM® 6 software (GraphPad Software, San Diego, CA, USA) and each point on the relevant curves represents the average percentage of colonies relative to control colonies, in three triple replications with the corresponding standard error (Mean ± SEM).

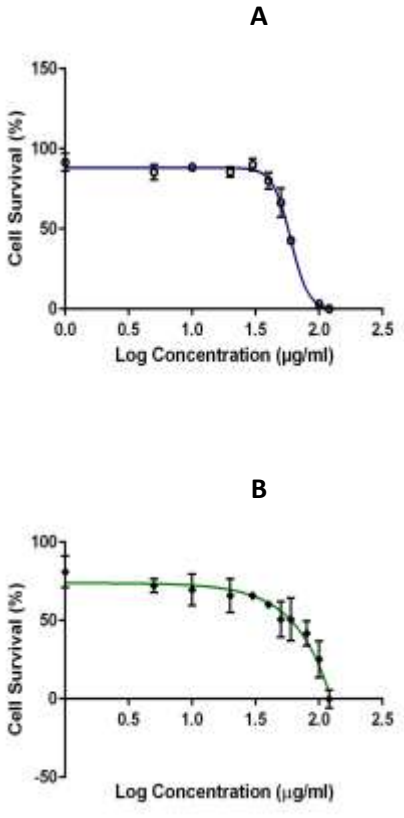


**Figure 1**. Effect of the *Mentha pulegium* L on (A) MCF7 and (B) A549 cell survival using clonogenic assay.

## 3. Results and Discussion

After counting the colonies in different groups and their statistical analysis by the GraphPad PRISM® software, the following results were obtained for the clonogenic assay (Figure 1).

As shown in this figure, graphs of cell survival percentage were plotted for each cell lines, $IC_{50}$ value of *Mentha pulegium L* extract is 59 ± 1.73 µg/ml for MCF7 and 48.86 ± 2.5 µg/ml for A549. According to MCF7 results, an inherent resistance is obvious up to a dose of 20 µg/ml in these cells following the exposure to the extract, and then at higher doses this resistance is broken and the cytotoxicity drops the number of colonies.

The status of cell surface growth and propagation receptors is different for MCF7 versus A549 cell line. Breast tissue cancer is a sex-related cancer with the well-known expression patterns of estrogen receptors (ER), progesterone receptors (PR), and the HER2 [22]. Information on the lung cancer indicates that this cancer is also sex-related, and sex hormones such as estrogen play a role in its incidence and progression [23]. The HER family of oncogenes is frequently overexpressed in lung cancer [24]; Feng *et al* stated that there is a relationship between COX 2 and HER2 and these are functionally related to each other by MEK/ERK pathway, which might affect cell proliferation and invasion via AKT signaling pathway in the non-small cell lung cancer (NSCLC) cells including A549

[25]. The level of expression of cell surface HER-2 in the NSCLC cell lines is moderate and lower than in the breast cancer cell lines [26]. However, other cell cycle progression receptors like EGFR on the A549 cell surface might provide a more favorable environment for the growth inhibition effects of the total extract of *Mentha Pulegium L.* That is why a cell cycle analysis and cytotoxic death mode experiments were designed and executed.

In this experiment, MCF7 and A549 cell lines were exposed to the three different concentrations of *Mentha pulegium L* total extract (30, 60 and 120 µg/ml) for 24 hours and then Annexin V/PI staining was performed as described in the method section.

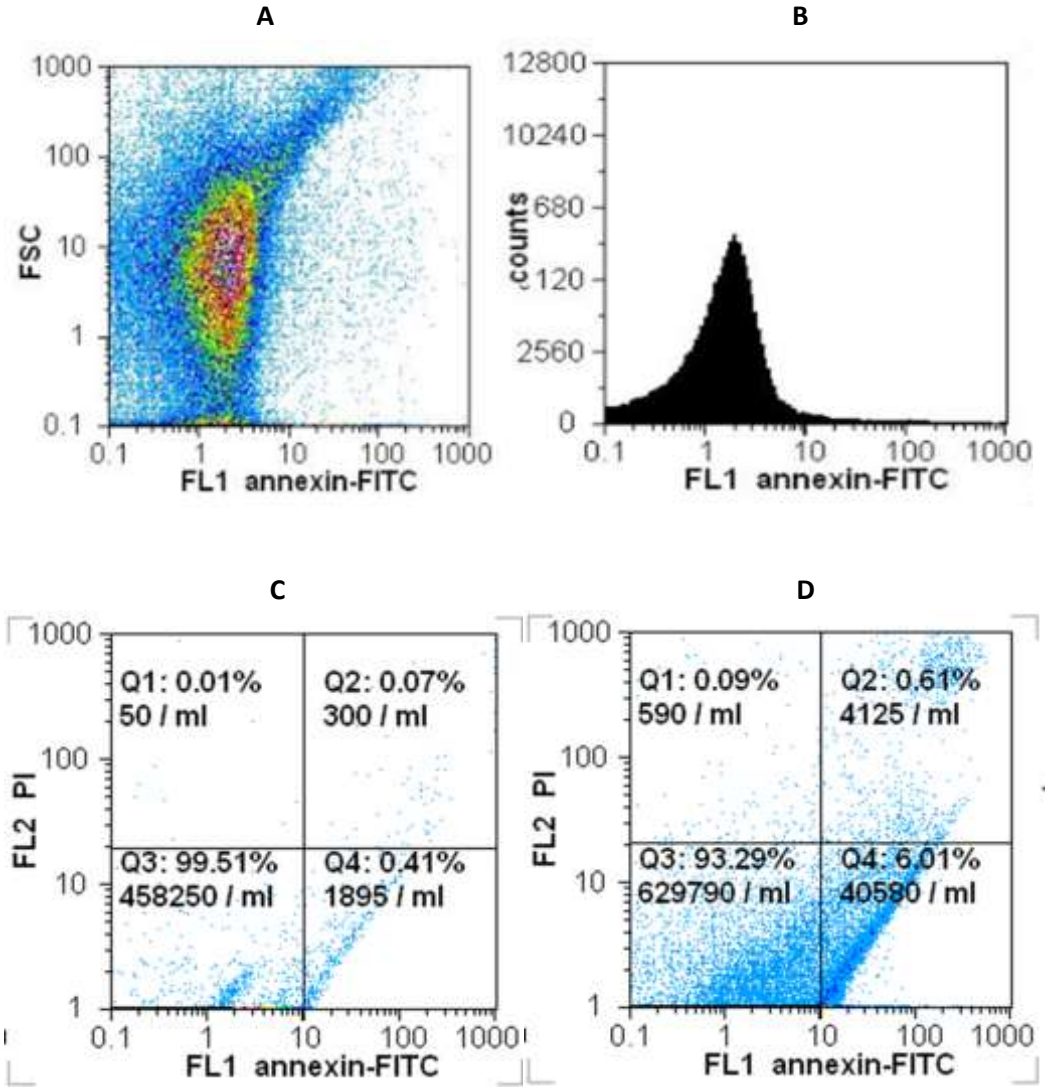


**Figure 2**. Flow cytometry pattern of MCF7 cells stained with Propidium Iodide (PI) and Annexin-FITC; Histogram of cells distribution (A), Cells distribution (B), quatrain separation of Control cells (C), and quatrain separation of cells exposed to Mentha Pulegium L total extract for 24 hours (D).

Figure 2 represents the flow cytometric distribution of recognized cells for any of these staining, which results are summarized in Figure 3. As is shown in Figure 3 C, MCF7 cell line are arrested at $G_2$/M phase of the cell cycle with the increase in the *Mentha pulegium L* concentration. This is in the cost of cells coming from the $G_1$/S phases as the number of cells in these two phases decreases with the increase in $G_2$/M phase leading to cells arrest in the $G_2$/M.

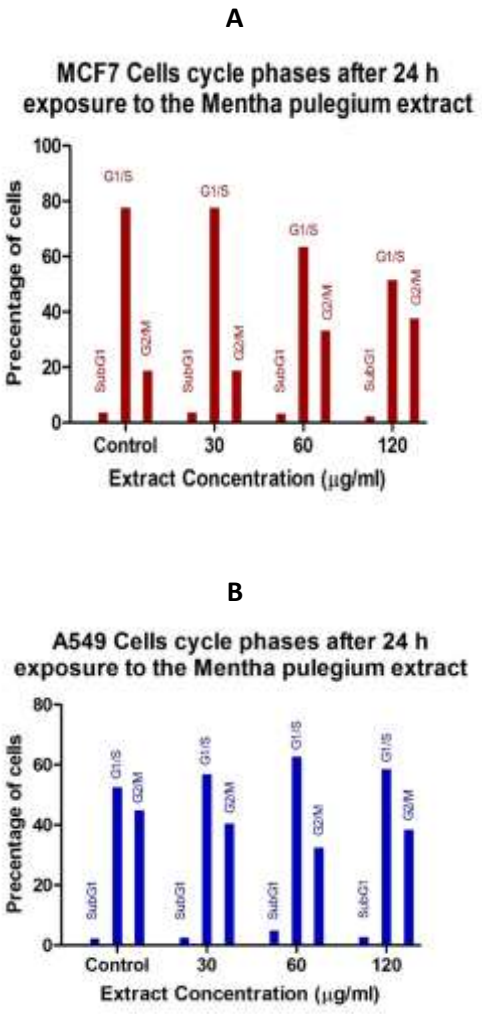


**Figure 3**. (A) MCF7 and (B) A549 cell cycle analysis after 24 hours exposure to the Mentha pulegium L total extract.

Figure 3 clearly shows no significant difference in the cell cycle phases distribution of MCF-7 cells after exposure to 30 µg/ml of *Mentha pulegium L* extract with that of the control group, precisely resemble the clonogenic assay of inherent resistant zone for this cell line up to this concentration. This inherent cellular resistance was broken by the increase in concentration from 30 to 60 µg/ml and further at 12 µg/ml. However, the sub$G_1$ phase did not show any significant changes at the different concentrations of the *Mentha pulegium L* extract compare to the control group, indicating that this extract might require a longer period of time to induce apoptosis in this cell line (Figure 3A).

On the other hand, there are up to 30 µg/ml intrinsic resistance in A549 cells, and from this concentration onwards the number of cells suppress in the $G_2$/M phases, so fewer cells leave $G_1$ and S toward the $G_2$/M phase of the cell cycle compare to the control group. As no or fewer cells are coming from the M phase, but cells population increases in the $G_1$ phase with of course no significant changes in the Sub-$G_1$ population, the conclusion might rise that cells exit cell cycle to remain in the so-called $G_0$ phase. Phase $G_0$ does not mean apoptosis and is a form of cellular resting state that might rise to the either of quiescence or senescence cells. We hypothesize that while quiescence cells are more like a reservoir tumor population in the body, the senescence cells might learn from the environment to produce some more resistant variant to the cytotoxic effect of the *Mentha pulegium L* extract in the future exposures (Figure 3B).

## 4. Conclusion

The total ethanolic extract of *Mentha pulegium L* grown in the Northern Iran has shown degrees of cytotoxicity on both of MCF-7 and A549 cell lines. The cytotoxicity of a single exposure to this compound is different for these cell lines, most likely related to the pattern of cell surface receptors distribution on these cell lines. Whatever is the mechanism of action for the *Mentha pulegium L* extract, the initial effect is on the cell cycle progression of these cells, which is of course different for any of these two cells. Further experiments with more repeated exposures to this extract might reveal the mechanism and explain the consequences of events, which is hypothesized and suggested in this article based on the initial observations. As a conclusion, promising results for a tissue specific therapy might be expected from this extract in future works.

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