



# Anti-cancer Effects of Methotrexate in Combination With *Melissa officinalis* on HeLa Cancer Cell Line

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## Abstract

**Objectives:** One of the well-accepted beliefs about natural products, considering the advances of recently appearing new edges and features of herbal medicine, is paying more attention to cancer treatments. However, they have not been properly studied with reasonable/reliable clinical trials in human subjects in most cases. Therefore, seeking *in vitro* effects of herbs like *Melissa officinalis* (MO) in cancer therapy to identify the involved possible mechanism in conjugation with configurative/morphological aspects of treated cells seems quite necessary. In this study, we evaluated the co-treatment effect of anti-cancer drug methotrexate (MTX) and MO on HeLa cancer cells.

**Methods:** MTT assay was applied to assess the quantitative cytotoxicity effect of both MTX and Mo. Apoptosis assay via flow cytometry was used to determine the amount of apoptotic and necrotic cells. To further investigate the anti-cancer effects, DAPI staining and DNA ladder assays are used qualitatively to detect changes in the nuclei of cells that are a sign of apoptosis occurring and morphological modifications of DNA.

**Results:** MTX and MO mixture showed high cytotoxicity and apoptosis rate compared to untreated cells. Furthermore, the morphological changes of MTX and MO mixture were more evident than that of single MO, MTX, and control groups.

**Conclusions:** These data regarding cell growth reduction and apoptosis induction in HeLa cancer cells showed that MTX and MO mixture can be an appropriate platform for cancer therapy.

**Keywords:** Methotrexate, *Melissa officinalis*, HeLa cancer cells, MTT assay, Flow cytometry

## Introduction

*Melissa officinalis* L. (MO), as one of the medicinal herbs, has a lot of traditional applications in covering wounds, infectious bites, ulcers, stress, anxiety, trachea-bronchitis, epilepsy, heart arrhythmias (1). Although it was used for functional beverages of human consumption through making lemon balm aqueous decoctions, recently, findings showed new features like insulin resistance properties (2), antimicrobials, preservatives (3), antifungal (4), antiviral (against HSV-1) (5) and antidepressant-like activities (6). It should be mentioned that MO with containing almost 30 compounds of essential oils outstripped other parallel herbal medicines (7). In terms of health-boosting compounds, several research articles have cast light on aspects of aromatic plants as excellent sources. Therefore, MO is a species of Lamiaceae family with aromatherapeutic treatments application (8,9). With more progress in threats and opportunities of herbal medicine with relevant biological proof, we would be able to develop the fundamental herbal antidepressant and anti-cancer drugs. However, this research still is newborn, and

recently produced data have been noticeable.

Folate antagonists were developed as the first antineoplastic agents (10). Recently, the combination of methotrexate (MTX) with other drugs has been considered one of the crucial medications for the treatment of alpha-viruses, parvovirus B19, hepatitis B/C virus (11), and rheumatoid arthritis, juvenile idiopathic arthritis, psoriasis (12). Moreover, after primary tumor surgery, breast cancer, brain tumors, lymphomas, hepatoma, and lung cancer generally underwent MTX treatments (13,14).

The progress in chemotherapy and radiotherapy for cervical cancer is still faced with failures based on the cumulative evidence (15), therefore, there have been suggestions with some regulators in gene network (like microRNAs) by traditional herbal medicine and treatment of various cancers through the down-regulation of their targets have received most cancer researchers attentions (16). Sometimes the anti-proliferative mechanism of MO on cells is through induction of apoptosis and formation of ROS. For example, carrying out the tests on T84 as well as HT-29, a tight monolayer of small intestine

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## Key Messages

- ▶ MTX and MO mixture showed high cytotoxicity and apoptosis rate, which supposed that the proposed mixture can be an appropriate platform for cancer therapy.

colon carcinoma cells suggest that the extracts of MO (rang  $\approx 600$  mg/mL) indirectly cause externalization of phosphatidylserine and cleavage of caspases 3 and 7 as a part of apoptosis process (17).

Cancer treatment with combinational therapy compared to a single drug provided several advances. Therefore, we desired to use combinational therapy of cancer via MTX and MO on HeLa cells in this work. The mixed solutions of MO components in specific amounts with MTX were used to evaluate cytotoxicity effects through MTT assay. For detecting nucleus abnormalities and fragmentation, a DAPI staining assay was used, and the apoptotic events and kind of cell death have been determined using flow cytometry measurements.

## Materials and Methods

### Chemical and Materials

MO was received from the pharmacy faculty of Tabriz University of Medical Sciences. 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was prepared from Sigma-Aldrich. The HeLa cell line was provided through the Pasteur Institute of Iran (the cell bank section). RPMI-1640 cell culture media, trypsin-ethylenediamine tetra acetic acid (EDTA) solution, fetal bovine serum (FBS), and penicillin/streptomycin were from Gibco Co (Dublin, Ireland). Dimethyl sulfoxide (DMSO) was obtained from Merck. The apoptosis detection kit, including Annexin V-FITC/PI, was purchased from Oncogene Research Products (San Diego, USA).

### Cell Culture and Cell Viability Assay

RPMI-1640 media supplemented with 10% fetal bovine serum and antibiotics (100 units/mL penicillin and 50 units/mL streptomycin) was used for culture of HeLa cancer cells and then stored and incubated at a constant temperature of  $\approx 37^{\circ}\text{C}$ , with a relative humidity of 95% and 5%  $\text{CO}_2$  concentration. To evaluate the cytotoxicity effect of MO, MTX and combined drugs (MO+MTX) on HeLa cancer cells MTT assay was used, which is based on the conversion of yellow tetrazolium salt to the violet formazan derivative by viable cells. After reaching appropriate confluency,  $8 \times 10^3$  cells were seeded in a 96 well plate with an initial volume of 200  $\mu\text{L}$  medium and incubated overnight. Specific concentrations of MO, MTX, and MO+MTX groups were used to treat cancer cells 24 and 48 hours. Next, 50  $\mu\text{L}$  of MTT solution (2 mg/mL) was added to each well after preparation in phosphate-buffered saline (PBS) buffer and incubated for another 4 hours. For dissolving the formed formazan crystals,

200  $\mu\text{L}$  of DMSO was poured over the cells. Finally, the spectrophotometric plate reader (BioTek Instruments Inc, Vermont, USA) was applied to measure UV absorbance at 570 nm. All experiments were carried out with three repetitions (18, 19).

### DAPI Staining Assay

To visualize the form and shape of the HeLa cancer cell nucleus DAPI staining test was applied. First, after seeding of  $1 \times 10^4$  cells in 96 well plates, the cells were incubated overnight to reach appropriate confluency. Next, the cancer cells were treated with MO, MTX, and combined MTX-MO groups based on IC50 concentration obtained from the MTT assay and incubated for 48h. Then, cells were fixed with 4% paraformaldehyde for 5-minute followed by another 5-minute incubation with Triton X-100 (0.1% (w/v)) to permeabilize cancer cells for staining. Finally, DAPI solution (1  $\mu\text{g}/\text{mL}$ ) was used to treat cells for 10 minutes. Morphological evaluation of prepared cells was performed with the fluorescent microscope.

### Flow Cytometry

Flow cytometry method with an accurately fast procedure provides a precious mechanism for the identification of cell death at multi-parametric measurements (20). Flow cytometry was used to determine apoptosis and necrosis proportions in treated cells with MTX, MO, and MTX-MO. The detached HeLa cancer cells were seeded in 6-well plates with a density of  $2 \times 10^5$  cells in each well and incubated overnight to reach 80 % confluency. Then the medium was depleted and treated with a fresh medium containing MO (400  $\mu\text{g}/\text{mL}$ ) and MTX (100  $\mu\text{g}/\text{mL}$ ) is separated and combined groups for 48 hours followed by detaching treated cells with trypsin after rinsing with PBS. After collecting the suspensions of cells, centrifugation was performed at 130 rad/s and then washed again with PBS. In a dark place, 200  $\mu\text{L}$  of Annexin V binding buffer was added to the cell pallets, and after that, 5  $\mu\text{L}$  of Annexin V/propidium iodide was used to stain the cells for 15 minutes. The fluorescence-activated cell sorting was used for analyzing stained cells.

### DNA Ladder Assay

As a complementary assay, a DNA ladder was accompanied to evaluate DNA fragmentation, an approving method for apoptosis in HeLa cancer cells. First, HeLa cancer cells were cultured in a 6-well plate ( $2 \times 10^5$  cell/well) and incubated for a day. Defined IC50 concentration of MO, MTX, and mixture of MO with MTX was used to treat HeLa cancer cells and incubated for 48 hours. Then, the harvested cells were kept for 1 hour within proteinase K and 1 mL lysis buffer at  $56^{\circ}\text{C}$ . Protein denaturation was done through 400  $\mu\text{L}$  of chloroform, and then total DNA was isolated to the upper phase of the suspension. Isolated DNA by absolute ethanol was extended at  $-20^{\circ}\text{C}$  and then centrifuged for 20 minutes at 12 000 rpm at  $4^{\circ}\text{C}$ . At the end, 2% agarose

gel containing 2 mL/100 mL of SYBER Green (1 hour at 70 V/30 mA) was used for electrophoresis of the obtained DNA pellet.

### Statistical Analysis

Statistical measurements were calculated and justified with GraphPad Prism software (version 6.01). Results were presented as mean  $\pm$  standard error using one-way ANOVA. The statistical significance was defined as a *P* value of less than 0.05.

## Results

### Cell Viability

MTT assay was conducted to evaluate the cytotoxicity and proliferation of HeLa cancer cells in the presence of MO, MTX, and MO+MTX groups. Different concentrations of MO and MTX, including 5, 10, 50, 100, 200, 400, 600, and 800  $\mu\text{g/mL}$ , were added to HeLa cancer cells to calculate the  $\text{IC}_{50}$ . After that  $\text{IC}_{50}$  dose of MTX was mixed with various concentrations of MO, ranging from 5 to 800  $\mu\text{g/mL}$ , for investigating the combined effect of MTX and MO on the growth of HeLa cancer cells. It can be seen that (Figure 1) mixture of MO with MTX reduced the proliferation of HeLa cancer cells up to 50% at 400 and 300  $\mu\text{g/mL}$  concentrations at 24 and 48 hours, respectively. In terms of MO and MTX solely, they were not as effective as the combined group (MTX+MO) in reducing the cell viability of HeLa cancer cells, especially in 24 hours. Therefore, simultaneous administration of MTX with various concentrations of MO was more efficient in decreasing cell proliferation than single-state of drug treatment. In a study conducted by Saraydin et al, single dosage administration of MO on MCF-7, MDA-MB-231 and MDA-MB-468 cells reduced cell viability in all three cancer cell lines with  $\text{IC}_{50}$  values of  $18 \pm 2.0$   $\mu\text{g/mL}$ ,  $17 \pm 1.4$   $\mu\text{g/mL}$ ,  $19 \pm 1.8$   $\mu\text{g/mL}$ , respectively (21).

### DAPI Staining Assay

The morphological changes of the HeLa cancer cell nucleus were investigated via DAPI staining assay to visualize abnormalities like shrinkage, fragmentation,

condensation, or even remodeling in the chromatin and DNA rings which are indications of apoptosis occurrence in treated groups in comparison to the negative control. Based on obtained results in Figure 2, the combination of MO and MTX (E) changed the appearance of the HeLa cancer cells nucleus. Therefore, DNA fracture and chromatin condensation were more evident than MTX (D) alone. However, MO (C) did not cause considerable changes in DNA structure compared to the negative control group (A).

### Flow Cytometry Assay

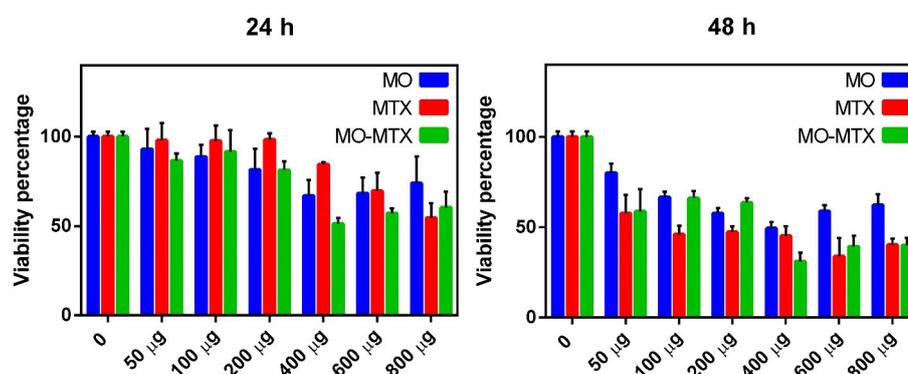
Calculation of the apoptotic cells percentage using Annexin V/propidium iodide (PI) staining method was done. If cells are positive for Annexin V-FITC but negative to PI, it is demonstrative of early apoptosis occurring, and cells positive to both Annexin V-FITC and PI-positive were classified as late apoptotic cells. Necrotic cells were just positive to PI. Living cells remained unstained. It can be seen in figure 3 that MO-MTX mixture induced apoptosis in HeLa cancer of about 34.70%; however, 28.4% of cells were necrotic after 48h. A single administration of MO caused apoptosis and necrosis up to 14.84% and 37.8%, respectively. Besides, 5.34% and 18.2% of HeLa cancer cells showed apoptosis and necrosis upon treatment with MTX.

### DNA Ladder Assay

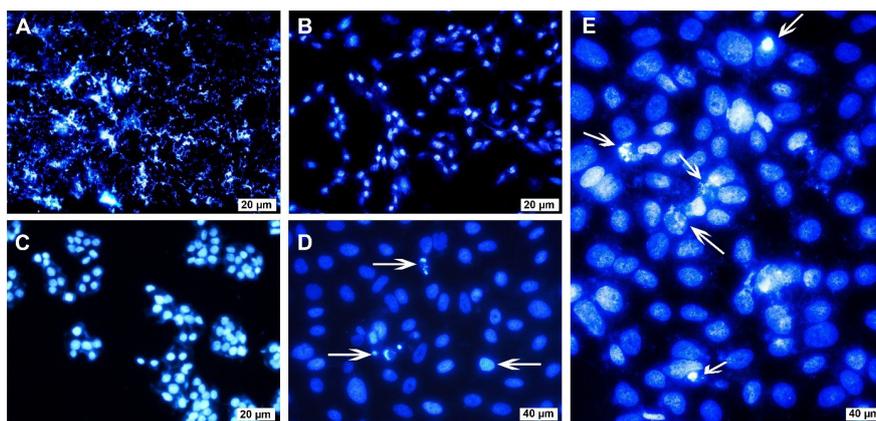
DNA ladder assay was used to distinguish the adverse effect of MO, MTX, and MO-MTX mixture on DNA. As it is obvious in Figure 3, MO and MTX caused gentle DNA fraction than the control group, while the MO-MTX mixture was more effective in DNA ladder formation.

## Discussion

In one study, single-dose administration of MO on MCF-7, MDA-MB-468, and MDA-MB-231 cells showed cytotoxicity in all three cancer cell lines with the  $\text{IC}_{50}$  values of  $18 \pm 2.0$   $\mu\text{g/mL}$ ,  $17 \pm 1.4$   $\mu\text{g/mL}$ , and  $19 \pm 1.8$   $\mu\text{g/mL}$ , respectively (21). The origin of these differences in  $\text{IC}_{50}$  concentration is associated with MO extraction



**Figure 1.** MTT Assay Plot for the Viability of the MO, MTX, and MO-MTX in Different Concentrations of MO as Subscribed on X-axes and Constant Concentration of MTX at 24 and 48 Hours Time-Points.



**Figure 2.** Labeled micrographs represent A) Negative control, B) Positive control, C) MO 400  $\mu\text{g/mL}$ , D) MTX, E) MO-MTX. Arrows show the induction of DNA deformation in nucleus of HeLa cells that is indicative of apoptosis occurring in cells ( $\times 40$ ).

methods and differences in cell line types. However, in another study, Sousa et al investigated the *in vitro* cytotoxicity of MO via MTT assay. They showed that MO oil had antiproliferative effects against various cancer cell lines, including Caco-2, MCF-7, HL-60, A549, K562, and a mouse cell line (B16F10) (22).

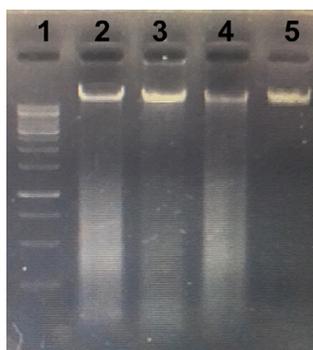
In the DAPI staining assay, there were qualitatively sensible changes in the morphology of apoptotic cells when treated with the MO-MTX group compared to the control group. In this group, the simultaneous presence of anti-cancer drugs causes severe apoptosis determined with arrows in Figure 2E. The number of apoptotic cells almost doubled in the co-treatment group.

In line with this study, Weidner et al examined the cytotoxicity effect of hydroethanolic extract of MO on

HT-29 colon carcinoma cells at the concentration of 600  $\mu\text{g/mL}$ . They showed that MO had a pro-oxidative impact rather than antioxidative effects due to ROS formation, resulting in the abnormal shape of the cell nucleus in the treated cells (17).

Figure 4 and Table 1 show the percentage of apoptotic and necrotic cells treated with MO-MTX mixture, 34.70%, and 28.4%, respectively, after 48 hours. In MO, 14.84% and 37.8% of the cancer cells experienced apoptosis and necrose, respectively. In HeLa cancer cells treated with MTX, 5.34% showed apoptosis properties, and 18.2% were necrotic. It showed that co-administration of MO and MTX has high efficiency in inducing apoptosis in HeLa cancer cells. Therefore, the apoptosis pathway was more dominant than other pathways such as necrosis. In other studies, the anti-tumoral effects of MO were assessed on various breast cancer cell lines including MCF-7, MDA-MB-468, and MDA-MB-23. The attained results verified that treating cells with MO increased the percentage of Annexin-positive cells. They concluded that MO had a cytotoxicity effect against three cancer cell lines in a dose-dependent manner (21).

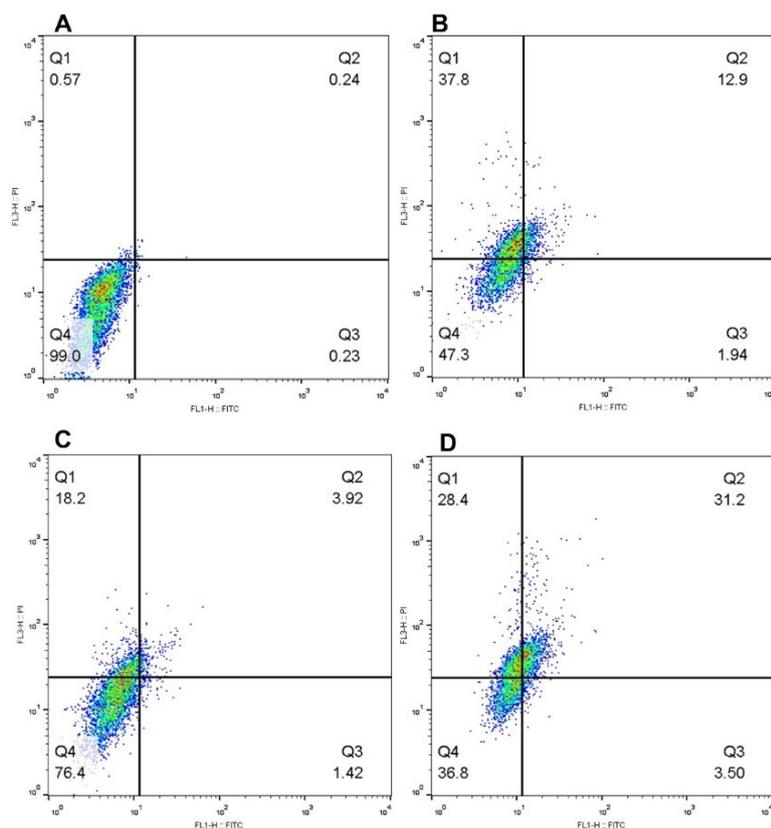
Furthermore, in another study, ethanolic extract of MO decreased the expression levels of pro-caspase 3 and induced the expression of cleaved caspase 3 in comparison with control cells. This result agrees with the flow cytometry assay output; the reduction in pro-caspase 3 expression level is an indicator of the activation of the caspase cascade in the late stages of apoptosis (23). In the end, DNA fragmentation in the nucleus of



**Figure 3.** DNA Ladder Assay, Illustrating DNA Fragmentation in HeLa Cancer Cells Upon Treatment With MTX (Lane 2), MO (Lane 3), MO-MTX (Lane 4). Lane 5 is a negative control, and Lane 1 is a DNA marker.

**Table 1.** Viable, Necrotic, Early and Late Apoptotic Rates in MO (400  $\mu\text{g/mL}$ ), MTX (100  $\mu\text{g/mL}$ ) and MO and MTX Mixture (400  $\mu\text{g/mL}$  + 100  $\mu\text{g/mL}$ ) Treated Cells After 48 hours

	Viable Cells (%)	Necrotic Cells (%)	Early Apoptotic Cells (%)	Late Apoptotic Cells (%)
Untreated control cells	99.00	0.57	0.23	0.24
MO treated cells	47.3	37.8	1.94	12.9
MTX treated cells	76.4	18.2	3.92	1.42
MO + MTX treated cells	36.8	28.4	3.50	31.2



**Figure 4.** Flow-cytometric analysis of HeLa cancer cells after treatment with MO, MTX, and MO+MTX during 48h. Labeled letters corresponds with A) Control, B) MO (400 µg/mL), C) MTX (100 µg/mL), and D) MO-MTX (400 µg/mL-100 µg/mL) groups.

HeLa cancer cells illustrated the increased cytotoxicity effect of MO in combination with MTX compared to the single administration of MO and MTX. Therefore, the combination of MO with conventional anti-cancer drugs like MTX can be considered a potential candidate for inducing apoptosis. However, further quantitative experiments are required to approve our observations.

### Conclusions

This study showed the high cytotoxicity of MO in combination with MTX. The anti-proliferative effect of the mixture of these two compounds was synergistic. However, MO and MTX alone were not as efficient as a mixture of MO and MTX to decrease cell viability at the same dose. The early/late stages of apoptosis induction in co-treatment were more than cells treated with just MO and MTX in HeLa cancer cells. Taken together, MO might offer a good strategy for treating human cervical cancer in combination with anti-cancer drugs. Moreover, this research probably would strengthen the outlook toward the use of synthetic anticancer drugs in combination with medicinal therapy that contains anti-depressive effects too.

The revelation of the probable anticancer mechanisms for medicinal antidepressants drugs bring about the related discussions of future studies and following challenges to

rethink the anticancer drug discovery.

By considering the current cervical cancer situation and available treatments, it seems that future studies should be embedded in cancer research, especially for the cervical cancer model of rodent animals simulating cervical cancer accompanied by induced depression condition. Probably comparison of the drugs' dual pharmacologic properties would produce a more satisfying result in terms of increasing clinical application chance in order to highlight the indexes of the quality of life of patients with both cancer and depression conditions. This is the first time a combinational therapy went through the conformational changes of the cell's nucleus *in vitro*.

### Authors' Contribution

PF: Formal analysis, investigation and writing original draft. MAK, RM, SH: Conceptualization. AJ, MG: Formal analysis and investigation. MdIG: Supervision and review & editing. JEND: Conceptualization, formal analysis, supervision and writing - review & editing.

### Conflicts of interest

Authors declare no conflicts of interest.

### Ethical Issues

There is no ethical issues to be declared.

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