

RESEARCH ARTICLE

Anti Proliferative Properties of *Melissa officinalis* in Different Human Cancer Cells

Akram Jahanban-Esfahlan¹, Sina Modaeinama², Mozghan Abasi³, Mehran Mesgari Abbasi⁴, Rana Jahanban-Esfahlan^{3,5*}

Abstract

Background: Medicinal plants, especially examples rich in polyphenolic compounds, have been suggested to be chemopreventive on account of their antioxidative properties. *Melissa officinalis* L. (MO), an aromatic and medicinal plant, is well known in this context. However, toxicity against cancer cells has not been fully studied. Here, we investigated the selective anticancer effects of an MO extract (MOE) in different human cancer cells. **Materials and Methods:** a hydro-alcoholic extract of MO was prepared and total phenolic content (TPC) and total flavonoid content (TFC) were determined by colorimetric assays. Antioxidant activity was determined by DPPH radical scavenging activity. MTT assays were used to evaluate cytotoxicity of different doses of MOE (0, 5, 20, 100, 250, 500, 1000 µg/ml) towards A549 (lung non small cell cancer cells), MCF-7 (breast adenocarcinoma), SKOV3 (ovarian cancer cells), and PC-3 (prostate adenocarcinoma) cells. **Results:** Significant ($P < 0.01$) or very significant ($P < 0.0001$) differences were observed in comparison to negative controls at all tested doses (5-1000 µg/ml). In all cancer cells, MOE reduced the cell viability to values below 33%, even at the lowest doses. In all cases, IC_{50} values were below 5 µg/ml. The mean growth inhibition was 73.1%, 86.7%, 79.9% and 77.8% in SKOV3, MCF-7 and PC-3 and A549 cells, respectively. **Conclusions:** Our results indicate that a hydro-alcoholic extract of MO possess a high potency to inhibit proliferation of different tumor cells in a dose independent manner, suggesting that an optimal biological dose is more important than a maximally tolerated one. Moreover, the antiproliferative effect of MO seems to be tumor type specific, as hormone dependant cancers were more sensitive to antitumoral effects of MOE.

Keywords: MOE - A549 - SKOV3 - MCF-7 - PC-3 - anti proliferative effects

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Introduction

Lemon balm, *M. officinalis* L. is one of the most used medicinal plants in Asia, Europe and the Mediterranean region. This plant is well known as a herbal tea for its aromatic, digestive and antispasmodic and sedative properties. The leaves emit a special fragrant lemon odour when bruised (Encalada et al., 2011). The chemical composition is essential oil, polyphenolic compounds: caffeic acid derivatives in large quantities, as well as Rosmaric Acid (RA), trimeric compounds and also some flavonoids such as luteolin-7-O-glucoside. Essential oil is considered to be the therapeutic principle mainly responsible for most of the activities mentioned; but plant phenolics, especially RA, are involved as well. The methanolic extracts of MO are more effective than aqueous extracts in term of neurological activities. (De Sousa et al., 2004; Encalada et al., 2011; Saraydin et al., 2012).

There are handful studies that evaluated antitumoral effects of MOE on a series of human cancer cells. In

study by Encalada in 2011, the authors investigated the antiproliferative effects of different fraction of 50% ethanolic and aqueous extract of MO on HCT-116 (human colon cancer cells) by MTT and NR assay. The 50% ethanolic extract showed significant differences after 72 h of treatment, reducing cell proliferation to values close to 40%, even the lowest dose tested (5 µg/ml). In the MTT assay, the same extract caused the lowest cell viability with 13% at a concentration of 1,000 µg/ml after 72h of treatment. Bioassay guided fractionation led to the isolation of an anti-proliferative compound, rosmarinic acid. In this study, the potent cytotoxic effects of MOE against HCT-116 cells was attributed to the presence of high amount of rosmarinic acid (1,000 µg/ml).

In another study by Saraydin et al in 2012, the authors investigated antitumoral effects of *Melissa officinalis* from Turkey on breast cancer cell lines (MCF-7, MDA-MB-468 and MDA-MB-23) *in Vitro* and *in Vivo*. *In vitro* apoptosis studies were performed by annexin V staining and flow cytometry analyses. Immunohistochemistry for Ki-67 and

¹Department of Nursing, Faculty of Maragheh, ²Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Urmia University, Urmia, ³Department of Medical Biotechnology, Faculty of Advanced Medical Sciences, ⁴Drug Applied Research Center, ⁵Student Research Committee, Tabriz University of Medical Sciences, Tabriz, Iran *For correspondence: jahanbanr@tbzmed.ac.ir

caspase 7 in the tumoral tissue sections of DMBA-induced mammary tumors in rats was also performed, along with TUNEL assays to detect apoptotic cells. MOE possessed cytotoxicity against three cancer cell lines, inducing increase in Annexin-positive cells. Moreover, MOE induced a significant reduction in the viability of MCF-7, MDA-MB-468, MDA-MB-231 cells in a dose-dependent manner. Crude extracts of MO showed cytotoxicity to three cancer cell lines with IC_{50} values $18 \pm 2.0 \mu\text{g/mL}$, $17 \pm 1.4 \mu\text{g/mL}$, $19 \pm 1.8 \mu\text{g/mL}$, respectively. Expression of caspase-7 protein and TUNEL positive cells were much higher in rats treated by MO, compared with the untreated control group, while expression of Ki-67 was decreased. Furthermore, in vivo studies showed that mean tumor volume inhibition ratio in MO treated group was 40% compared with the untreated rats. These results indicated that MO extracts have antitumoral potential against breast cancer (Saraydin et al., 2012).

In the course of our interest in biological applications and toxicity profile of this plant, this paper focused on study of the anti-proliferative activity of methanolic extract of *Melissa officinalis* from Iran on different human cancer cells.

Materials and Methods

Preparation of Extracts

Plant collected from Tabriz, East Azerbaijan Province, Iran, in Jun 2014. The Leaves were shade dried and grinded into powder with mortar and pestle. The prepared powder was kept in tight containers protected completely from light. Extraction of hydroalcoholic extract (HAE) was carried out according to previous protocols (Sineh Sepehr et al., 2012; Valiyari et al., 2013) by macerating 100g of powdered dry plant in 500mL of 80% ethanol for 48h at room temperature. Then, the macerated plant material was extracted with 80% ethanol solvent by percolator apparatus (2-liter volume) at 25°C. The plant leaves extract was removed from percolator, filtered through Whatman filter paper (NO. 4), and dried under reduced pressure at 37°C with rotatory evaporator. The concentrated leaves extracts of the plant were dissolved in Phosphate Buffer Saline (PBS) and was filtered to obtain a stock solution of 10 mg/mL. Subsequently, the substocks with concentrations ranging from 5-1000 $\mu\text{g/ml}$ was prepared from stock and further used in MTT assay.

Determination of Total phenolic content (TPC)

Total phenolic content was determined with Folin-Ciocalteu Reagent (FCR) according to the a described method (Singleton and Rossi, 1965) with some modifications. Briefly, 0.5 ml of each phenolic extract was mixed with 2 ml of 7.5% sodium carbonate, and then the mixture was allowed to stand at room temperature for 2 min. After addition of 2.5 ml ten-fold Folin-Ciocalteu reagent, the mixture was incubated in the dark room for 30 min. The absorbance was measured at 720 nm by using a spectrophotometer. The results were expressed as equivalent mg of Gallic acid per 100 g of fresh mass (mg GAE/100 g FM). A standard curve for Gallic acid was plotted under the same conditions as the studied samples.

All determinations were performed in triplicates.

Determination of Total flavonoid content (TFC)

Total flavonoid content of the extracts were assayed by the colorimetric method described by other authors (Zhishen et al., 1999; Jahanban-Esfahlan et al., 2012), with minor modifications. CME (250 μl) was mixed with 1.25 ml of distilled water and 75 μl of a 5% NaNO_2 solution. After five minutes, 150 μl of a 10% $\text{AlCl}_3 \cdot \text{H}_2\text{O}$ solution, 500 μl of 1 M NaOH and 275 μl of distilled water were added to the mixture. The absorbance of the mixture was measured at 507 nm. The results were expressed as equivalent mg of Quercetin per 100 g of fresh mass (mg Q/100 g FM) and compared with the Quercetin standard curve, which was made under the same conditions. All determinations were performed in triplicates.

DPPH free radical scavenging activity

The DPPH radical scavenging activity was determined as described methods (Brand-Williams et al., 1995) with some modifications. Various volumes of extracts (30, 50, 70 and 100 mL) were added to 1mL of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) solution (0.1 mM in methanol) and the reaction mixture shaken vigorously. After incubation at room temperature for 10 min, the absorbance of this solution was determined at 517 nm, by using a spectrophotometer. The antioxidant activity was expressed as IC_{50} values, which were calculated by non-linear regression with a one phase exponential association equation using GraphPad Prism version 6.0.

Cell culture

SKOV3 (human ovarian carcinoma), MCF-7 (human breast adenocarcinoma), PC-3 (Human prostate adenocarcinoma), A549 (lung non small cell cancer cells) were obtained from the Pasteur Institute (Tehran- Iran). Cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (10 U/ml), streptomycin (10 $\mu\text{g/ml}$) and 0.2 mM sodium pyruvate. Cultures were incubated in the presence of 5% CO_2 at 37°C and 100% relative humidified at MOsphere.

MTT assay

The cells were seeded in 96-well plates with a density of 1×10^4 cells/well/200mL and incubated for 24h at 37°C and 5% CO_2 . The cells were treated with different concentrations of solvent extracts (5, 20, 100, 250, 500, 1000 $\mu\text{g/ml}$). Paclitaxel (plant-derived chemotherapeutic anti-cancer drug from *Taxus brevifolia*) was used as a positive control. Untreated cancer cells was used as negative control (0 $\mu\text{g/ml}$, vehicle alone). After 72h treatment, 10mL of MTT reagent was added to each well. The plates were incubated at 37°C and 5% CO_2 for 4h. Then, 100 mL of the solubilization solution was added to each well and followed by incubation overnight at 37 C to dissolve formazan crystals. Finally, absorbance was read using an ELISA plate reader at a wavelength of 570 nm. The percentage of cytotoxicity and cell viability were calculated using following equation: % Cytotoxicity=1-(mean absorbance of treated cells/mean absorbance of negative control) and % Viability=100- % Cytotoxicity.

Statistical analysis

All the data represented in this study are mean \pm SEM of three identical experiments made in three replicate. Statistical significance was determined by analysis of variance, followed by LSD test and p-value ≤ 0.01 was considered significant. All analyses were conducted using the SPSS 16.

Results and Discussion

In the recent decades, although so much success accomplished in cancer treatment, however it still remains among the most common killers in the world (Jahanban Esfahlan et al., 2011). universal cancer burden rises to 14.1 million new cases in 2012 and striking increase in breast cancers must be addressed. Furthermore, according to the 2014 cancer statistics in United States, Among men, cancers of the prostate, lung and bronchus, and colorectum will account for about 50% of all newly diagnosed cancers, as prostate cancer alone will account for 27% (233,000) of incident cases in men. At the other hand, cancers of breast, lung and bronchus, and colorectum, accounting for one-half of all cases in women. Breast cancer alone is expected to account for 29% (232,670) of all new cancers among women. Overall, cancers of the lung and bronchus, prostate, breast, and colorectum continue to be the most common causes of cancer death. These 4 cancers account for almost half of the total cancer deaths among men and women, with more than one-quarter of all cancer deaths due to lung cancer. An urgent need in cancer control today

is to develop effective and affordable approaches to the early detection, diagnosis, and treatment of cancer (Abbasi et al., 2014b; Siegel et al., 2014).

Among a plethora of options for cancer treatment and prevention, natural products especially those rich in antioxidants, have always being regarded as a safe agents that effectively could scavenge the free radicals and so protect cells form their hazardous effects (Jahanban-Esfahlan et al., 2010; Abbasi et al., 2014c). In this way, *Melissa Officinalis* shows a high content for divergent polyphenolic and flavonoid compounds.

In study by Encalada, et al in 2011, Hydroalcoholic (HAE) and Aqueous extract (AQE) of MO from Barcelona-Spain were analyzed. For AQE, % TPC was determined as 1,980.54 mg/100 g FM and % TFC was determined 627 mg/100 g FM. DPPH IC₅₀ was 17.11 μ g/ml. For HAE, % TPC was determined as 3407.32 mg/100 g FM and % TFC was determined 927 mg/100 g FM. DPPH IC₅₀ was 11.01 μ g/ml (Encalada et al., 2011). Hydroalcoholic extract (HAE) of MO from Azerbaijan, Iran cultivators showed a considerable amount of %TFC and %TPC comparable to that of Spain Cultivators. In this way, TFC was determined 415.7 mg Q/100g FM and %TPC was 1506.713 mg GAE /100g FM. The antioxidant capacity is described quantitatively by the concentration

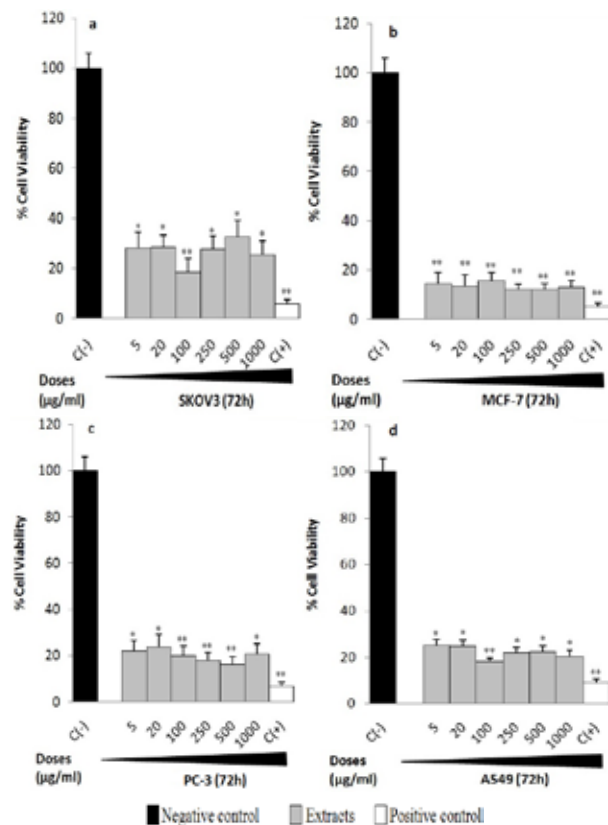


Figure 1. MO Extract Inhibit Different Human Cancer Cell Proliferation. *p<0.01; **p<0.001, compared to the negative control

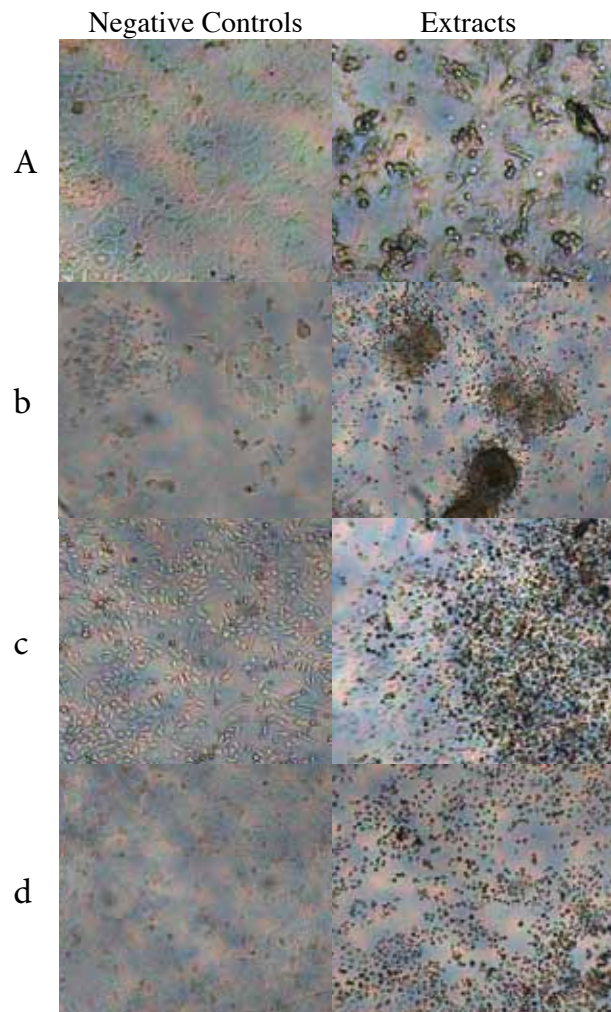


Figure 2. Cytotoxic Effect of MOE on Human SKOV3 (a), MCF-7 (b), PC-3(c) and A549 (d) Cell Lines after 72h Exposure

Table 1. Growth Inhibition (72 h)

Doses ($\mu\text{g/ml}$)	SKOV3	MCF-7	PC-3	A549
1000	74.5 \pm 5.2	87.25 \pm 4.2	79.6 \pm 5.2	79.46 \pm 5.1
500	67.29 \pm 5.5	88.12 \pm 6.6	83.7 \pm 3.0	77.45 \pm 4.3
250	72.4 \pm 4.8	88.08 \pm 5.4	82.0 \pm 6.4	78.04 \pm 4.1
100	81.3 \pm 6.1	84.5 \pm 8.3	80.0 \pm 8.1	81.58 \pm 4.9
20	71.4 \pm 8.2	86.54 \pm 5.4	76.29 \pm 5.2	75.16 \pm 3.7
5	71.7 \pm 7.6	85.6 \pm 4.3	78.0 \pm 7.1	74.87 \pm 3.4
Mean \pm SEM	73.09 \pm 4.4	86.67 \pm 1.1	79.93 \pm 3.7	77.8 \pm 4.7

of antioxidant required to scavenge 50% of DPPH•, which is referred as IC₅₀. This value was obtained from the graph of the percentage of scavenged radical versus the concentration of antioxidant. There is a reverse relation between DPPH IC₅₀ and TPC and TFC. In our study, DPPH IC₅₀ was obtained at 2.9 $\mu\text{g/ml}$ which indicates to the high antioxidant activity of Persian MOE compared to MO extract from Spain. In course of our interest on toxicological properties of different medicinal plants towards aggressive tumors (Abbasi et al., 2014a), in following study, we investigated the selective cytotoxicity of MO extract on a series of human cancers including ovarian cancer cells (SKOV3), breast adenocarcinoma cells (MCF-7), prostate adenocarcinoma cells (PC-3) and lung non small cancer cells (A549) by MTT assay. In course our interest on treatment of aggressive tumors; we specially selected these cancer cells because each displays a high propensity for metastasis in vivo. It is shown that MCF-7 cells form tumors when injected into athymic nude mice. These tumors are able to metastasize to lungs, liver and spleen. MCF-7 cells secrete into the culture media collagenases able to lyse types I and IV collagens (Shafie and Liotta, 1980). At the other hand, PC-3 cells have high metastatic potential to bone compared to other prostate cell lines such as DU145 cells which have a moderate metastatic potential and to LNCaP cells which have low metastatic potential (Sanchez-Sweatman et al., 1998). Lung non small cell carcinoma is a rapid growth tumor with high propensity for invasion and metastasis. This cell line frequently use for induction of lung metastasis in mice (Shindo-Okada et al., 2002). Similar to MCF-7, SKOV3 cells are among the aggressive and also the most common gynecologic cancers in women, however the special behavior of SKOV3 differ it from other ER+ cancer cells such as MCF-7. Both MCF-7 and SKOV3 have the receptors for estrogen (ER+) and basically they proliferate in response to estrogen, however practically ER+ SKOV3 cells are irresponsive to both estrogen and its analogues and this feature makes it a good candidate for studying the mechanism by which ER+ cells became unresponsive/resistance to estrogen and so anti-estrogenic therapies (Esfahlan et al., 2011). As some polyphenols and especially some well known flavonoids such as chrysin, genistein, and quercetin, could interfere with aromatase activity and so could hinder synthesis of estrogen which act as a main growth promoting factor on cancer cells (Gnoatto et al., 2008), so we used both hormone responsive and irresponsive cells to discern the selective effects of high polyphenolic extract of MO on

these cells.

The results of following study showed that in all doses, significant or very significant difference was observed between treated and untreated controls in a dose independent manner (Figure 1,2). In all cell lines, 5 $\mu\text{g/ml}$ of MO extract resulted in >50% growth inhibition in treated cells which account for the high toxicity profile of hydroalcoholic extract used in our study which requires a lower IC₅₀ compared to other extracts (De Sousa et al., 2004; Encalada et al., 2011) (see also Table 1).

In case of ovarian cancer cells, the maximum growth inhibition of 81.3% was obtained at doses 100 $\mu\text{g/ml}$, value close to positive control (20 $\mu\text{g/ml}$ Toxol used as positive control and it caused to 93.76% growth inhibition of SKOV3 cancer cells) meanwhile dose 500 $\mu\text{g/ml}$ of MO extract caused to the lowest growth inhibition of 67.29%. The mean growth inhibition in these cells was 73.09%.

In MCF-7 cancer cells, the maximum growth inhibition of 88.08% and 88.12% was obtained at doses 250 $\mu\text{g/ml}$ and 500 $\mu\text{g/ml}$, respectively, value comparable to that of 20 $\mu\text{g/ml}$ Toxol which caused to 95.1% growth inhibition of MCF-7 breast adenocarcinoma cells. The lowest growth inhibition of 84.5% was obtained at dose 100 $\mu\text{g/ml}$ of MO extract. The mean growth inhibition was 86.67%.

In PC-3 prostate adenocarcinoma cells, MO extract caused to the maximum growth inhibition of 83.7% at doses 500 $\mu\text{g/ml}$. In this cell line, 20 $\mu\text{g/ml}$ Toxol caused to 93.1% growth inhibition of PC-3 cancer cells meanwhile dose 20 $\mu\text{g/ml}$ of MO extract caused to the lowest growth inhibition 76.29%, respectively. The mean growth inhibition in these cells was 79.93% (Table 1, Figure 2).

In case of A549 cancer cells, the maximum growth inhibition of 81.58% was obtained at dose 100 $\mu\text{g/ml}$. 20 $\mu\text{g/ml}$ Toxol caused to 90.25% growth inhibition of A549 cells. In these cells, dose 5 $\mu\text{g/ml}$ caused to the lowest growth inhibition of 74.87%. The mean growth inhibition in these cells was 77.8% (Table 1, Figure 2).

In all cancer cells, MO extract reduced the cell viability to values below 33%, even the lowest doses. In this regard, MCF-7 breast cancer cells were the most responsive cells to antiproliferative effects of MOE with a maximum mean growth inhibition of 86.67% vs. 79.93%, 77.8% and 73.09% in PC-3, A549 and SKOV3 cells, respectively. It seemed that SKOV3 ovarian cancer cells were the least responsive cells to cytotoxic properties of MOE compared to the other studied cancer cells. This finding could be partly explained by different responsiveness of studied cancer cells to the anti-estrogenic activity of the rosmarinic Acid (RA). The RA is one of the main constituents of HAE extract of MO and it could interfere with aromatase activity and so estrogen synthesis (Balunas et al., 2008). As SKOV3 cells are ER+ but their proliferation is not estrogen dependent, so these cells are least sensitive to antiproliferative activity of MOE compared to other estrogen and androgen sensitive cancer cells including MCF-7, PC-3 and A549 cancer cells.

Our results indicated that hydroalcoholic extract of MO possess a high potency to inhibit proliferation of different tumors cells in a dose independent manner, suggesting that an optimal biological dose is more important and relevant than a maximally tolerated one. Moreover, the

antiproliferative effects of MO seems to be tumor type specific, as hormone dependant cancer cells showed a high responsiveness to antitumoral effects of this extracts.

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