

Full Length Research Paper

Cytotoxic effect of *Mentha spicata* aqueous extract on cancerous cell lines *in vitro*

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***Mentha spicata* is a herb with several biological properties. Cytotoxicity of essential oils of *M. spicata* on some cancer cells has been reported. In this study the cytotoxicity of aqueous extract of *M. spicata* on two tumor cell lines (Wehi-164 fibrosarcoma and U937 leukemic monocyte) has been evaluated *in vitro*. Wehi-164 and U937 cells were separately cultured in RPMI with 10% FBS. Then the cells at logarithmic growth phase were incubated in the presence of different concentrations of aqueous extract of *M. spicata* (0.1 to 10 mg/ml) at 24, 48 and 72 h periods. The cell proliferation was assessed with trypan blue dye exclusion and MTT assays. Aqueous extract of *M. spicata* significantly reduced the proliferation of Wehi-164 and U937 cells dose and time- dependently. The LD 50 values of *M. spicata* extract were 5.97, 4.63 and 4.77 mg/ml for the Wehi-164 cells and 5.6, 5.3 and 4.84 mg/ml for the U937 cells, after 24, 48 and 72 h treatment respectively. Aqueous extract of *M. spicata* showed cytotoxic effect in mouse fibrosarcoma Wehi-164 and human monocytic U937 cells. Thus, *M. spicata* could have potential anti-tumor activity. *In vivo* studies as well as identification of effective components of *M. spicata* with anti-cancer activity and their exact mechanism of action could be useful in designing new anti-cancer therapeutic agents.**

Key words: *Mentha spicata*, cytotoxicity, cancer.

INTRODUCTION

Herbal medicines are widely used for treatment of many diseases and a lot of medicinal plants with anti-tumor activities are available (Kamatenesi et al., 2011; Afolayan et al., 2010; Gathirwa et al., 2011). The protective effect of herbs rich diets against various cancers has been proposed by several epidemiological studies (Kaefer and Milner, 2008; Mehta et al., 2010). Cytotoxic effects of numerous herbal extracts against cancerous cells have been reported (Bisi-Johnson et al., 2011). *Mentha spicata* is a herb belonging to the *Mentha* genus in the Labiatae family (Choudhury et al., 2006). The beneficial effects of this plant in treatment of many gastro-intestinal disorders have been well documented (Rokaya et al., 2010). Also, the anti-microbial, anti-inflammatory and anti-tumoral properties of *M. spicata* have been shown (Pearson et al., 2010; Zu et al., 2010; Hussain et al., 2010). The cytotoxic effects of essential oils from *M. spicata* leaves

on some cancer cell lines have been revealed *in vitro* (Zu et al., 2010; Hussain et al., 2010). In addition the antigenotoxic effects of aqueous fraction of *M. spicata* have been attributed to its modulatory actions on lipid peroxidation (LPO) and antioxidant enzymes (Arumugam and Ramesh, 2009). Moreover the cytotoxicity of essential oil of another *Mentha* species (*Mentha pulegium*) on some cancer cell lines has been shown (Shirazi et al., 2004). There are many reports on biological activities of some compounds derived from herbs (Bisi-Johnson et al., 2011; Ma et al., 2011). Accordingly, many biological activities including antioxidant (Agata et al., 2010), anti-tumor (Furtado et al., 2008), immunomodulatory (Yun et al., 2003) and anti-inflammatory (Swarup et al., 2007) effects of Rosmarinic acid (a polyphenolic carboxylic acid found in *M. spicata* have been reported.

Evaluation of biological effects of phytochemicals on tumor cells could be useful to establish more potent chemopreventive agents (Karikas, 2010).

To the best of our knowledge, no well-documented study on the toxic effect of *M. spicata* in fibrosarcoma or

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leukemia has been published. At the other hand the current therapeutic strategies for fibrosarcoma as well as leukemia have not been very successful.

In the present study the cytotoxicity of aqueous extract of *M. spicata* leaves on two tumor cell lines (Wehi-164 fibrosarcoma and U937 leukemic monocyte) has been assessed.

MATERIALS AND METHODS

Reagents

RPMI-1640 medium, penicillin, streptomycin, trypan blue (TB) was from sigma (USA). Fetal calf serum (FCS) was from Gibco (USA). M.T.T. was purchased from Merck (Germany). *M. spicata* leaves were purchased from Pakan Bazr company from Esfahan province of Iran (the species was identified and authenticated by Dr. Shams). Microtiter plates, flasks and tubes were from Nunc (Falcon, USA).

Extract preparation

150 g of *M. spicata* dried leaves were boiled in 3 L distilled water for 2 h. Then solution was filtered and dried by evaporation. The extract was dissolved in RPMI-1640 and filtered by 0.2 µm filter and stored at -20°C until use in experiments. The extract was diluted in culture medium to prepare the required concentrations before use.

Cell lines

Mouse fibrosarcoma cells [Wehi-164 (NCBI C200)] and human leukemic monocyte [U937 (NCBI C130)], were obtained from NCBI (National Cell Bank of Iran, Pasteur Inst. of Iran, Tehran). The cells were maintained in RPMI-1640 medium supplemented with 10% FCS at 37°C in 5% CO₂.

Cell culture and treatment

The cells were cultured in RPMI-1640 medium supplemented with 10% FCS, penicillin (100 IU/ml) and streptomycin (100 µg/ml) at 37°C in 5% CO₂.

The cells were seeded at a density of 2×10^4 cell/well and then incubated with different concentrations of *M. spicata* (0.1-10 mg/ml) for 24, 48 and 72 h. Cytotoxicity was expressed as the percentage of viable cells at different doses of *M. spicata* extract. All experiments were done in triplicate.

Cell proliferation assay

To evaluate the effect of different concentrations of drug on viability of leukemic cell lines, we used trypan blue dye exclusion (TB test) (Moldeus et al., 1978) and MTT assay (Mossmann, 1983).

Trypan blue dye exclusion test

Principle of trypan blue dye exclusion test is exclusion of dye by viable cells and taking it up by dead cells. Viability is evaluated by direct counting of viable and dead cells. Percentage of the number of viable cells to the total number of cells is considered as viability percentage.

MTT assay

In MTT assay the conversion of yellow water soluble MTT to a blue-insoluble formazon was assessed according to the method developed by Mosmann (1983). At the end of incubation time, 20 µl of MTT solution (5 mg/ml in PBS) was added to each well and incubated at 37°C for 4 h. Subsequently, the medium was depleted and then 100 µl of the isopropanol-HCl solution (0.04 N), was added to each well. So the insoluble formazon derivative was dissolved and absorbance at 570 nm was measured using a microplate reader (ICN Flow Titertech Multiscan plus, USA). The results were expressed as cell numbers per control.

LD 50 determination

The 50% lethal dose (LD(50)) was calculated by M.T.T. based dose-response curve as the concentration at which 50% cell death rate occurred relative to the untreated cells.

Statistical analysis

Effect of the *M. spicata* extract on tumor cells was performed in three independent experiments and the results were expressed as mean ± SEM. Statistical comparisons between groups were made by analysis of variance (ANOVA), $P < 0.05$ was considered significant. Test of multiple comparison of Tukey was applied (5%) for statistically significant differences. The software SPSS 11.5 and Excel 2003 were used for statistical analysis and graph making respectively.

RESULTS

Toxicity of *Mentha spicata* extract on Wehi-164 cells

Cytotoxic effect of different concentrations of *M. spicata* aqueous extract on Wehi-164 fibrosarcoma cells at three time intervals are shown in Figure 1A and B. A and B represent the result of trypan blue dye exclusion and MTT assays respectively. *M. spicata* significantly decreased proliferative responses of Wehi-164 fibrosarcoma cells in both staining methods in all time intervals dose-dependently ($P < 0.05$) (Figure 1A and B). The results illustrated in Figure 1A and B showed that *M. spicata* aqueous extract significantly decreased the proliferation of Wehi-164 fibrosarcoma cells at ≥ 2.5 mg/ml concentration after 24 h incubation compared with untreated control cells ($P < 0.05$). *M. spicata* aqueous extract cytotoxicity on Wehi-164 cells at ≥ 2.5 mg/ml concentration was significantly increased with time in this order: 72 and 48 h > 24 h (Figure 1A and B). M.T.T assay represented a typical dose-response curve with LD50s of 5.97, 4.63 and 4.77 mg/ml after 24, 48 and 72 h treatment of Wehi-164 cells, respectively.

Toxicity of *Mentha spicata* extract on U937 cells

Cytotoxic effect of different concentrations of *M. spicata* aqueous extract on human monocytic U937 cells at three

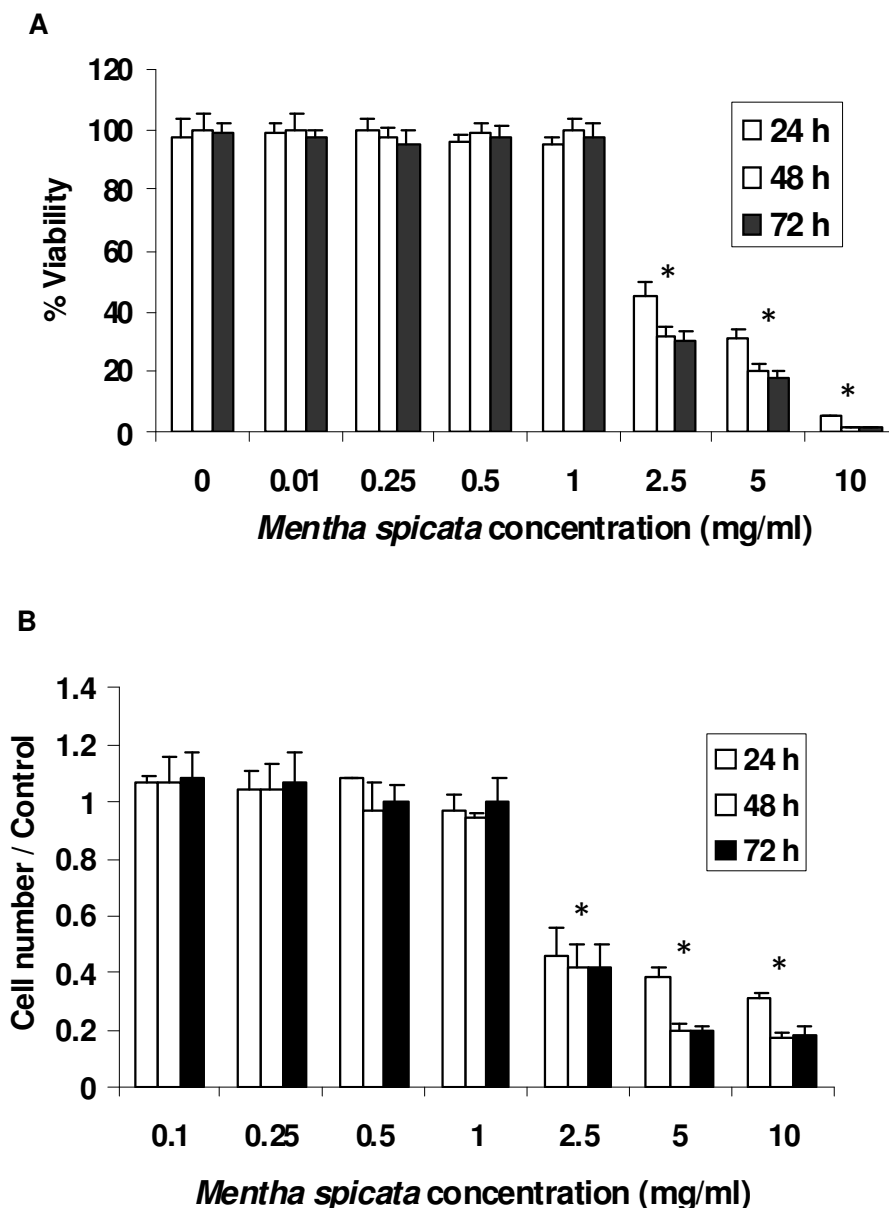


Figure 1. Cytotoxicity of *Mentha spicata* extract on Wehi-164 cells. The Wehi-164 cells were treated with different concentrations of *Mentha spicata* extract (0.01 to 10 mg/ml) for 12, 24 and 48 h. The results are presented as % of viability demonstrated by trypan blue dye exclusion (TB) test (A) and cell number/ control demonstrated by MTT assay (B). Data are mean \pm SEM of triplicate cultures. n = 3; P < 0.05 was considered significant.

time intervals are shown in Figure 2A and B). A and B represent the result of trypan blue dye exclusion and MTT assays respectively. *M. spicata* significantly decreased proliferative responses of U937 cells in both staining methods in all time intervals dose-dependently (P < 0.05) (Figure 2A and B). The results depicted in Figure 2A and B showed that *M. spicata* aqueous extract significantly decreased the proliferation of U937 cells at ≥ 2.5 mg/ml concentration after 24 h incubation compared with untreated control cells (P < 0.05). *M. spicata*

aqueous extract cytotoxicity on U937 cells at ≥ 2.5 mg/ml concentration was significantly increased with time in this order: 48 and 72 h > 24 h (Figure 2A and B). M.T.T assay represented a typical dose-response curve with LD50s of 5.6, 5.3 and 4.84 mg/ml after 24, 48 and 72 h treatment of U937 cells, respectively.

DISCUSSION

Cytotoxicity of some medicinal plants has been well

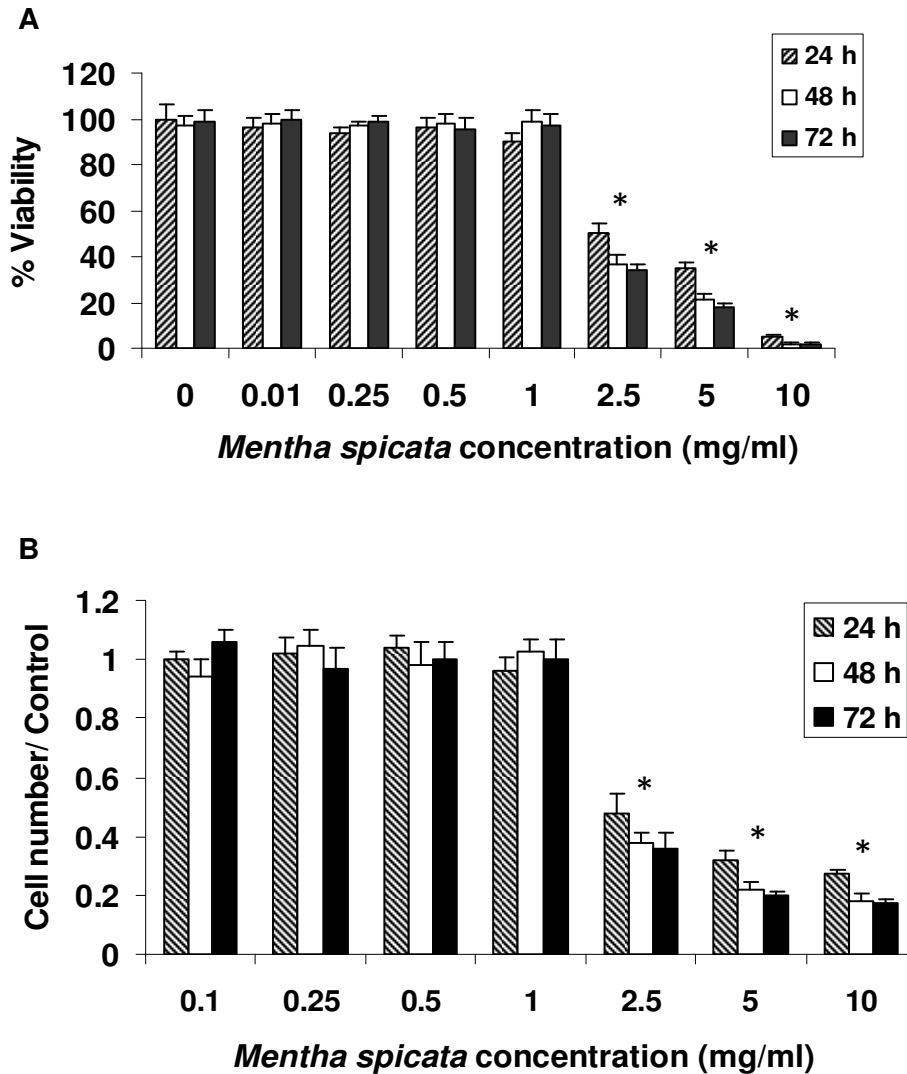


Figure 2. Cytotoxic activity of *Mentha spicata* extract on U937 cells. The U937 cells were treated with different concentrations of *Mentha spicata* extract (0.01 to 10 mg/ml) for 12, 24 and 48 h. The results are presented as % of viability demonstrated by trypan blue dye exclusion (TB) test (A) and cell number/ control demonstrated by MTT assay (B). Data are mean \pm SEM of triplicate cultures. n = 3; P<0.05 was considered significant.

known (Kaefer and Milner, 2008; Afifi-Yazar et al., 2011; Sertel et al., 2011). According to the results of this study, the aqueous extract of *M. spicata* leaves has cytotoxic effects on both human and mouse carcinoma cell lines. This cytotoxicity was dose and time dependent shown at ≥ 2.5 mg/ml concentration of the extract at 24 h incubation time onwards. The cytotoxic effect at 48 and 72 h incubation time was significantly more than 24 h but there was no significant difference in cytotoxicity between 48 and 72 h incubation time. The pattern of *M. spicata* cytotoxicity was similar for wehi-164 and U937 cells.

The cytotoxicity of Labiatae herb family including the *Mentha* genus has been reported (Hoffman, 1999). Moreover the *Mentha* preparations have been used for

therapy of human cervical cancer (Briggs, 1989; Duke, 2001). The cytotoxic effect of essential oil of *M. pulegium* (another species of *Mentha* genus) on ovarian adenocarcinoma (SK-OV-3), human malignant cervix carcinoma (Hela) and human lung carcinoma (A549) cell lines has been shown by other investigators (Shirazi et al., 2004). However, they did not use aqueous extract in their study. Furthermore, they used clonogenic and neutral red (NR) assays for assessment of cytotoxicity, while we studied the effect of aqueous extract of *M. spicata* species and used trypan blue dye exclusion and M.T.T methods for evaluation of its cytotoxicity. In Shirazi et al. (2004) study, the metanolic extract of *M. pulegium* (at the concentration ≤ 1000 μ g/ml) did not show any

cytotoxicity. This result of Shirazi et al. (2004) is similar to us as in our study we did not see any cytotoxic effect at the concentrations ≤ 1000 $\mu\text{g/ml}$ of the *M. spicata* extract. In our study the cytotoxic effect of *M. spicata* extract was shown at ≥ 2.5 mg/ml . According to the results of our study on cytotoxicity of *M. spicata* aqueous extract, there are some toxic agents in *M. spicata* with hydrophilic nature.

In our study, the LD50s of *M. spicata* aqueous extract for Wehi-164 cells at 24, 48 and 72 h were 5.97, 4.63 and 4.77 mg/ml and for U937 cells were 5.6, 5.3 and 4.84 mg/ml respectively. In this study the LD50s of *M. spicata* aqueous extract for Wehi-164 fibrosarcoma and U937 leukemia were found to be very close to each other. In Shirazi et al. (2004) study, the LD 50s of *M. pulegium* essential oil on different human cancer cell lines were between 14.10 and 59.10 $\mu\text{g/ml}$. The discrepancy between our results and Shirazi et al. (2004) may be due to the different cell lines, *Mentha* and type of extract used.

Shirazi et al. (2004) used ovarian adenocarcinoma (SK-OV-3), human malignant cervix carcinoma (Hela) and human lung carcinoma (A549) cell lines but we used mouse fibrosarcoma Wehi-164 and human monocytic leukemia U937 cells. Different cells have different sensitivities to drugs (Shirazi et al., 2004). Besides we used aqueous extract of *M. spicata* while Shirazi et al. (2004), utilized essential oils of *M. pulegium*. The aqueous extract ingredients are not as the same as that in essential oils. El Babilii et al. (2011) reported that essential oil of oregano (a herb of the mint family) leaves was nontoxic (contrast to Shirazi et al. study, 2004) study, and that between oregano leaves extracts, the aqueous extract had the maximum antioxidant effect.

Anti-inflammatory effect of essential oils of *M. spicata* has been reported (Pearson et al., 2010). As fibroblasts and macrophages (used in this study), have important role in inflammation (Barron et al., 2011; Ichioka et al., 2011), anti-inflammatory effects of *M. spicata* may be in part due to its cytotoxic effects on inflammatory cells. In addition, inflammation has an important role in pathology of fibrosarcoma (Glinghammar et al., 2011) and therapeutic potential of aspirin analogues (as non-steroidal anti-inflammatory drugs) in chronic lymphocytic leukemia has been reported (Pepper et al., 2011). Besides, the current therapeutic strategies for leukemia and fibrosarcoma have not been very successful. Thus, *M. spicata* preparations could have potential implications in designing novel therapeutic methods for fibrosarcoma/leukemia. According to our results, *M. spicata* aqueous extract could be cytotoxic for tumor cells. In order to prevent the probable side effects, studies of *M. spicata* aqueous extract on normal cells and tissues are recommended.

The cytotoxic concentration of *M. spicata* aqueous extract on the cell lines used in this study was ≥ 2.5 mg/ml . In Shirazi et al. (2004) study, different patterns of

M. pulegium essential oil effects on different cell lines has been shown. For determination of the patterns of the sensitivity of different cells to *M. spicata* aqueous extract, additional *in vitro* studies are warranted. Besides, further investigations are required to determine the molecular mechanisms of *M. spicata* cytotoxicity as well as isolation and characterization of *M. spicata* constituents mediating anti-cancer activity. Moreover, *in vivo* studies are necessary to support the *in vitro* tests and planning the intelligent therapeutic strategies.

Conclusion

According to the results of this study, aqueous extract of *M. spicata* leaves has cytotoxic effects on mouse fibrosarcoma (Wehi-164) as well as human monocytic leukemia (U937) cells. Therefore, *M. spicata* could have potential implication in designing novel therapeutic methods for fibrosarcoma/ leukemia and possibly other malignancies. Further investigations are required to determine the molecular mechanism(s) of *M. spicata* aqueous extract cytotoxicity as well as isolation and characterization of its constituents mediating cytotoxic effects.

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