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Relative Cytotoxicity of Fractionated Extract of Arial Parts of *Mentha Pulegium* on Three Cancer Cell Lines

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ABSTRACT

Medicinal herbs are significant sources of chemotherapeutic drugs and play a vital role in the prevention and treatment of cancer. *Mentha pulegium* L. from Labiatae family was traditionally used as an anticancer agent. In this study, aerial parts including leaves of this plant were extracted by methanol and fractionated extracts have been produced by petroleum ether, ethyl acetate, acetone, methanol and distilled water. For the purpose of cytotoxic evaluation of methanolic extract and its fractions on human ovary carcinoma cells (C13), human hepatocarcinoma cells (HepG2) and human lung carcinoma cells (A549), clonogenic assay was performed. Briefly, 200 cells were seeded in each well of 6 well plates in RPMI 1640 with 10% FBS media. After 24 hours incubation, 0-50µg/ml of methanolic extract and its fractions were exposed to the cells. Finally, colonies with more than 50 cells were counted after 7 days. In each case, a control row was set by the exposure of cells to compounds-free solvents. LC_{50} values were calculated using nonlinear regression analysis on Graphpad prism® software. The result showed that the methanolic extract and its fractions are cytotoxic on all three studied human carcinoma cell lines at different degrees. Human ovary carcinoma cell line (C13), which is resistant to many other chemotherapeutic agents (e.g. cisplatin), is the most sensitive cell line to methanolic extract and its fractions compared to two other cell lines. Further complementary cellular and animal studies are recommended for these anticancer candidates.

Keywords: Cancer, *Mentha pulegium*, Clonogenic assay, Cytotoxicity, C13, HepG2, A549

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INTRODUCTION

Nowadays, achievement of drugs and chemicals which are able to suppress the out of control growth and proliferation of cancer cells is the goal of many cellular investigations. Scientific consideration and test of traditionally used herbs for the treatment of different malignancies could be considered as a very valuable source for new chemotherapeutic drugs. Some reports on the use of Labiatae species for the treatment of cancer are available [1-4]. *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 [5]. It also grows naturally in Golestan and Gilan, Provinces in the north of Iran [6] 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 [7], antiflatulent, carminative, antispasmodic, expectorant, antitussive, diuretic, menstruate [8], insect repellent and anti-inflammatory [7]. Traditionally, total decoction of this herb was used for the treatment of different kinds of cancers such as gingival [9], colon, pudenda, spleen, belly, stomach and uterus [10]. Furthermore, there are some publications confirming the cytotoxic activity of its essential oil against different human cell lines [11]. Most of the previous publications have correlated the pharmacological effect of *M. pulegium* to its pulegone content [12-16] but reviewing the literatures has shown that *M. pulegium*'s constituents depend on the region of cultivation and there are some variations in its constituents in different countries [17-19]. Studies on Iranian *Mentha pulegium* show that in many cases, it contains much less pulegone than the other countries' *Mentha pulegium* [17, 20]. In this study, clonogenic assay has been used to define the cytotoxicity of total and fractionated extract of Iranian *Mentha pulegium* on three human carcinoma cell lines.

MATERIALS AND METHOD

Unless otherwise specified, Chemicals were purchased from Sigma alderich (USA) and Merck (Germany) companies.

Plant material

Aerial parts including leaves of *Mentha pulegium* L. was collected from Bandar-Anzeli (Province of Gilan, Iran) during the flowering period in spring 2009 and authenticated by M. Kamalinezhad. A voucher specimen was deposited at the Herbarium of the Pharmacognosy Department, School of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran. The plant was dried in the room with active ventilation at ambient temperature.

Preparation of the extracts and fractions

The dried powdered material of aerial parts including leaves (300 g) was macerated in methanol 80% at room temperature for 72 hours. After filtration, the macerate (extract) was concentrated in vacuo at 40 ˚C using a Rotary Evaporator (Hiedolph,Germany).The dried extract (total methanolic extract) was subjected to the fractionation with different solvents i.e. petroleum ether, ethyl acetate, acetone, methanol and distilled water (in the order of increasing polarity) by maceration technique. The solvents were completely removed under reduced pressure at 40˚C (except petroleum ether which is flammable). The dried extracts were dissolved in dimethylsulfoxide (DMSO) to reach the final concentration of 50mg/ml as the stock samples [21]. For the cellular applications the stock solutions were diluted in sterile RPMI 1640 cell culture media. The cells were exposed to different concentrations of 2.5, 5, 10, 15, 20, 25, 37.5, and 50µg/ml of the total methanolic extract and its fractions.

Cell cultures

Human HepG2 hepatocarcinoma cell line (NCBI Code C158), human lung carcinoma A549 cell line (NCBI Code C137) and human ovary carcinoma C13 cell line (NCBI Code C446), were purchased from Pasture Institute of Iran, Tehran, Iran. Cells were cultured in RPMI 1640 medium (Gibco BRL, USA) supplied with 10% fetal bovine serum and 1% penicillin-streptomycin at 37ºC in humidified incubator with 5% CO_2. In all of the experiments the exponentially growing cells were used, which were prepared by at least three passages of the initial seeding of the frozen stock.

Clonogenic assay

Clonogenic assay was performed as described by Hickson *et al.* with some modifications [22]. Cells were harvested with trypsin and seeded in 6-well plates (SPL Lifesciences®, Korea) with the appropriate concentration to give 200 to 300 cells per well. The plates were incubated overnight allowing the cells to attach to the surface of the wells. After 24 hours the cells were exposed to the eight concentrations of each extract which were mentioned above, in triplicate. In order to normalize the resulting data, three controls were set by exposing the cells to compounds-free solvent in each set of experiment. The plates were incubated at 37º C in 5% CO_2 atmosphere humidified incubators. After 7 days incubation the medium was removed and formed colonies were fixed with 96% ethanol for 10 minutes and stained with 0.4% Methylen blue for 30 minutes. Finally the plates were gently washed with water and air-dried. Colonies containing at least 50 cells were manually counted with microscope.

Statistics

The median lethal concentration (LC_{50}) of each extract was calculated by plotting the percent of cell survival vs. logarithm of extract concentration using nonlinear regression analysis in GraphPad

PRISM® Software. One-way analysis of variance (ANOVA) followed by Dunnett's post hoc test was used to assess significant differences ($P<0.05$) between different concentrations of extracts with controls. All the statistical analysis was performed using GraphPad PRISM® Software (GraphPad Software, San Diego, CA, USA).

RESULTS AND DISCUSSION

The cytotoxicity results of *M. pulegium* total extract on C13, HepG2 and A549 cell lines are shown in figure1A. As shown in this figure Significant decrease in C13 ($P<0.001$), HepG2 ($P<0.001$) and A549 ($P<0.05$) cell survival was observed with concentrations more than 2.5, 2.5 and 10µg/ml of total methanolic extract. figures 1B to 1F represent the cytotoxicity results of petroleum ether, ethyl acetate, acetone, methanol and distilled water fractions of the total extract of *M. pulegium*, respectively.The results indicate that Petroleum ether fraction significantly decreases C13 ($P<0.001$), HepG2 ($P<0.001$) and A549 ($P<0.001$) cell survival with concentrations more than 2.5, 2.5 and 37.5µg/ml (Figure 1B). Ethyl acetate fraction significantly decreases C13 ($P<0.001$), HepG2 ($P<0.001$) and A549 ($P<0.01$) cell survival with concentrations more than 10, 37.5 and 37.5µg/ml (Figure 1C). Figure 1D shows that Significant decrease in C13 ($P<0.001$), HepG2 ($P<0.01$) and A549 ($P<0.01$) cell survival was observed with concentrations more than 10, 10 and 15µg/ml of acetonic fraction. As shown in figure 1E Methanolic fraction significantly decreases C13 ($P<0.05$), HepG2 ($P<0.01$) and A549 ($P<0.01$) cell survival with concentrations more than 2.5, 10 and 15µg/ml and figure 1F shows that significant decrease in C13 ($P<0.001$), HepG2 ($P<0.001$) and A549 ($P<0.05$) cell survival was observed with concentrations more than 15, 2.5 and 2.5µg/ml of water fraction. As shown in graphs A to F most extracts exhibited a noticeable concentration-dependent cytotoxicity in the three mentioned cell lines but the general toxicity pattern of each extract is unique in different cell lines. It may be predicted that there are different target sites for the active ingredients of *M. pulegium* extract in each cell line which is the reason of unique cytotoxicity patterns. Based on the result shown in table 1, the total methanolic extract and its fractions are cytotoxic on all three studied human carcinoma cell lines at different degrees. In reference to the reported classification [23], total extract of the aerial parts of *M. pulegium* and its fractions can be categorized as potentially toxic on all three cell lines. These data are in agreement with Rahimifard *et al*. publication in 2011 which has tested the cytotoxic properties of Iranian *M. pulegium* essential oil and total methanolic extract on Hela (human malignant cervix carcinoma), Hep2 (human laryngeal carcinoma) and Vero (green African monkey kidney) cell lines using MTT

assay. Their results have also showed that both methanolic extract and essential oil have cytotoxic properties on these three cell lines [24].

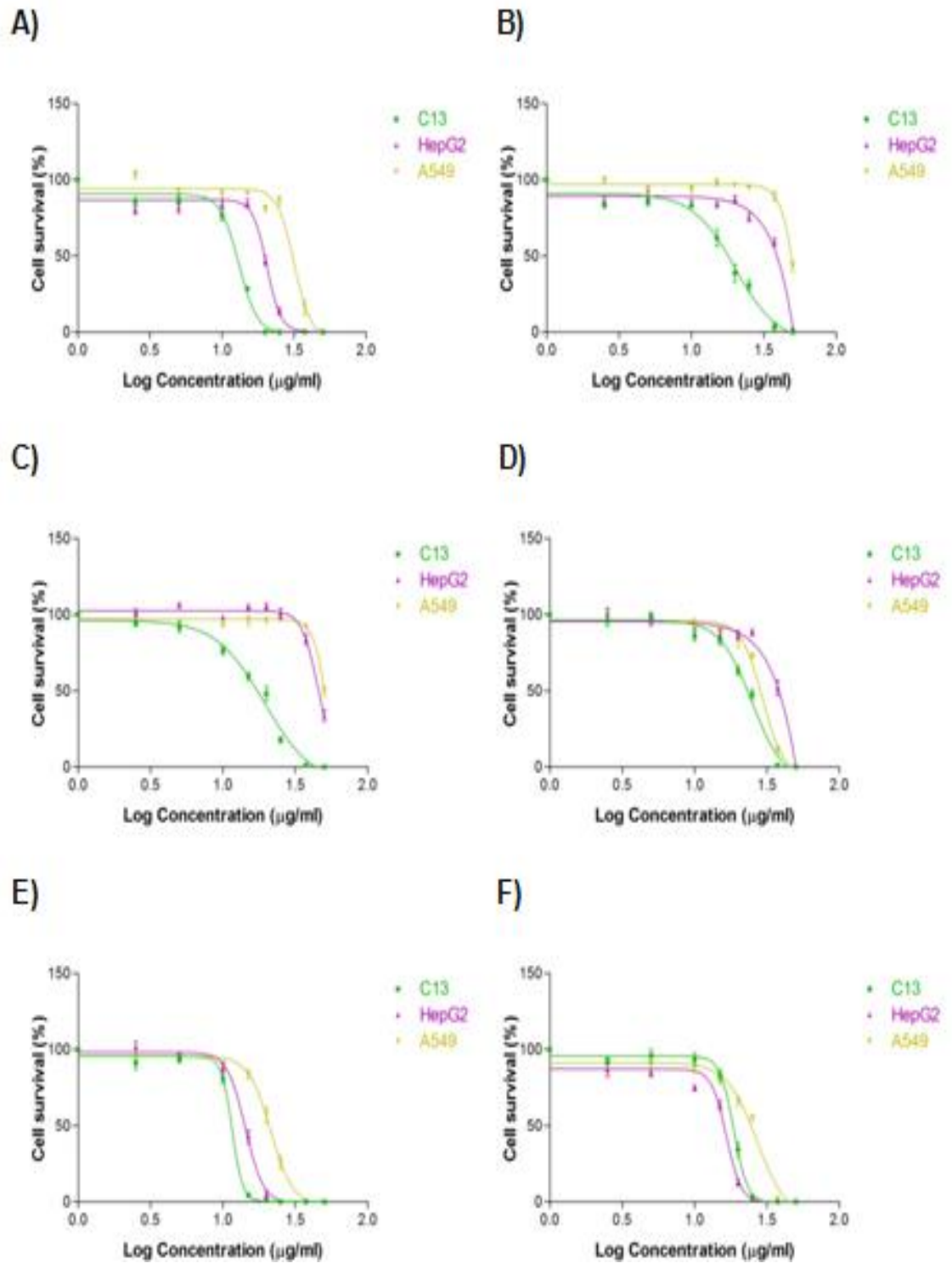

Figure 1. Effect of *Mentha pulegium* total methanolic extract and petroleum ether, ethyl acetate, acetone, methanol and distilled water fractions on C13, HepG2 and A549 cell survival using colonogenic assay.

Table 1: Mean LC_{50} ± SEM values of *Mentha pulegium* methanolic extract and its fractions using clonogenic assay. LC_{50} ± SEM values were calculated using nonlinear regression analysis on Graphpad prism® software.

LC_{50} (µg/ml)	Total Methanolic Extracts	Petroleum Ether Fraction	Ethyl Acetate Fraction	Acetonic Fraction	Methanolic Fraction	Water Fraction
A549	13.11±1.030	20.09±1.060	19.33±1.046	24.84±1.032	11.58±1.020	18.65±1.044
C13	20.47±1.023	39.81±1.013	44.66±1.051	37.00±1.039	14.30±1.023	16.59±1.016
HepG2	31.66±1.016	51.73±1.056	47.71±1.415	29.47±2.570	21.39±1.016	26.96±1.020

Considering the IC_{50} values shown in table 1, the methanolic fraction is more cytotoxic than the water fraction in all three cell lines. So it could be concluded that beside solubility, there might be another factor which is influencing the fractions component and that might be physicochemical characteristics of *M. pulegium* cytotoxic ingredient which seems to be more solubility-compatible with methanol than water. As shown in the results, human ovary carcinoma cell line (C13) is the most sensitive cell line to the total methanolic extract, followed by petroleum ether, ethyl acetate, acetone and methanol fractions. However, the C13 cell line is mostly sensitive to the cytotoxicity caused by the water fraction that might be due to the existence of some other toxic active ingredients which are physicochemically more soluble in water than methanol. Human lung carcinoma cell line (A549) is the most resistant cell line to the *M. pulegium* extracts in this study. In spite of the inherent resistance of lung cells to the most of available anticancer chemotherapeutic agents, we have noticed a relatively acceptable cytotoxic effectiveness of *M. pulegium* extracts on this cell line. According to our results, regardless of minor differences, the cytotoxicity of *M. pulegium* extract fractions increases by enhancing the solvents' polarity in all three studied cell lines. This finding is in contrary to the general belief that the cytotoxicity of *M. pulegium* is mainly related to its pulegone (low polarity molecule) content [12-16]. Our results suggest that some other polar compounds might be responsible for *M. pulegium*'s cytotoxicity collected from the northern part of Iran and examined on these three cancer cell lines. Pehlivan Krakas *et al.* study on antitumor activity of *Mentha pulegium* in 2012 supports our hypothesis; in this study the antitumor activity of this herb was correlated with the polarity of the three different aqueous, ethanol and methanol extracts and they have also reported that the aqueous extract had the most antitumor activity [25].

CONCLUSION

Our results indicate that *M. pulegium* Extracts, especially those extracted in the methanolic solvent may be proper anticancer candidates for at least three studied human cell lines. *Mentha pulegium*

extracts are particularly cytotoxic to the human ovary carcinoma cell line (C13) which is resistant to many other common chemotherapeutic agents (e.g cisplatin). It is noticeable that the majority of previous investigations have correlated the cytotoxicity of *M. pulegium* to its pulegone content [12-16], being the most dominant compound (up to 99%) in the essential oil of plants grown in many other countries [15]. There are some reports indicating that the Iranian *M. pulegium* contains much less pulegone [17, 20]. Our results have shown an increase in cellular toxicity of the *Mentha pulegium* grown in Iran with polarity of the fractions, hypothesizing the presence of some other polar toxic chemicals in the fractions. The present results may elucidate the traditional use of *M.pulegium* extract in the treatment of cancers. Based on these results, further phytochemical investigations on the polar fractions of this herb are recommended to identify the antitumor ingredient(s).

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