DARU Volume 12, No. 3, 2004

EVALUATION OF NORTHERN IRAN *MENTHA PULEGIUM L.* CYTOTOXICITY

FARSHAD H. SHIRAZI, NEDA AHMADI AND MOHAMMAD KAMALINEJAD

Department of Toxicology/Pharmacology, Faculty of Pharmacy, Shaheed Beheshti University of Medical Sciences, Tehran, Iran.

ABSTRACT

In vitro tests could be a valuable tool for the evaluation of medicinal plants' cytotoxicity. One of the most frequently used Iranian traditional plants is *Mentha Pulegium* from Labiatae family. In the present study, essential oil and the methanolic extract of *Mentha pulegium*, were analyzed for cytotoxicity on human ovary adenocarcinoma SK-OV-3, human malignant cervix carcinoma Hela, and human lung carcinoma A549 cell lines. Two different assays of clonogenic and neutral red (NR) were used for evaluation of cytotoxicity. Although the methanolic extract of *Mentha pulegium* did not show any cytotoxic effects, the essential oil of this plant proved to be a potent cytotoxic agent on the above three cell lines. According to the clonogenic assay, LD_{50}s of the essential oil on SK-OV-3, Hela and A549 cell lines are 14.10, 59.10 and 18.76 µg/ml, respectively. Our findings suggest that *Mentha pulegium* essential oil might be considered as a potentially toxic agent on human cancer cell lines, and a possible candidate for human cancer chemotherapy. However, further biological tests on the efficacy and side effects of this plant are necessary before its use in human.

Keywords: *Mentha pulegium*, Cytotoxicity, SKOV3, Hela, A549, Cell culture.

INTRODUCTION

Plants in traditional medicine for the treatment of various illnesses are widespread and a number of naturally produced herbal formulations are available for cancer patients. While many of these herbal medicinal candidates may not rise to a significant healing outcome, and some could even be potentially toxic and dangerous in human, demands and applications of formally accepted and/or informal herbal drugs are experiencing increase popularity. Therefore 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.

Mentha pulegium L., popularly known as "Khalvash", is consumed mainly for its antiseptic, insect repellent, carminative, antispasmodic, diaphoretic and anti-inflammatory properties in Iran (1). Traditionally, total decoction of this herb was used for the treatment of fibrosis and cervical tumors (2). However, to the best of our knowledge, no well-documented investigation on the cytotoxicity of this herbal medication in cancer cells has been reported. Although, the total decoction and infusion of this plant has a traditional application, its essential oil ingredients have never been considered before.

This paper describes the results of our study on the cytotoxicity of *Mentha pulegium* aerial parts essential oil, as well as its methanolic extract on three different human carcinoma cell lines.

MATERIALS AND METHODS

Preparation of extract and essential oil

M. pulegium aerial parts were collected from Rasht, Gillan, one of the northern provinces of Iran, by ethnic groups. Samples of the plants used for this study were identified by M. Kamalinejad and kept for record in the Toxicology/Pharmacology lab, to be used in the herbarium of the faculty of Pharmacy, Shaheed Beheshti University of Medical Sciences, Tehran, Iran. Both of the extract and essential oil were prepared from the aerial parts including leaves of this plant. Methanolic extract of the dried sample in concentration of 100 mg/ml was prepared using Soxhlet apparatus using 80% methanol as solvent, and sterilized by the use of the biological 0.22 µm membrane filters. This extract was further diluted in sterile DMEM/F12 cell culture media for cellular application. The cells were treated by different concentrations of: 5, 25, 50, 100, 150, 200, 400, 600, 800, and 1000 µg/ml of the methanolic extract. For essential oil experiments, Clevenger-type apparatus was used to prepare a 0.6 ml essential oil from 100 g dried sample in double distilled water. The essential oil was separated from the surface of water and dehydrated using sodium sulfate. The density of

Correspondence: Farshad H. Shirazi, Department of Toxicology/Pharmacology, Faculty of Pharmacy, Shaheed Beheshti University of Medical Sciences, Tehran, Iran,E-mail: Dr_fshirazi@yahoo.ca

the prepared essential oil was calculated 0.938 mg/ml. Essential oil was diluted by the use of 96% ethanol and added to cell culture media to prepare different concentrations of 9.38, 18.76, 23.45, 28.14, 37.52, 46.90, 56.28, 65.66, 70.35, and 93.80 µg/ml.

Cells

Human ovarian adenocarcinoma SK-OV-3, human malignant cervix carcinoma Hela, and human lung carcinoma A549 cell lines were obtained from Pharmacology lab, Ottawa Regional Cancer Center (Ottawa, Canada). Cells were cultured in DMEM/F12 medium (Gibco BRL, USA) supplied with 10% fetal calf serum at 37 ºC in humified incubator with 5% CO_2. All cellular experiments were carried out in triplicates. To examine cells' growth in this condition, 30,000 cells per well in 6 well petridish were seeded and growth curve were drawn for each cell line. All experiments were performed on the exponentially growing cells, which were prepared by minimum three passages of the initial seed of frozen stock.

Clonogenic assay

Cells from the above three different cell lines were harvested with trypsin, washed with medium, and plated in quadruplicate onto 60 mm plastic tissue culture dishes (Falcon Co., USA) at a density of 500 cells/dish in 4 ml culture medium. The cells were incubated overnight and allowed to attach on the surface of the dishes.

Cells were then exposed to the above-mentioned concentrations of *M. pulegium* methanolic extract, and/or essential oil for 24 hours at 37ºC. Three controls were used to normalize the resulting data. Quadruplicate set of dishes treated with saline, another set was treated with extract and/or solvents of the essential oil without the herbal ingredients as controls for experiments. The medium was then aspirated and cells were twice rinsed with saline and then the fresh medium was added to each dish. Each experiment was performed in triplicates. After 7-14 days incubation (based on the cell line growth parameters) the medium was aspirated, and cells were fixed and stained with tripan blue dye. Colonies of cells containing at least 50 cells were counted under a microscope. Percentages of colonies for each concentration compared to the appropriate control were assigned as the measurement of cytotoxicity for different concentrations (3).

Neutral red (NR) assay

The Borenfreund and Puerner method for measurement of the inhibition of the cell growth was used for this purpose (4). In this method, the vital dye neutral red is absorbed and is only converted enzymatically in living cells' lysosomes. Cells were harvested and diluted to a concentration of $2x10^4$ cells/well containing 150 µl culture media. After 24 hours of incubation, different concentrations of the herbal extract and/or essential oil were added to different wells. Medium containing herbal ingredient were mixed with 150 µl of neutral red solutions and incubated for 3 hours. Wells were then rinsed with phosphate buffered saline (PBS) and cells were then decolorized with 150 µl of distaining solution (glacial acetic acid:96% ethanol:water, 1:50:49 v/v) for 10 minutes. Ninety six wells plates were then shacked and the absorbance of solution in each well was measured at 540 nm with a microplate reader (5).

Statistical analysis

A bi-directional analysis of variance (ANOVA) with three replicates and two variations factors (concentrations of extracts and/or essential oil, and cytotoxicity test) was carried out. In addition to a Student's t-test of concentrations, two-way ANOVA was used to compare different points for each cytotoxicity curve.

RESULTS

The results of *M. pulegium* methanolic extract and essential oil cytotoxicity on SK-OV-3 cell line are shown in tables 1 and 2. Tables 3 and 4 represent the cytotoxicity results of *M. pulegium* essential oil on Hela and A549 cell lines, respectively. In each table, clonogenic and NR results (mean±SEM) are listed as the percentages of colonies survived after exposure to different concentrations of essential oil, in comparison with the control. Group values of p in the respected columns for each row are the result of ANOVA in comparison to the control group.

Neutral red assay was not able to produce a proper dose-response pattern for the cytotoxicity of these cell lines. Clonogenic assay, however, represented a typical dose-response curve with IC_{50}s of 14.4, 59.1 and 18.76 µg/ml for the essential oil of SK-OV-3, Hela and A-549 cell lines, respectively. As they are shown in tables 3 and 4, each of these three cell lines presents a unique pattern in their related- survival curves resulting from the clonogenic assay cytotoxicity experiment. On the basis of the IC_{50} ($t_{(4)}$=114.1; $p<0.0001$, table 5), there was no significant statistical correlation between cytotoxicity values resulting from the essential oil and the methanolic extract survival curve in SK-OV-3 cell line.

DISCUSSION

It is well known that chemicals and herbal medicines may produce toxic effects. However,

the concentration, and risk-benefit profile are of prime importance. In cancer treatment, any healing suggestion is of a big hope; patients follow it optimistically and scientists look into it seriously.

In terms of dosing issue, and its consequent problems of possible adverse effects, final resolution comes from animal and clinical experiments. However, categorization of natural products' cytotoxicity degrees, and kind of guideline has been provided in 1999 (table 5) (6).

Based on results presented in this paper, *M. pulegium* cytotoxic materials are hydrophobic in nature and the essential oil of aerial parts is cytotoxic on all three studied human carcinoma cell lines at different degrees. In reference to the reported classification (6), essential oil of the aerial parts of *M. pulegium* can be categorized as potentially toxic on all three investigated cell lines. The IC_{50}s for human ovarian adenocarcinoma SK-OV-3, and human lung carcinoma A549 are very close to the range of

Table 1. Percentage of SK-OV-3 cells survived after exposure to the different concentrations of the methanolic extract of *M. pulegium*. One-way ANOVA p values are compared to the control; N.R.: result of neutral red assay; CLN: result of clonogenic assay; CON: concentration in µg/ml; numbers represents mean±SEM.

CON	N.R.	p value	CLN	p value
0	100±3.60	-	100±4.98	-
5	103.34±3.26	>0.05	97.25±4.98	>0.05
25	116.32±1.84	<0.05	94.38±8.58	>0.05
50	121.34±3.18	<0.01	85.80±8.58	>0.05
100	123.01±2.18	<0.001	65.77±4.98	<0.01
150	126.37±2.34	<0.001	31.45±4.98	<0.001
200	128.78±3.26	<0.001	0	<0.001
400	132.21±5.60	<0.001	0	<0.001
600	133.40±3.18	<0.001	0	<0.001
800	134.00±3.93	<0.001	0	<0.001
1000	134.50±1.26	<0.001	0	<0.001

Table 2. Percentage of SK-OV-3 cells survived after exposure to the different concentrations of *M. pulegium* essential oil. One-way ANOVA p values are compared to control; N.R.: result of neutral red assay; CLN: result of clonogenic assay; CON: concentration in µg/ml; numbers represents mean±SEM.

CON	N.R.	p value	CLN	p value
0	100±15.15	-	100±4.56	-
9.38	120.33±3.21	>0.05	90.30±6.45	>0.05
18.76	127.28±5.08	>0.05	27.95±2.15	<0.001
23.45	127.82±4.81	<0.01	19.35±4.30	<0.001
28.14	128.89±4.81	<0.001	1.08±1.08	<0.001
37.52	133.70±3.48	<0.001	0	<0.001
46.90	134.77±3.74	<0.001	0	<0.001
56.28	128.35±4.55	<0.001	0	<0.001
65.66	124.61±2.94	<0.001	0	<0.001
70.35	121.93±3.21	<0.001	0	<0.001
93.80	116.59±3.74	>0.05	0	<0.001

Table 3. Percentage of Hela cells survived after exposure to the different concentrations of *M. pulegium* essential oil. One-way ANOVA p values are compared to control. N.R.; result of neutral red assay; CLN: result of clonogenic assay; CON: concentration in µg/ml; numbers represents mean±SEM.

CON	N.R.	p value	CLN	p value
0	100±0.56	-	100±24.30	-
9.38	117.80±2.10	<0.001	90.45±4.05	>0.05
18.76	118.30±2.10	<0.001	86.40±2.70	>0.05
23.45	118.58±2.20	<0.001	84.60±6.30	>0.05
28.14	120.50±1.30	<0.001	72.90±1.60	>0.05
37.52	123.70±0.70	<0.001	72.00±4.80	>0.05
46.90	113.50±0.70	<0.001	62.30±11.70	<0.01
56.28	110.50±0.80	<0.001	60.30±12.00	<0.00
65.66	107.60±0.70	<0.001	37.80±2.70	<0.001
70.35	104.90±0.80	<0.01	35.10±2.70	<0.001
93.80	102.70±0.80	>0.05	28.40±1.40	<0.001

Table 4. Percentage of A549 cells survived after exposure to the different concentrations of *M. pulegium* essential oil. One-way ANOVA p values are compared to control. N.R.; result of neutral red assay; CLN: result of clonogenic assay; CON: concentration in µg/ml; numbers represents mean±SEM.

CON	N.R.	p value	CLN	p value
0	100±7.22	-	100±1.75	-
9.38	87.70±12.40	>0.05	60.60±6.31	<0.001
18.76	75.13±6.57	<0.01	51.51±5.25	<0.001
23.45	60.42±10.07	<0.001	46.45±2.67	<0.001
28.14	49.09±4.78	<0.001	43.42±1.01	<0.001
37.52	41.45±8.11	<0.001	40.39±1.01	<0.001
46.90	37.59±4.60	<0.001	39.39±1.75	<0.001
56.28	33.72±6.31	<0.001	15.15±3.50	<0.001
65.66	28.00±4.14	<0.001	5.03±1.01	<0.001
70.35	25.90±4.17	<0.001	0±0	<0.001
93.80	25.29±3.42	<0.001	0±0	<0.001

Table 5. Classification of the cytotoxicity for natural ingredients (Balantyne, 1999).

Category	IC_{50}
Potentially very toxic	IC_{50}<10µgmL^{-1} (million cells mL^{-1})
Potentially toxic	10µgmL^{-1}<IC_{50}<100µgmL^{-1}
Potentially harmful	100µgmL^{-1}<IC_{50}<1000µgmL^{-1}
Potentially non toxic	IC_{50}>1000µgmL^{-1}

potentially very toxic agents. Although *M. pulegium* essential oil can be considered optimistically as a candidate for the treatment of above three cancers, the pattern of its cytotoxicity varies on different tissues. Based on previous publications on the survival curve algorithm for anticancer agents (7), there is almost no resistance to *M. pulegium* essential oil cytotoxicity in human ovarian adenocarcinoma SK-OV-3 cell line (table 2); survival curve presents a dramatic sharp negative slope for SK-OV-3 cell line, even by addition of a very diluted amount of *M. pulegium* essential oil. These results represent SK-OV-3 human ovarian cell line, which is resistant to many other most used chemotherapeutic agents (e.g. cisplatin), and is a very sensitive cell line to *M. pulegium* essential oil. Although, human lung carcinoma A549 cell line population declined dramatically after exposure to *M. pulegium* essential oil in the same concentrations as for SKOV3, a kind of presumably active resistant overcame this pattern soon, however, at the concentration of 50 µg/ml or higher of *M. pulegium* essential oil the cellular defense mechanisms were destroyed. Human cervix carcinoma Hela, shows kind of passive resistance to *M. pulegium* essential oil based on the related survival curves. On the basis of the survival curve for this cell line, whatever mechanisms presented in Hela cells were challenged by *M. pulegium* essential oil cytotoxicity at any concentrations that was added to the cell culture medium.

The clonogenic assay was trusted as the cut off point for the cytotoxicity measurement. The length of time required to gain results in this assay (about 10-14 days) concludes all different cellular mechanisms (arrest, resistance, passive or active defenses, repair an so on) in response to the test agents. Neutral red assay (NR), on the other hand, not only presents a more rapid idea on the degree of the cytotoxicity of the test agent, but also clarify whether cytotoxicity is related to the cell membrane integrity, and/or lysosomal enzymes. From the results of this investigation it appears NR method failed to agree with the clonogenic assay results for SKOV3 and Hela cell lines, possibly because *M. pulegium* essential oil cytotoxicity was not related to the cell membrane damage and the activity of the cellular lysosomal enzymes. This conclusion is not valid for A549 cell line. The IC_{50} of *M. pulegium* essential oil is calculated 28.14 µg/ml for A549 cell line in NR assay which is categorized in the range of potentially toxic substance for the human lung carcinoma. This pattern would suggest cellular membrane and/or lysosomal structure, as the site of action for *M. pulegium* essential oil in A549 cell line.

The herbal medications of Labiatae family, including the genus Mentha, are famous for their cytotoxic effects (8). Mentha family decoction or infusion have been used for the treatment of human cervical cancer (9,2). *M. pulegium* has also been reported to have insect repellent, antiseptic, emmenagogue, carminative, anti-spasmodic and diaphoretic characteristics (1). This herb has also been used for the treatment of flu and cold syndrome (10). The genotoxicity and

insecticide properties of *M. pulegium* has been investigated and this plant due to the presence of pulegone in its extract has been introduced as a potent insecticide (11).

Our results indicate that *M. pulegium* essential oil is a good anticancer candidate for at least three human cell lines. Its degree of toxicity suggests the application of a very diluted amount of the essential oil of the aerial parts for cancer treatment, which might prevents it adverse effects on other human tissues. Most of the previous publications have correlated the cytotoxicity of *M. pulegium* to its pulegone, which is present up to 90% in plants grown in many other countries. It has been reported that the Iranian *M. pulegium* contains less than 0.1% pulegone (12). *Mentha pulegium* growth in Iran may therefore contain some other chemicals responsible for its cytotoxicity. This herb has popular uses in Iran, by itself or as food additive. It may therefore be assumed that although the direct application of these toxic agents on cells are potentially very toxic, but they are sensitive to the gastro-intestinal environment so that the oral consumption of the aerial parts of this plant does not cause any complications in human. Based on these results, further investigations on the fractionation of *M. pulegium* essential oil for finding the most cytotoxic agents and their chemical stabilities are recommended.

ACKNOWLEDGMENT

This work was supported by Shaheed Beheshti University of Medical Sciences, Islamic Republic of Iran. Authors would like to express their appreciation from Dr. Rakesh Goel, medical research scientist at pharmacology lab, Ottawa Regional Cancer Centre, Ottawa, Canada.

REFERENCES

1. Marderosian AD. Peppermint. In: Marderosian AD, ed. The review of natural products. USA: Facts and Comparisons; 2001. pp. 465-466.
2. Duke JA. Mentha pulegium L. (Lamiaceae) – Pennyroyal. In: Duke JA. Handbook of medicinal herbs. Boca Raton: CRC Press, Florida, USA. 2001. pp. 307-308.
3. Von Hoff D D, Forseth B, Warfel L E. Use of a radiometric system to screen for antineoplastic agents: Correlation with a human tumor cloning system. Cancer Res. 1985; 45:4032-4038.
4. Borenfreund E, Puerner J A. Toxicology determined in vitro by morphological alterations and neutral red absorption. Toxicol. Lett., 1985; 29:119-123.
5. Barils FA. Continues cell lines as a model for drug toxicity assessment. In: Castell JV, Gomez-Lechon MJ, eds. In vitro methods in pharmaceutical research. USA Academic Press, 1997, pp. 41-42.
6. Gad Shayne C. (1999) Alternatives to in vivo studies in toxicology. In: Balantyne B., Marrs T., Syversen T, eds. General and applied toxicology. Vol (1). Grove's dictionari's Inc., USA. 1999. p.178.
7. Stewart D J. Cisplatin resistance in lung cancer and dose-response curve shape. Proc. Am. Assoc. Cancer Res., April 10-14, Philadelphia, PA, USA, 1999: #1239.
8. Hoffman EJ. Plant biochemistry and cancer. In: Hoffman EJ. Cancer and the search for selective biochemical inhibitors. Boca Raton: CRC Press, Florida, USA. 1999, p.76.
9. Brigges C J. Pennyroyal a traditional medicinal herb with toxic potential, Can. Pharm. J. 1989; 122:369-372.
10. Skidmor-Roth L. Primary chemical components of pectin and their possible actions. In: Mosby's handbook of herbs and natural supplements. Mosby Inc., St. Louis, MO, USA. 2001, pp. 671-674.
11. Franzios G, Mirotsou M, Hatziapostolou E. Insecticidal and genotoxic activities of mint essential oils., J. Agric Food Chem., 1979; 45: 2690-2694.
12. Amin G H, Salehi Surmaghi M H, Shahidi F, Alimirzaii R. Survey of pulegone as a toxic compound in *Mentha pulegium*. 14th Iranian Congress of Physiology and Pharmacology, 16-20 May, Tehran University of Medical Sciences, Tehran, Iran, 1999; p.503.