Indian Journal of Experimental Biology
Vol. 60, October 2022, pp. 753-758
DOI: 10.56042/ijeb.v60i10.47280

Antiproliferative and cytotoxic activities of *Mentha x piperita* L. essential oil in non-small cell lung cancer cells

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Received 09 March 2021; *revised 27 October 2021*

Among 33 types of listed cancers worldwide, lung cancer with 2.2 million cases (12.2% of total cancer cases) ranks second next only to breast cancer. Globally, Turkey, with overall rate of 40.0 (41,264 cases), ranks 5th among the top 10 countries in lung cancer. Currently used therapeutic agents and approaches have considerable side effects, and hence, there is a need for alternative agents for effective management of lung cancer. In this study, we explored the *in vitro* cytotoxic, antiproliferative and proapoptotic activities of *Mentha x piperita* L. (peppermint) essential oil in human non-small cell lung cancer (A549) cells. Cell viability was determined by MTT assay, morphological changes were determined by confocal microscopy and apoptosis promoting action was determined by flow cytometry technique. Peppermint essential oil found to effectively decrease the viability of non-small cell lung cancer cells and IC_{50} value was detected at low concentrations (2.12%) for 24 h. In addition, peppermint essential oil was found to alter the morphology of A549 cells, leading to changes that could describe programmed cell death. Apoptosis was the triggered cell death by *Mentha x piperita* essential oil. Results reveal that *Mentha x piperita* essential oil has antiproliferative and anticarcinogenic properties which could be attributed to the bioactive phytochemical contents and has the potential to be used as an anticancer agent and chemotherapeutic drug.

Keywords: Anticancer activity, Apoptosis, Growth supression, Peppermint

Cancer is a major health problem worldwide, second cause of death in United States and first ranked in frequency for men in Turkey. Globally, lung cancer which ranks second with 12.2% of total listed 33 types cancers (2.21 of 18.1 million cases) causes 1.8 million deaths accounting for 18% of 9.9 million cancer related fatalities in total. Though the incidence appear to decline in 2022, with 350 deaths per day, lung cancer is reported to be the leading cause of cancer deaths in US. Turkey, with overall rate of 40.0 lung cancer incidence, ranks 5th globally, and among men, it leads with a score of 74.8. In lung cancer deaths, Turkey ranks 4th with overall mortality rate of 35.9. Most of the lung cancer statistics comprise of small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). Approximately, 13% of all lung cancers are SCLC, and 84% are NSCLC[1-5]. Although all countries spend large budgets in combating cancer, cancer continues to exist as a disease that significantly affects human health and quality of life.

The tumor that occurs with the abnormal uncontrolled proliferation of the lung parenchyma and bronchial tree cells is called lung cancer and does not cause a specific complaint in the patient until it reaches advanced levels[6]. After the tumor grows, it metastasizes primarily to the brain, bone tissues, liver, and adrenal glands via lymphatic or blood route. It has been reported that the progression of lung cancer is determined by receptor activity in the cell membrane and intracellular signal transduction pathways that regulate many biological processes like apoptosis[6]. Lung cancers are classified as small cell and non-small cell lung cancers in clinical studies[7]. Approximately, 80-85% of the cases are NSCLC[8,9].

The treatment of lung cancer, surgery, radio-therapy, and chemotherapy options are applied in single or different combinations according to the stage of the disease. With new approaches of adjuvant therapy and agents availability, remarkable innovative advancements have been made recently which made significant clinical impact in patients with lung cancer[10]. Despite such progress in various treatment options, there is an increasing need for novel therapy agents especially for in advanced stage cases[2].

There are many studies showing that plant secondary metabolites can be used as anticancer

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agents[11-13]. *Mentha x piperita* L. (Fam. Lamiaceae), commonly called peppermint, 30-90 cm tall, rhizome and perennial herbaceous plant, leaves 4-9 cm long and 1.5-4 cm wide with reddish-dark green veins, blunt tip, coarsely toothed edges, petiole sparsely hairy, flowers are spike, 6-8 mm long, purple in colour is also known for its medicinal value[14-17]. It is a good source of phenols, terpenes and tannins. Essential oil from *M. piperita* contains mainly menthol and menthone, and alsosabinene, b-myrcene, α-terpinene, limonene, g-terpinene, linalool and menthyl acetate[18,19]. It possess antimicrobial properties[19] and also reported to be effective against breast cancer (MCF-7)[20], colon, prostate, lung and brain cancer cells[21-23].

Mint essential oils are reported to show cytotoxicity, proapoptotic activity and to decrease migration and invasion capability of various cancer cell lines, as well as to induce senescence phenotype[15]. Anticarcinogenic properties of *Mentha x piperita* leaves have been determined to show radioprotective activity in a mouse skin tumor model and various cell lines[25-28]. A study found that *M. piperita* extract has the potential to reduce the risk of non-small cell lung cancer[11].

In this study, we have attempted to determine the *in vitro* antiproliferative, cytotoxic and proapoptotic activities of essential oil of *Mentha x piperita* grown around Eskişehir on non-small cell lung cancer (A549) cells.

Materials and Methods

Preparation *Mentha x piperita* essential oil

Essential oil derived from *Mentha x piperita* L. leaf grown in Turkey was used in this study. The plant material was collected from local producers in Eskişehir and brought to the laboratory in sterilized containers. The carefully cleaned leaves were dried, milled and then strained through a 2 mm sieve into standard size particles. The essential oil was obtained by steam distillation using Clevenger apparatus and was stored in dark bottles at 4°C until it was used[15].

Gas chromatography/Mass spectrometry (GC/MS)

For identification of constituents of *Mentha x piperita* essential oil was used Gas Chromatography/ Mass Spectrometry (GC/MS) (Agilent 7890B GC 5977B Mas Selective Detector System, 250 injection temperature) and Gas Chromatography (Agilent 7890B GC System, 250 injection temperature) method was used for determining of relative percentages of the ingredients[15].

Chemicals

A549 non-small lung cancer cell line was obtained from the American type culture collection (ATCC). Fetal bovine serum, penicillin–streptomycin, dimethyl sulfoxide (DMSO), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were obtained from Sigma-Aldrich (USA), and Roswell Park Memorial Institute medium (RPMI-1640) was obtained from GIBCO (USA).

Cell culture and determination of antiproliferative, cytotoxic and proapoptotic activities

Non-small cell lung cancer cells were cultured in 25 cm^2 cell culture flasks in RPMI-1640 medium containing 10% serum (Fetal bovine serum/FBS) and 1% penicillin/streptomycin at 37°C, 5% CO_2 and appropriate humidity conditions in an incubator. Passaging of the proliferated cells was carried out at a complete confluency routinely to their sixth passage. The proliferated cells were used in the experiments in the 6^{th} passage at a confluency of 85%[15].

MTT assay for cytotoxicity

A549 cells were seeded in 96-well cell culture plates at 5×10^3 cells per well. The cells seeded on the plate were incubated in an incubator with 5% carbon dioxide at 37°C. *Mentha x piperita* essential oil was diluted serially in a concentration range of 0.3-10 (%) and A549 cells were incubated with the essential oil for 24 h. At the end of the incubation period, 20 µL of MTT dye (5 mg/mL) was added to the wells and incubated at 37°C for 2 h. After the incubation, the liquid in the plate was replaced with DMSO (200 µL) and plates were read at 570 nm wavelength in the HTX Synergy (Bio-Tek, USA) plate reader. The untreated A549 cells were considered as control group. Separate viability values for each concentration were calculated according to the control group. The concentration that showed 50% cytotoxic effect according to the control was accepted as the IC_{50} cytotoxic concentration. The IC_{50} concentration of the applied substance on the cells was calculated with the Excel program using the obtained viability values[15]. Experimentations were performed in triplicates.

Determination of morphological changes in cells by confocal microscopy

Morphological changes caused by *Mentha x piperita* essential oil on non-small cell lung cancer cells were examined by confocal microscopy method.

Cells to be examined under confocal microscope were initially seeded in 6-well plates in triplicates at a density of 3×10^5 cells/well and incubated for 24 h with the IC_{50} concentration of the essential oil. At the end of the 24 h incubation, the media were removed, the cells were washed in phosphate buffer (PBS) and fixed in glutaraldehyde. After fixation, the cells were washed with PBS again, and double staining was applied with acridine orange and phalloidin dyes[15]. The morphological changes in the cells were examined and visualized using a confocal microscope (Leica TCS-SP5 II) using Leica Confocal Software Version 2.00 software.

Flow cytometric analysis

The phosphatidylserine translocation on the outer side of cell mebrane as an indicator of early apoptosis was evaluated by annexin V staining technique on a flow cytometer. The test was realized as it was described by manufacturer of the kit (Anexin V kit, Merck, Millipore, Hayward, CA, USA). Briefly, A549 cells were seeded in 6-well plates (5×10^5 cells/well) and incubated with IC_{50} value of the agent for 24 h. After the incubation period cells were harvested by trypsinization and centrifuged at 1200 rpm for 5 min. Centrifuged cells were resuspended in complete medium (100 µL) and annexin V reagent (100 µL) was added to each test tube. All samples were incubated at room temperature 20 min (in dark) and analyzed on Muse™ Cell Analyser (Merck, Millipore, Hayward, CA, USA)[15]. The experimentation was performed in triplicates.

Statistical analysis used in the evaluation of the results

GraphPad prism 6.0 package program was used for statistical evaluations, and the significance of the data was determined using one-way ANOVA and post-hoc Tukey test.

Results

Determination of cell viability by MTT assay, determination of morphological changes in cells by confocal microscopy and flow cytometric analysis are given under three separate main headings. Firstly, the ingredients of *Mentha x piperita* essential oil were determined (Table 1). MTT assay was performed to evaluate the cytotoxicity of *M. piperita* essential oil and calculated the IC_{50} concentration of essential oil on A549 cells. Then, the cells were treated with the IC_{50} concentration of *M. piperita* essential oil for 24 h and conducted confocal microscopy analysis for observing the morphological alterations. Finally, annexin V assay was performed to determine the death mode of A549 cells.

Table 1 — Components of *Mentha x piperita* essential oil

Compound*	Relative Percent (%)
α-Pinene	0,6
β-Pinene	1,4
Sabinene	0,9
Myrcene	0,8
Limonene	12,8
1.8-Sineol	8,7
β-Burbonen	1,5
β-Caryophyllin	2,0
trans-Dihydrocarvone	6,4
cis-Isodihydrocarvone	0,9
α-Terpineol	1,1
Neodihydrocarveol	1,1
Carvone	55,8
Caryophyllin oxide	0,5

*P <0.05 (%)

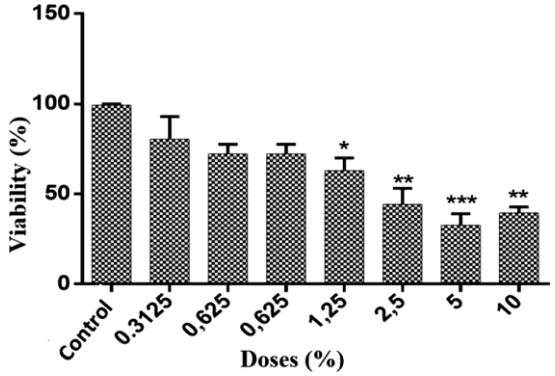

Fig. 1 — Viability inhibition (24 h) data of *Mentha x piperita* essential oil to non-small cell lung cancer (A549) cell lines. [Significance values were determined as $P \leq 0.044$ *; $P \leq 0.005$ **; $P \leq 0.0007$ ***with one-way ANOVA and post-hoc Tukey test of GraphPad prism 6.0 statistical package]

Determination of cell viability decrease by MTT assay

The results of the MTT assay revealed that the decreased viability of NSCLC A549 cells in a dose-dependent manner when compared with the control cells during the 24 h application period. The highest viability decrease was detected at concentrations of 5 and 10%. The decreased viability at the lowest dose was recorded at the lowest level (Fig. 1). Separate viability values for each dose were calculated according to the control group and the results obtained were found to be statistically significant. The IC_{50} concentration of peppermint extract inhibiting the viability of lung cancer (A549) cells was determined as 2.12% for 24 h.

Determination of morphological changes in cells by confocal microscopy

Confocal microscopy findings indicated that cell nucleus preserved its integrity and no changes

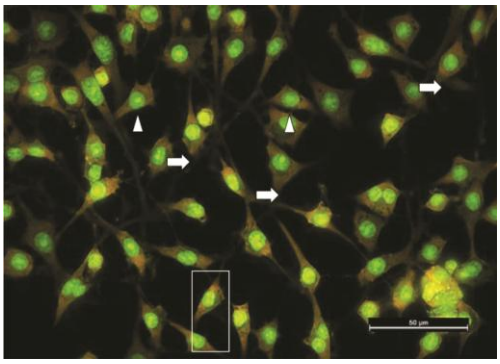

Fig. 2 — Confocal microscopy images of non-small cell lung cancer (A549) untreated group cells. [Arrowhead, Compact nucleus; Rectangle, fusiform shaped cell A549; and Arrow, cytoskeleton]

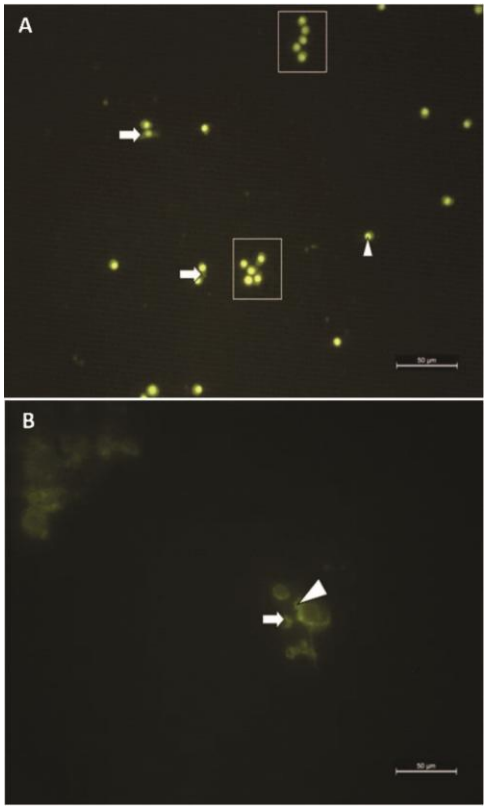

Fig. 3 — Confocal microscope images of A549 cells treated with IC_{50} value of *Mentha x piperita* essential oil for 24 h. (A) Arrow-Cytoskeleton, Arrowhead-shrunken cell nucleus, Square-skeleton fragmented and shrunken A549 cells; and (B) Arrowhead-shrunken cell membrane, Arrow-chromatin condensation.

occurred in the cytoskeleton in the control group of A549 cells that were not exposed to the essential oil (Fig. 2).

In A549 cells treated with the IC_{50} concentration of *M. piperita* essential oil for 24 h, it was determined that the cells shrank by losing the fusiform shape. In addition, the cytoskeleton was fragmented. Chromatin

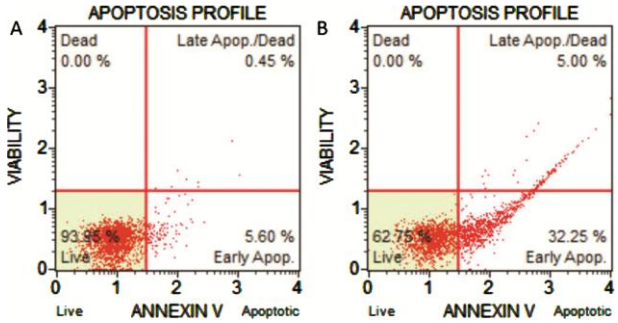

Fig. 4 — Apoptosis profiles of non-small cell lung cancer cells (A549). (A) Untreated A549 cells (Live cells: 93.95%; Early apoptotic cells: 5.60%, Late apoptotic cells: 0.45% and Dead: 0.00%); and (B) A549 exposed to IC_{50} value of *Mentha x piperita* for 24 h (Live cells: 62.75%; Early apoptotic cells: 32.25%, Late apoptotic cells: 5.00% and Dead: 0.00%)

condensation and nuclear shrinkage were also detected in the experimental group (Fig. 3).

Annexin-V analysis findings

Apoptotic profiles of A549 cells were evaluated by flow cytometry technique. Data revealed that untreated cells were alive 93.95% and total apoptotic cell percentage of this group was detected to be approximately 6%. A549 cells exposed to essential oil of *M. piperita* were found to be alive at a percentage of 62.75%. The total apoptotic cell percentage of these cells was determined as 37.25% (Fig. 4).

Discussion

Mentha x piperita extract has the potential to reduce the risk of non-small cell human lung cancer, and also effective on the expression of aromatase enzyme in human NSCLC cells[11]. However, the effect of peppermint essential oil on non-small cell lung cancer has been studied here in this study.

MTT assay results indicated that *M. piperita* essential oil exposure reduced the viability of A549 cells in a dose-dependent manner. The IC_{50} concentration was detected as 2.12% for 24 h. This finding imply to the high cytotoxicity of the *Mentha x piperita* essential oil on non-small cell lung cancer cells for a short application period of 24 h. It was determined by this research that *M. piperita* essential oil effectively reduced the viability of non-small cell lung cancer (A549) cells and caused programmed cell death by changing the morphology of the cells. This effect may occur as a result of a synergistic effect by the secondary metabolites contained in peppermint essential oil or caused by one of the compounds contained in the essential oil. Futher studies are required to understand the mechanism of action.

In addition, *M. piperita* essential oil was found to alter the morphology of non-small cell lung cancer (A549) cells, leading to changes that could describe programmed cell death. Confocal microscopy data obtained, indicated morphological changes as shrinkage and dysintegration of nucleus, chromatin and cytoskeleton (Fig. 3) whereas untreated cells were compact with no changes in the morphology (Fig. 2).

Proapoptotic activities of mint essential oils were reported to have apoptosis inducing activity and to cause upregulation of Bax and p53 genes as well as to modulate TNF, IL-6 and 8 in a variety of cancer cells[24]. Similarly in our study, apoptotic profiles of A549 cells exposed to the IC_{50} concentration of *M. piperita* essential oil were obtained and evaluated in comparison to the untreated cells. The apoptotic profile of control cells indicated the percentage of viable, early apoptotic and late apoptotic/necrotic cells as 93.95, 5.60 and 0.45%, respectively. Following treatment, the percentage of live, early apoptotic and late apoptotic cells were observed to be 62.75, 32.25 and 5.00%, respectively (Fig. 4). No necrotic cells were detected both in control and test cells. Total apoptotic cell percentage of test cells was detected to be 37.25% that imply to the decreased viability by apoptotic cell death. Based on the obtained data, new deeper and further investigations may be conducted and it may be possible to synthesize effective pharmaceutical compounds by purifying new herbal agents from *Mentha x piperita* essential oil.

Conclusion

Results of this study have demonstrated that *Mentha x piperita* essential oil effectively reduced the viability and caused apoptosis of non-small cell lung cancer (A549) cells demonstrating its antiproliferative, cytotoxic and proaoptotic properties, and thereby its potential as an anticancer agent and chemotherapeutic drug. Further investigations are required to understand the mechanism of action and ultrastructural changes in the cells.

Acknowledgement

This study was presented at the regional final with Project No 1139B422001065 within the scope of “2242 - University Students Research Project Competitions, 2020/1 term”.

Conflicts of interest

Authors declare no competing interests.

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